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Combined preventive preparations against wound infections

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COMBINED PREPARATIONS FOR THE ACTIVE PREVENTION OF WOUND INFECTIONS (p.3-18)

Numerous data in the literature of recent times on the pathogenesis and immunity in case of wound infections prove the fact that the most effective and prospective direction in the combat of these infections is the solution of the problems of active immunization. If we reckon that in the pathogenesis of tetanus, of gas gangrene and of the staphylococcic wound infection the leading factor is the poisoning by the corresponding toxins, this determines of course not only the pathway of immunization and the immunogenic factor but also the end result of immunization- the creation of a permanent and stable immunity.

The gas gangrene itself is a quickly spreading necrosis of the muscles, and it is accompanied by considerable edema which gave reason for OKLI to call this process an edematous myonecrosis. AIKET and DIBL(1956) think that the basic lesion is provoked by the exotoxin of the Cl.perfringens,- the myonecrosis is provoked by the action of the alpha-toxin(lecithinase). The myonecrosis, due to the direct action

upon the muscular fiber by the exotoxin of the Cl.perfringens, but not to the action of the collagenase upon the reticular structure and upon the fibrous tissue of the endomysium. The disappearance of the endomysial collagen connective tissue is the result of the action of hyaluronidase. The reticulin is stable. In the injured muscles, the nutrition of the tissue with blood is partly or completely impaired. The anastomotic blood supply is insufficient to prevent a necrosis of the muscles which develops in the anoxic parts. The amount of haemoglobin at the sites of the blood extravasation is quickly diminished, the aerobic oxidation stops, and the oxidation-reduction potential is lowered, and the pH of the muscles will drop. Favorable conditions arise for the growth of the anaerobic bacteria.

The gravity of the evolution of the process is complicated by the following changes in the white blood count:- the polymorphonuclear leukocytes (p.4) perish in the anoxic tissues. The serum therapy and the medicamentous therapy is of small effectiveness under such conditions, since, in consequence of the anoxia, of the edema and of the thrombosis of the vessels, the introduced specific antibodies and medicinal substances are unable to penetrate in sufficient amount to the site of the lesion and they cannot stop the development of the pathological process. Under such conditions, the active immunization may show itself as the most effective and most prospective means.

In this way, for the prevention of wound infections(at least,for the most important ones among them), the most prospective method seems to us the active immunization with anatoxins for the ultimate end to produce chiefly an antitoxic, but also an antibacterial immunity. As it is well known, the employment of the tetanus anatoxin for active immunization meant a new stage in the combat against tetanus. Numerous works of domestic and foreign investigators have shown the feasibility of a reinforced active immunization against tetanus by means of the tetanus anatoxin, and its advantages above the serum prophylaxis(G.RAMON et al.; E.V.GLOTOVA & O.YA.OSTROVSKAYA; A.V.PONOMAREV et al.; P.F.ZDRODOVSKII; B.V.VOSKRESENSKII & O.I. NIKOLAEVA, I.I.ROGOZIN, D.D'ANTONA, and others). The effectiveness of active immunization with the tetanus anatoxin was corroborated during the Second World War:- among 100,000 wounded persons who had received full courses of vaccinations only a few cases of sickness were observed due to tetanus.

In the pathogenesis of gas gangrene, caused by Clostridium perfringens, the

basic importance is attached to the toxin of this microbe. However, as it had been established by a number of investigators(S.N.MUROMTSEV,OKLI), the generalized development of the infection with serious universal poisoning is accompanied by a vehement proliferation of the microbes at the site of their intrusion and by their quick penetration into the blood,and later-- almost into all the organs and tissues of the organism. In connection with this, the question arises about the significance of the antimicrobic [illegible] factor in the mechanism of immunity against the Clostridium perfringens, and about the appropriateness of including microbic components in an antigen predestined for active immunization against gas gangrene caused by the perfringens bacillus.

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The literature is not very large on this question. WEINBERG and others, in a series of investigations(1927- 1929) had obtained the reinforcement of anti-infectious substances of the antiperfringens serums after the inclusion of microbic bodies of the Type A Clostridium perfringens in the antigen meant for immunization. Similar results were obtained by LAKHIERI(1938) and VINCENT(1939).

The cited works do not determine,however, the role of the antibacterial factor in the mechanism of the passive antiperfringens immunity (p.5) since the different effectiveness of the obtained sera could be conditioned not only by the presence or the absence of the antibacterial factor, but also by their different contents in antitoxin, which has been also later convincingly shown by O.I.LEVKOVICH(1945) LEVKOVICH proved that the effectiveness of the antiperfringens sera which were prepared by means of introducing various antigens(of microbic bodies, of toxin and anatoxin) had depended only upon the level of the obtained antitoxic titre.

G.B.VYGODCHIKOV,Z.M.VOLKOVA,S.A.ZELEVINSKAYA and I.A.LARINA set themselves the task to study the possibility of increasing the immunogenic properties of the perfringens anatoxins by means of including in them different protein fractions of the microbic cell of Clostridium perfringens and to show the role of the antibacterial factor in the active immunity against gas gangrene which was provoked by the Type A Cl.perfringens. As antigens for the immunization of the animals,they used concentrated purified sorbed perfringens anatoxin; different protein fractions of the microbic bodies of the Type A Cl.perfringens; a mixture of anatoxin with microbic fractions which they prepared by the methods elaborated by N.V.KHOLCHEV(1952) for the obtaining of the protein fractions of staphylococci.

For getting the first protein fraction, they extracted dry microbic bodies three times by means of cold distilled water. They cooled the obtained extract at 4°C, and subjected it to precipitation with a 20% solution of trichloracetic acid at pH 3,7 - 4,0. They collected the obtained precipitate of protein by means of centrifugation in a little volume of water(distilled) at pH 7,0, and then subjected it to lyophilic exsiccation(first fraction). This procedure is similar to the precipitation of the toxin from the bouillon filtrates of the cultures of staphylococci.

For getting the second fraction, they poured a weak solution of alkali(0,05-normal solution of NaOH) over the microbic mass which remained after the extraction of the first fraction, at the rate of 50-100 ml per 1 gram of dry mass.

They precipitated the extract with acetic acid at pH 4,0 - 4,2. The outfallen precipitate was collected by centrifugation, dissolved in a 0,05-N solution of NaOH, and subjected to a second precipitation at pH 4,0 - 4,2. Again, the obtained precipitate was separated by means of centrifugation; a suspension was made of it in a small volume of distilled water at pH 7,0, and it was exsiccated by the lyophilic method(second fraction). By the extraction method, this fraction hathers the part of the microbic nucleoproteids.

For getting the third fraction, to the centrifugate which was left over after the removal of the second fraction, 20% trichloro-acetic acid was added at pH 1,0 - 0,9, and with the same the rest (p,6) of the protein contained in the centrifugate was precipitated. The obtained precipitate was collected by centrifugation, it was dissolved in a small volume of distilled water, and exsiccated by the lyophilic method(third fraction). The method of isolation of this fraction is similar to the obtaining of the specific protein of staphylococci(VERVEI).

In all the prepared protein fractions the physico-chemical properties were studied, and the toxicity was determined on white mice by means of intravenous injection, and on rabbits by means of intracutaneous injection.

All prepared microbic fractions and concentrated sorbed anatoxins were utilized for the immunization of animals. Fortyfive rabbits were arranged for the immunization experiment. The immunization was done twice(two-shot) at an interval of twenty days.

Fifteen days after the second injection, the content of antitoxin was deter-

mined in the blood of all animals. Five months after the immunization, a sistate revaccination followed with the same doses of antigens which had been introduced at the first injection. Fifteen days after the revaccination, the antitoxin content of the blood of the revaccinated animals was determined, and the strength of immunity was tested by a direct experiment, by means of infecting a spore culture of the perfringens bacillus. The infection was done intramuscularly into the rear paw, with the spores of B. perfringens, activated with 0.1 ml of a 50% solution of $CaCl_2$.

The conducted studies permitted to draw the following conclusions:

1). The immunization of the rabbits with the concentrated sorbed perfringens anatoxin causes in the organism of the animals the formation of a considerable amount of antitoxin which fully protects them from experimental gas gangrene caused by the perfringens bacillus. The addition of various microbic fractions to the anatoxin provokes the formation of bacterial antibodies, but it does not help the increase of the antitoxin production.

2). The immunization of the rabbits with anatoxic protein fractions of the microbic cells of the perfringens bacillus will provoke in the immunized animals the formation of agglutinin-precipitins, of complement-binding antibodies and, evidently, it imparts a slightly marked anti-infectional immunity to separate animals.

3). The basic protective factor in the immunity against gas gangrene due to the perfringens bacillus appears to be the antitoxin. The antibacterial factors which are detectable to a slight degree are just playing a secondary role. Sufficient content in antitoxin in case of the absence of antibacterial components (p.7) will fully protect the animals from experimental gas gangrene, even under the harsh conditions of the experiment(infection with sporal culture, activated by 0.1 ml of a 50% solution of calcium chloride).

The first investigations about the active immunization of people have been carried out in 1915 by WEINBERG who had prepared an antiperfringens vaccine, and had used this vaccine for the treatment of wounds in case of phlegmone(cellulitis) of an indolent course. In the subsequent years, WEINBERG and his coworkers had used, in addition to the vaccine, anacultures and anatoxins for immunization. The results which the WEINBERG School achieved had been just slightly satisfactory. WEINBERG thought that the immunization against gas gangrene is " a difficult task".

In further works of ZELEVINSKAYA(1935), PENFOL'D and TOLKHORST(1937), PLUMER (1939), KOLMER(1942), STEWART(1942), ROBERTSON and KEPPI(1949), DOUDI(1942), ZELEVINSKAYA, VOLKOVA & KONSTANTINOVA(1944), ZELEVINSKAYA & EBERT(1944), MILAN, LOGAN & TAITL(1944), LOGAN & TAITL(1945), KOMKOVA(1946), ALTMEYER, CULBERTSON, TAITL & LOGAN (1947), BERNHEIMER(1947), WATERS AND MOLON(1949), PLETNEVA(1952), FOVTUMOVICH(1953-1954), and of other authors, it was proved that, in the majority of cases, the immunization with native(= natural) anatoxins gave unsatisfactory results in relation to the immunity against B. perfringens, too. ZELEVINSKAYA, VOLKOVA and BULATOVA established that the purification and concentration of the perfringens anatoxins with the aid of ammonium sulfate or with the salt of heavy metals(Cd) permits the obtaining of considerably stronger antigens than with the native anatoxins. The possibility has been also found that immunity can be experimentally produced against tetanus and gas gangrene due to the perfringens bacillus and to the edematiens bacillus by the use of a composite preparation for the immunization.

As it can be seen from the available literature, the slight efficiency of research in relation to the specific prevention from gas gangrene is connected with the difficulties of getting preventive preparations(anatoxins) which would have sufficient antigenicity and immunogenicity. However, ADAMS(1947), TAITL & LOGAN (1947), thanks to the bivalent immunization and the revaccination of volunteers with perfringens plus edematiens anatoxins, have detected perfringens antitoxin in the strength of from 0.05 to 2 antitoxic units, and edematiens antitoxin in the strength of from 0.1 to 1 antitoxic units(1947), in the blood of the inoculated persons. BERNHEIMER(1947), as a result of a combined immunization with the anatoxins of perfringens and edematiens and septicus, after the revaccinations had obtained the titres of perfringens antitoxin from 0.1 to 2.0 A.U. (antitoxic units), of the edematiens antitoxin from 0.2 to 1.0 A.U., and of the antitoxin of Vibrio septicus from 0.1 to 1.0 A.U. in the blood of the inoculated individuals.(p.8) On the other hand, according to the data of ADAMS(1947), TAITL & LOGAN(1948), and BERNHEIMER(1947), the use of precipitated and concentrated anatoxins allowed to get positive results under the condition of a compulsory performance of revaccination in a period of 6 to 12 months after the immunization. This has been also pointed out by MAIES() who thought that a stimulating dose is required some time after the initial injection for a high level of immunity.

The modern status of knowledge about the antigens and the broad development of immuno-chemical research makes it possible to considerably strengthen the range of action of the preventive preparations by employing purified and concentrated preparations simultaneously against several infections. This circumstance has a profound importance for the active immunization against wound infections.

What kind of conditions are then required and which are satisfactory for the development of this really new stage in the field of practical immunology?

The production of immunogenic complex preparations for active immunization against wound infections is tied up with 1) the investigation and study of new nutrient media which are best for the formation of toxins, and the production of the toxins; with 2) a study of the process of detoxication of the toxins which are obtained on the above indicated media, for the purpose of getting anatoxins; with 3) the elaboration of the methods of purification and concentration of the toxins and anatoxins which, in the presence of a small amount of the preparation, will permit to reach the maximum immunological effect as a result of the active immunization; with 4) the study of the process of sorption of the anatoxins for the purpose of strengthening their antigenic and immunogenic properties.

Considerable interest is concentrated on the results of the elaboration of a new method for getting anaerobic toxins by means of the breeding of a culture of anaerobe germs in cellophane bags; the method had been undertaken by S.A.ZELEVINSKAYA, E.A.GIL'GUT, N.S.KASHINTSEVA, I.B'LANOVA, E.V.VLASOVA and G.E.FRUMKINA.

It was shown that, at the cultivation of toxigenic strains of anaerobes, the culture in the cellophane bag may get "dialyzed" toxins, considerably exceeding in strength the toxins which can be obtained in case of the direct seeding of cultures into the nutrient medium. The tetanus toxins which are prepared by this method were ten to hundred times stronger, the edematiens toxin 10 to 40 times stronger, and the septic vibrio toxin 5 to 10 times stronger, the perfringens toxins 2 to 4 times stronger than the toxins made by the usual methods. It has been established that the maximum peak of the toxin formation in the cellophane bag comes later than in the case of a cultivation of the strains (p.9) on ordinary nutrient media:- for the tetanus and the botulinus toxin of Type B--on the 9th day; for the edematines--on the 6th day; for the perfringens-- on the 5th day. In the process of incubation a lysis of the microbic bodies takes place. At the end of the incubation, their al-

most complete disintegration has been observed.

At staining by MOROZOV's method, flagella(cilia) are easily destated in the Clostridium botulinum and the Clostridium edematiens. Among the lysed forms, punctuate ciliated forms have been observed. It has been proved that the "dialyzed" toxins, at the addition of 0.3 - 0.4% formalin, will change into anatoxins in a shorter period of time than the ordinary toxins. The anatoxins obtained from the "dialyzed" toxins possess higher antigenic and immunogenic properties. Their immunological effectiveness is several times higher than of the ordinary anatoxins. A serious shortcoming of the elaborated method has been the difficulty of creating the conditions which are required for getting "dialyzed" toxins in large volume for the purpose of wholesale production.

The native preparations, even those of considerable antigenic power, which are prepared on meat media of hardly definable and of changing chemical composition, are sufficiently effective in themselves(for instance, the native tetanus anatoxin). Yet, they do not satisfy the basic requirements that can be claimed for the components of the complex preparations, which requirements consists above all in high purity and high concentration so that in a small volume the highest possible amount of full-value antigens should be included.

In connection with the study of the indicated questions, the collective team of the Department of Wound Infections of the N.F.GAMALEI Institute of Epidemiology and Microbiology of the Medical Science Academy of the U.S.S.R., with the jointly combined work of the Biochemical Department(Chief:- V.A.BLAGOVESHCHENSKII) and the Department for the Preparation of Culture Media(Chief:- I.V.VINOGRADOVA) have subjected to elaboration and have studied the immunizing properties of the combined preparations for active immunization against tetanus, gas gangrene and staphylococcic infection.

We are now proceeding to the description of the investigations the purpose of which has been the comparative evaluation of the nutrient media used for the production of the toxins and anatoxins.

The organization of the scientific works in this direction has been entirely necessary since the ordinarily used culture media composed of expensive meat products, in addition to this factor of cost, are very inconvenient when it is the question to purify the produced toxins and anatoxins from the ballast substances. Moreover, the meat nutrient media are very heterogenous as regards their composition

and, owing to their complexity, they can be hardly standardized.

(p.10) -- For the production of the tetanus toxins, KASHINTSEVA, GIL'GUT and BULANOVA used the media of RAMON and GLUZMAN; for the production of the perfringens toxins, ZELEVINSKAYA, VOLKOVA and LARINA used a medium of the tryptic digestion of meat and the meat-fungal medium of ZELEVINSKAYA and VINOGRADOVA in which the amount of meat was considerably decreased and a fungal protease has been used as a ferment; for the obtention of the edematiens toxins, VLASOVA used a medium of the peptic digestion of meat.

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By using for the production of toxins such media which had a caseine hydrolysate for their basic substance the following results have been obtained:

When the toxicity of the filtrates of the tetanus bacillus cultures which can be produced on Ramon's and Gluzman's media are compared with the toxicity produced at seeding the very same strain(KOLLE No.8) on bouillon of a caseine hydrolysate, then one may become convinced of the advantage of the latter medium.

The average titre of the produced toxins reached 3,000,000 in one ml of the filtrate of the culture, exceeding six times the titres of the toxins which can be produced on Ramon's medium, and three times the titres of the toxins produced on Gluzman's medium. The maximum formation of the toxin on the meatless media is after 6-7 days of incubation at 36°C, i.e., somewhat later than on the meat media on which the peak of toxin production had frequently occurred already after 3 to 5 days of cultivation. It is possible that this can be explained with a retardation of the commencement of the growth(in comparison with the cultures on the meat media).

For the production of the perfringens toxins we delayed with two variants of the meatless media: 1) fish-casein hydrolysate, and 2) caseine hydrolysate.

The titres of the toxins obtained on these media, in case of seeding upon them the strains BR_6K, were 2.5 times higher than those obtained on POUP's media, and 1.5 times higher than on the meat-fungal media. With the cultivation on caseine hydrolysates, the growth of the culture is extremely intensive, and it is accompanied by an abundant formation of gas.

The same regularities were noticed at the production of the edematiens toxins. On the caseine media, in case of the seeding of the No.794 Strain, the toxins were six times stronger than those toxins obtained on a medium of the peptic digest of meat, and three times stronger than on the fish hydrolysate. Edematiens toxins of

high power were also produced on media which did not contain corn extract. However, in such a case, the multiplication of the culture developed very slowly; sometimes, visible growth was noticed 2-3 days after the seeding only. At the addition of corn extract to the medium, the intensity of the growth was not less than on the meat media.

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(p.11) The neutralization of the toxin-stimulators of gas gangrene and of tetanus produced on different nutrient media occurs quicker in case of the cultivation on caseine media. The neutralization of tetanus toxins produced on meatless media occurs in a considerably shorter period of time. For their full neutralization, with the addition of 0.4% formalin, a 15-day' stay in the thermostat at 37°C is sufficient instead of the 21-25 days required for the neutralization of toxins obtained on meat media.

The perfringens toxins, as well as the tetanus toxin, when prepared on meatless media, are rather quickly changed into anatoxins, particularly when media of the fungal protease ferment are used for their preparation. While for the full neutralization of the toxins which are obtained on pancreatic meat media and on meatless media, after the addition of formalin, it is necessary that they should stay in the thermostate at 37°C for a period of 21 days, then for the neutralization of the toxins which are produced on caseine-fungal media nine days are sufficient.

The quick change into anatoxins is of considerable advantage since under these conditions, of course, the produced anatoxin is less liable to denaturation.

The harmlessness of the anatoxins as tested on guinea pigs, and thereafter the anatoxins were subjected to purification and to concentration. The purification was carried out according to the method worked out by V.A.BLAGOVESHCHENSKII and by his coworkers, namely by the precipitation with HCl at the isoelectric point, after a preliminary salting out with a 20-25% solution of common salt. The further purification of the concentrated tetanus anatoxins was achieved by adsorption to aluminium hydroxide, with the subsequent elution of the anatoxins. The application of this method allowed the production of highly purified tetanus anatoxins containing 20,000 - 50,000 binding units(ES) per 1 mg of protein nitrogen. Later on, it has been proved that the method of precipitation with acetone in the cold, with strict control of the pH and of the ionic potency, a highly purified perfringens, edematiens and tetanus anatoxin was successfully produced, completely pigmentless and of

a high level of binding units(ES) per 1 mg of protein nitrogen.

The antigenic properties of the native and of the concentrated anatoxins were determined in experiments in vivo on mice for the tetanus and for the edematiens toxins, and, in a reaction with the emulsion of egg yolk, in experiments in vitro for the perfringens anatoxins.

The anatoxins received from toxins which were produced on meat media contained considerably smaller amounts of binding units(ES) than the anatoxins received from toxins produced on meatless media. As an average, the tetanus native anatoxins which were prepared (p.12) on meat media, contained 33-50 binding units, the native anatoxins on meatless media -- 100-300 binding units, the purified concentrated tetanus anatoxins -- 2000 - 5000 binding units. The edematiens anatoxins which were produced on meat media contained 4 binding units, on meatless media -- 15 - 30 binding units, the concentrated ones -- 400 - 800 binding units. The perfringens anatoxins on meat media contained 2 binding units , on meatless media -- 2 - 4 binding units, the concentrated ones -- 60 - 100 binding units.

The sterile anatoxins, containing 60-80 binding units of perfringens, 400 - 800 binding units of edematiens, 2000 - 5000 binding units of tetanus were sorbed to aluminium hydroxide.

From the purified concentrated and aluminium-hydroxide-sorbed anatoxins , the trianatoxin was prepared by means of their combination at definite ratios so that one ml should contain 25 binding units of perfringens, 4 binding units of edematiens, 200 binding units of tetanus. The Al_2O_3 content of the ready preparation was equal to 5 mg %.

As a preservative, 0.25% phenol or merthiolate(1:10,000) has been added to the trianatoxin. The trianatoxin , after being poured into vials, was checked for sterility and for harmlessness by means of injecting it into two guinea pigs(each receiving 5 ml subcutaneously).

The immunizing properties of the trianatoxin were investigated on a great number of laboratory animals(small animals):- guinea pigs , mice and rabbits.

The guinea pigs, both those who were immunized with a single shot of 3 ml trianatoxin and a double shot(two shots) each time with 1 ml of trianatoxin, proved to be resistant to 1 and 2 M.L.D.(minimum lethal dose; in Russian :Dlm)of perfringens toxin. Five of the control pigs which received 1 M.L.D. of toxin died in 24-48 hours. The guinea pigs which were twice inoculated with 1 ml of trianatoxin at an

interval of 20 days and, 30 days after the second injection were intramuscularly infected with 1-2 and 5 M.L.D. of the B.perfringens culture in a 50% solution of $CaCl_2$, proved to be completely protected from 1 and 2 M.L.D. of the STAT culture; from 15 pigs which were infected with 5 M.L.D. of the culture ten remained alive, five died in 48-72 hours under the symptoms of gas gangrene; five control pigs which were infected with 1 M.L.D. of the culture died in 24- 48 hours.

All guinea pigs which were immunized either with a single shot of 3 ml of trianatoxin or with two shots of 1 ml trianatoxin proved to be resistant to 50 and 100 M.L.D. of the edematiens toxin.

The guinea pigs which were immunized with 1 ml of the trianatoxin with two successive shots proved to be resistant to 1 and 5 M.L.D. of the B.edematiens culture. The control pigs died in 24-48 hours.

With the research of immunity at the inoculation of the tetanus toxin, the guinea pigs which were immunized with a single 3 ml injection (p.13) of trianatoxin or with two shots of 1 ml of trianatoxin proved to be resistant to the inoculation of 500 to 10,000 M.L.D. of the tetanus toxin.

In this manner, the immunization of the guinea pigs with trianatoxin proved to be effective in regard to all three of its components.

The immunization of white mice with trianatoxin which was sorbed to aluminium hydrozide showed its high immunogenic properties. The white mice which were inoculated with a single shot of the sorbed trianatoxin in the amount of 0.5 ml and were examined 30 days later for their resistance to the perfringens toxin(intravenously), to the edematiens toxin(intramuscularly) and to the tetanus toxin(subcutaneously) were resistant at the rate of the following: -to 10 M.L.D. of perfringens toxin, to 1 M.L.D of edematiens toxin, and to 10 M.L.D. of tetanus toxin.

The white mice which were inoculated with two shots of trianatoxin at an interval of 20 days between the injections, each injection being 0.5 ml, proved to be resistant to the intravenous introduction of 5 - 10 - 20 - 50 M.L.D. of the perfringens toxin. All mice which received 5 - 10 - 20 M.L.D. remained healthy. Out of 5 mice which received 50 M.L.D., 2 died and 3 remained healthy; all the 5 mice which were given 100 M.L.D. died. Positive results were also reached in respect to the creation of an immunity against an infection with cultures of the corresponding agents. The white mice which were given two shots of 0.5 ml of sorbed trianatoxin

at an interval of 20 days, one month after the last injections were found to be resistant to an infection with 1 - 5 M.L.D. of the B. perfringens culture, to 1 - 5 M.L.D. of the B. edematiens culture, and to 5 M.L.D. of the culture of the tetanus bacillus.

For the determination of the optimum dosages of the trianatoxin and of the most advantageous intervals between the injections, which will contribute to the peak accumulation of the antitoxins in the blood of the inoculated animals, a few groups of rabbits were also immunized.

The results of the observation about the dynamics of the antitoxin formation in the rabbits, which were immunized with two shots of trianatoxin and then revaccinated, have proved that, twenty days after the injection of 1 ml or 0.5 ml of trianatoxin, formation of antitoxin takes place against all the three components of the trianatoxin. Five days after the first injection, the antitoxins cannot be detected even in a minimum amount. Fifteen days after the second injection, the average titre of the perfringens antitoxin in the blood serum of the rabbits is considerably raised, and it even reaches 2.5 A.U. (antitoxic units; Russian: AE) in one ml; the titre of the tetanus antitoxin was equal to 4 antitoxic units, and the titre of the edematiens antitoxin reached 8 A.U.

Fortyfive days after the second injection, a reduction of the antitoxin level was noticed against perfringens and against edematiens (p. 14) and a slightly smaller drop in the titre of the tetanus antitoxin.

The obtained data indicate that the second injection at the initial immunization has a great importance in regard to the increase of the antitoxin level in the blood of the inoculated animals. It could be also successfully established that 1) the reduction of the dose of the antigen to its half also decreased the titre of the antitoxins in the blood of the inoculated animals, especially against perfringens as the weakest component which enters into the composition of the trianatoxin; 2) a more favorable interval between the injections seemed to be the interval of 20 days, in contrast to the five-day interval.

The revaccination of the rabbits which have been immunized with trianatoxin was done six months after the initial immunization. All together 50 rabbits were subjected to revaccination. Before the injection, blood was taken from all rabbits, and in a mixture of the sera the titres of the antitoxins were determined before the revaccination.

The average titre of the perfringens antitoxin equalled 0.15 A.U. in 1 ml, the titre of the edematiens antitoxin was 0.75 A.U., that of the antitetanus antitoxin was 0.15 A.U. in one ml. starting from the data in the literature which show that the revaccination--which can be done with non-sorbed antigens-- is accompanied by a higher formation of antitoxin, for the revaccination of this series the rabbits were separated into two portions. One group was inoculated with one ml of non-sorbed trianatoxin, and the other group-- with one ml of sorbed anatoxin. After the revaccination, blood was taken from the rabbits at the 3rd, 7th and 15th days and 1, 2 and 6 months after the revaccination.

The conclusion can be made that, three days after the revaccination with non-sorbed trianatoxin, the antitoxin level in the blood of the revaccinated rabbits was almost twice as high as in the rabbits inoculated with the sorbed antigen; 7 to 15 days after the vaccination, the antitoxin level was at its peak, and it remained at an identical level in both groups; after a month, a reduction followed in both groups. After two months, in the rabbits revaccinated with non-sorbed antigen, the perfringens antitoxin titre was twise as low as in those rabbits which were revaccinated with sorbed antigen; after 6 months, the titres of the perfringens, edematiens and tetanus antitoxins were twice as low in the animals revaccinated with sorbed antigen as in those animals revaccinated with non-sorbed antigen. The results of the study of the immunizing properties of the trianatoxin in the animals allowed us, and also other investigators, to study the reactogenicity and the antigenic properties of this prevaration on volunteer persons. The initial immunization was done with two shots of trianatoxin, each of 1 ml amount, and containing 25 binding units of perfringens, 40 binding units of edematiens and 200 binding units of tetanus .

(p.15) The results of vaccination and revaccination of the human volunteers with the purified sorbed trianatoxin proved that the reactogenicity of this preparation is but slight; serious local and general reactions were absent; moderate and slight reactions did not exceed those which have been noticed in persons inoculated with other bacteriological prevarations. The immunization of trianatoxin is accompanied by the formation of the corresponding antitoxins in the blood of the inoculated individuals. The perfringens antitoxin is detected in 92% of the inoculated persons 15 days after the inoculations, with amounts from 0.05 to 1 A.U.; the edematiens

antitoxin(from 0.05 to 1 A.U.) in 78% of persons; the tetanus antitoxin(from 0.25 to 1 A.U. or higher) in 100% of the inoculated individuals.

Sixty days after the inoculation, at a titre of from 0.05 to 1.0 A.U., the perfringens antitoxin has been detected in 78% of the inoculated persons; the edematiens antitoxin(from 0.05 to 1 A.U.) in 98%, and the tetanus antitoxin(from 0.5 to 1 A.U.) in 98%. (sic!).

After the revaccination, which is done 6 months later with 1 ml of trianatoxin, the perfringens, edematiens and tetanus antitoxins were found in all investigated sera:- the perfringens antitoxin from 0.1 to 3 A.U. in 1 ml; the edematiens, from 0.5 to 10 A.U. in 1 ml; and the tetanus antitoxin, from 0.5 to 20 A.U. in 1 ml.

In this way, it was proved:- the revaccination which was done after 6 months has considerably increased the titre of the antitoxins. It can be assumed that the antitoxin content in 1 ml of the blood serum is sufficient in the majority of cases to protect the individual from infection with tetanus or with gas gangrene.

As a result of the researches of ZELEVINSKAYA, VOLKOVA, GIL'GUT, LARINA, VLASOVA, KASHINTSEVA and BLAGOVESHCHENSKII, from the purified concentrated anatoxins of the perfringens, edematiens and tetanus toxins, preparations of trianatoxin sorbed to aluminium hydroxide have been made which possess high antigenic and immunogenic properties.

The facts which were established as the result of the mentioned investigations about the absence of an immunological competition between the components that enter into the composition of the trianatoxin, and the good immunological effectiveness of the combined preparations and their transmissibility in the immunization experiments permitted that we, after using and perfecting the elaborated methods, suggest a preparation for the active immunization against tetanus and against the agents of gas gangrene(B. perfringens, edematiens, Vibrio septicus)--this is the tetranatoxin (tetra-anatoxin). The dynamics of the antitoxin formation were studied in rabbits immunized and revaccinated with sorbed tetra-anatoxin.

(p.16) Rabbits were immunized with one shot of 1 ml of tetra-anatoxin and with two shots of the same amount given at an interval of 20 days. This amount of 1 ml contained 25 binding units of perfringens anatoxin, and 15 binding units of Vibrio septicus anatoxin, 40 binding units of edematiens anatoxin and 200 binding units of tetanus anatoxin. Revaccination was made after 6 months.

Twenty days after the first injection, the perfringens antitoxin reached a titre of 0.25 A.U., the septicus antitoxin reached 0.5 A.U., the tetanus antitoxin itre reached 2.0 A.U., the [STAT] edematiens antitoxin titre reached 3.0 A.U. On the 15th day after the second injection, the individual titres of the antitoxins rose very similarly to that which we had already observed in the examination of the dynamic function of antitoxin formation after the immunization with the trianatoxin. At the 45th day, the titre of all antitoxins was reduced, and it continued to drop until the time of revaccination which was done 6 months later. Between the 3rd and the 7th days after the revaccination, a sharp increase was discovered in the titre of all antitoxins; the smallest elevation of the titre was observed in the B. septicus amtitoxin; the titre of the perfringens antitoxin increased considerably, and the titres of the edematiens and tetanus antitoxins increased very sharply. It should be also underlined that the revaccination showed an identical influence upon the elevation of the antitoxic titres(edematiens, tetanus and perfringens) in both groups of the rabbits whether immunized with one shot or with two shots.

We have investigated the immunogenicity of the tetra-anatoxin on guinea pigs and on white mice. We immunized 63 guinea pigs with a single shot of 3 ml of tetra-anatoxin, and we examined them as to the strength of immunity 30 days after the immunization, by means of toxins and cultures of the perfringens, edematiens, septic vibrio and tetanus bacilli.

It was proved that all pigs inoculated in the experiment tolerated 1 M.L.D. of the perfringens toxin. Pigs receiving 5 M.L.D. of the perfringens toxin died as well as the controls; guinea pigs proved to be resistant to 50 M.L.D. of the edematiens toxin and to 1 M.L.D. of the Vibrio septicus toxin.

Guinea pigs examined in regard to resistance to the tetanus toxin tolerated from 500 to 100,000 M.L.D. of the toxin, with the death of the controls at 1 M.L.D. of the toxin.

Forty guinea pigs immunized with the tetra-anatoxin were grouped in 4 groups, and examined 30 days after the immunization concerning their resistance to 1 M.L.D. of the corresponding cultures. All pigs remained alive, while all the controls died.

After the immunization of white mice with a dose of 0.5 ml of tetra-anatoxin which dose contained 12.5 binding units of perfringens anatoxin, 20 binding units of edematiens anatoxin, 7.5 bidning units of Vibrio septicus anatoxin, and 100 binding units of tetanus anatoxin, the mice were examined as to their resistance to

various amounts of minimum lethal doses of the appropriate toxins. The mice showed resistance to 5 M.L.D. (p.17) of the perfringens toxin, to 2 M.L.D. STAT the edematiens toxin, to 2 M.L.D. of the vibrio septicus toxin, and to 50 M.L.D. of the tetanus toxin. At the examination of white mice which were immunized with two shots (at 20-day interval), it was found that the mice were resistant to 20 M.L.D. of the perfringens toxin, to 10 M.L.D. of the edematiens toxin, to 10 M.L.D., of the Vibrio septicu toxin, and to 100 M.L.D. lf the tetanus toxin.

At the investigation of the white mice immunized under the same conditions, the following was discovered in regard to their resistance to from 1 to 5 M.L.D. of the corresponding cultures. After a single shot immunization, the mice showed resistance to 5 M.L.D. of the perfringens culture, to 1 M.L.D. of the edematiens culture, to 1 M.L.D. of the septicus culture, and to 1 M.L.D. of the culture of the tetanus bacillus. After two-shot immunizations, at 20 days' interval, the mice were resistant to 50 M.L.D. of all the above mentioned cultures. The control mice died, from 1 M L.D. of the culture.

In this manner, the immunization of the guinea pigs and of the white mice with the tetra-anatoxin has created in them an immunity of sufficient power both against the corresponding toxin and against the corresponding culture.

In a study of the preparation on volunteers, the transmissibility (tolerability) of the tetra-anatoxin proved to be the same as that of the trianatoxin. The immunological researches of the blood sera of the vaccinated persons showed that the dynamics of the antitoxin formation were the same as at the immunization with the trianatoxin in respect to the components of perfringens, edematiens and tetanus anatoxins. The level of the titres of the antitoxin of the Vibrio septicus was the same as that of the perfringens antitoxin.

It must be said that we were unable to observe any immunological competition between the antigens under these conditions.

The results obtained with the tetraanatoxin gave us a possibility to study also another combined preparation--- the purified sorbed penta-anatoxin in the composition of which we find the anatoxins of perfringens, edematiens, tetanus(in the same dosage as for the trianatoxin)as well as Types A and B of the botulinus anatoxinx.

For the study of the dynamics of the antitoxin formation, the penta-anatoxins were introduced subcutaneously in two shots at 20 days between the injections.

As a result of this research it has been established that the dynamism of antitoxin formation in regard to the components perfringens, edematiens and tetanus was the same as we have earlier observed at the immunization with the tri- and tetra-anatoxins; however, in regard to the botulinus components the titres of the antitoxins were small, which was evidently in direct connection with the insufficient dosage of the botulinus antigens included by us in the composition of the penta-anatoxin.

(p.18) For the elucidation of the question whether here we were dealing with an immunological competition between antigens or our dosage of the botulinus component was insufficient, we arranged an experiment under other conditions when, with the maintenance of the earlier dosage of the perfringens, edematiens and tetanus anatoxins as components of the penta-anatoxin, the amounts of the botulinus A and B anatoxins were raised up to 200 binding units for an injection.

The following results were obtained. Twenty days after the first injection of the penta-anatoxin, the blood serum of the rabbits contained 3 A.U. of the antitoxins of tetanus and edematiens as an average; the titre of the perfringens anti-toxin reached 0.25 A.U., the same were also the titres of the botulinus A and B antitoxins. Fifteen days after the second injection, the titres of the antitoxins of tetanus and edematiens were up to 10 A.U.; the titre of he botulinus B antitoxin reached 5 A.U., the titre of the botulinus A reached 4 A.U., and the titre of the perfringens was 1 A.U. In this way, it could be successfully shown that, with sufficient dosage(of the antigen) the introduction of the botulinus A and B antitoxins into the composition of the penta-anatoxin assures a considerable increase in the titres of the botulinus antitoxins and it does not oppress the formation of the other antitoxins called for by the other components of the penta-anatoxin.

The obtained data permitted for us to develop the researches for the study of the immunogenicity of the tri- and penta-anatoxins in combined preparations with the polyantigen of the Gamalei Institute for Epidemiology and Microbiology which contains antigens of the microbes of the group of intestinal infections.

CONCLUSIONS:

1) The utilization of less complex(non-meat) nutrient media permits to obtain toxins of sufficient power which are capable to be quickly neutralized and to change into anatoxins.

2) The worked out methods of purification and concentration of the anatoxins permitted the production of preparations of high degree of purity which contain STAT sufficient immunogenicity in a small amount.

3) The separate components in the combined preventive preparations(with the dosages suggested by us) do not show the phenomenon of immunological competition in the compositions of the tri-, tetra- and penta-anatoxins.

4) The further trends of the investigations are:

a) study of other polyvalent preparations for active immunization;

b) reinforcement of the separate components in the combined preparations against wound infections(B.perfringens and B.septicus).

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(p.19)

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CELLULAR REACTION AND REPRODUCTION OF RIBONUCLEIC ACID IN THE LYMPHATIC NODES IN CASE OF IMMUNIZATION WITH COMBINED PREPARATIONS AGAINST INFECTION (p.19-28)

(p.19) The study of the reaction of the regional lymph nodes to the subcutaneous injection of the new combined preventive preparations against wound infection, the reaction to the trianatoxin and to the penta-anatoxin which were worked out in the N.F.GAMALEI Institute, is of interest from both a practical and a theoretical point of view.

The illumination of this question has an important meaning for the judgment on the reactogenicity of the preparation and for the study of the processes which occur in the lymphatic nodules at the time of immunogenesis.

By the investigations of VYGODCHIKOV, VOLKOVA, ZELEVINSKAYA, KASVINTSEVA, VLASOCA and GIL'GUT(1957) the high immunogenic properties of the trianatoxin and of the tetraanatoxin have been proved, and their practical lack of reactogenicity was shown in experiments on animals and at immunization of volunteers. The obtained results allowed the use of the trianatoxin for the immunization of people with positive findings(PONOMAREV, BRYZGALOVA, 1956; KONDRAT'EV, 1956).

From the large number of published works, it is known that the formation of the antibodies(antitoxins) happens basically in the lymphatic nodes which are regional to the site of the injection of the antigens, and in the spleen. McMASTER and HUDACK(1935), and thereafter EHRICH and HARRIS(1942) have already established that the injection of an antigen will provoke the formation of agglutinins in the regional lymph nodes.

Later on, many investigators corroborated these findings by utilizing various methods; their confirmations were direct or indirect(cf. the review articles: FORSHTER, 1955; PARNES, 1957; UCHITEL', 1957; EHRICH, 1955, and others). In recently published works(GINDIN and FORSHTER, 1958; (p.20) STENDER, STRAUCH & WINTER, 1958) new data were brought forward about the formation of agglutinins in the lymph nodes.

A work which is devoted to the study of the reaction in the regional lymph nodes after the introduction of polyanatoxins against wound infections does not exist in the literature. The present investigation is devoted to this problem.

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Materials and methods

For the immunization of the animals two combined preparations against wound infection have been used:- the trianatoxin, composed of the anatoxins of tetanus, perfringens and edematiens, and the tetra-anatoxin which is composed of the trianatoxin and the anatoxins of botulinus Type A and Type B. In one ml of trianatoxin there are 25 binding units of perfringens anatoxin, 40 binding units of edematiens anatoxin, and 100 binding units of tetanus anatoxin. One ml of penta-anatoxin contains 25 binding units of perfringens, 40 binding units(B.U.) of edematiens, 100 B.U. of tetanus, and 100 B.U. of botulinus A and B anatoxins.

The immunization was carried out subcutaneously into the right lateral surface of the trunk of the rabbits.

In the experiment of immunization, 42 rabbits were used, each weighing about 2.5 to 3 Kg. The animals were divided into six groups. The rabbits of Group I obtained a single subcutaneous shot of 1 ml of pentaanatoxin. The rabbits of Group II received correspondingly a single injection of 1 ml of trianatoxin. The rabbits of Group III served as controls, and they received a subcutaneous shot of 1 ml of standard aluminium hydroxide. For the purpose of studying the nature of the reaction to a second immunization--which, as well known, gives reinforcement to the formation of the antitoxin--, the remaining three groups received correspondingly the same preparations but in two shots, keeping a one-month interval between the injections.

On the 2nd, 5th and 10th day, and after 3 and 4 weeks, a rabbit of each group was killed by means of air embolism, and after 5 weeks, two rabbits were killed in each group.

The lymph nodes regional to the site of the injection(the right axillary nodes and the right inguinal nodes) and the homolateral controls to these nodes(left axillary and left inguinal nodes) were extracted and fixed in alcohol, or in a mixture of alcohol/formalin, and they were treated with the usual histological methods, and, for the detection of ribonucleic acid(RNA) they were treated according to the method of BRASCHE.

Own investigations

Own investigations

Two days after the single shot of the immunizing preparations, a distinctly manifest reactive process is observable in the regional lymph nodes. The lymphatic (p.21) glands are enlarged, slightly congested and edematous. During the following three days, these processes continued in development:- the lymph nodes became enlarged still more, and their medullary substance became swollen. At the tenth day, this process has become so strong that the size of the lymph nodes was almost twice larger than the control nodes. At this time, the hyperemia was reduced.

During the three following weeks, the dimensions of the regional lymph nodes gradually diminished, but they did not return to their normal condition, not even 35 days after the injection(period of the observation time).

FIG.1: LYMPH NODE: Small lumps of ribonucleic acid(on the photograph in black) in the cytoplasm and in the nucleoli of the reticulum cells(indicated with arrows). Methyl green-pyronin staining.

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In the control animals which were given aluminium hydroxide only the development of the mentioned processes was delayed, and it did not reach such an intensity as in the animals subject to experimentation.

By the histological and histochemical examinations, it was discovered that already two days after the immunizing injection, parallel with the hyperemia and the dilatation of the sinuses, a marked proliferation of the reticulum cells occurred in the parenchymal cords, and they were transformed into large(coarse) little differentiated cells with large nuclei, with poor chromatin, and with a contracted thin (narrow) belt of the cytoplasm, saturated with ribonucleic acid, and with nucleoli rich in ribonucleic acid.

(p.22) These cells were similar to the hemocytoblasts or plasmoblasts. The reactive centers of the secondary nodules were still small at this time, and their lymphoblasts and reticulum cells were rich in ribonucleic acid.

During the following three days, all these processes became strengthened:- in the parenchymal cords the number of hemocytoblasts and of plasmoblasts increased, and the ripening of many of them into lymphoblastic and plasma cells was noticed. At this time(on the 5th day after the immunization) the size of the preexisting follicles sharply increased, and a large number of new folliculi appeared. In them, intensive proliferation of the lymphoblasts and of the reticulum cells occurred,

which is accompanied by, or which conditions, a heavy reproduction of ribonucleic acid in their cytoplasms and nuclei. The number of the small lymphocytes becomes less on this account, and the folliculi are transformed into very large reactive centers.

On the 10th day after the immunization, further enrichment of the parenchymal cords occurred with lymphoblasts, plasma cells, and small lymphocytes rich in ribonucleic acid. The reactive centers remained strongly enlarged as before.

FIG. 2: LYMPH NODE: plasmoblasts with cytoplasm and nucleoli rich in ribonucleic acid (indicated by the double-contour arrows), and plasma cells (indicated by arrows). The ribonucleic acid is intensively black on the photograph. Staining with methyl-green-pyronin.

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(p. 23) Between the 10th and the 24th days after the immunization, the proliferation of the lymphoblasts and plasmoblasts in the parenchymal cords was less marked. In these cords, the number of plasma cells has somewhat increased, and the number of small lymphocytes has sharply increased, with a narrow thin belt of the cytoplasm, containing more or less ribonucleic acid. At the end of the observational period (after 35 days), reduction of the size of the reactive centers occurred, but their cells remained rich in ribonucleic acid as before. The entire lymph node remained hyperplastic chiefly due to the small lymphocytes with their cytoplasm rich in ribonucleic acid, and due to the reactive centers.

FIG. 3: (No legend).

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In the control rabbits which received only aluminium hydroxide the above described changes were at first manifested very little, and they became more noticeable on the 5th and the 10th day of immunization, but even at that time they did not reach such a size as they had reached in the rabbits which were given immunizing preparations, on the 2nd and the 5th day after the injection.

The second immunization (revaccination) which was performed one month after the first has again provoked an intensive reproduction of the ribonucleic acid, a pro-
(p. 24) liferation of the lymphoblasts and of the plasmoblasts in the at this time still hyperplastic tissue of the lymph node. Therefore, on the second day after the re-immunization, the lymph node in its size was roughly the same as it had been on the 5th day and later after the initial immunization. One the 10th to 20th days, a slight weakening was noticed in the above indicated process.

The second injection of the aluminium hydroxide, as the first one also, provoked a by far weaker process in the lymph nodes. However, this process was also manifested in the reproduction of the ribonucleic acid(in a small number of cells), in the formation of a small number of plasmoblasts and of plasma cells, and in a slight increase in the size of only a few separate reactive centers.

From the described observations, it is evident that in the regional lymph nodes a single-shot immunization with the penta-anatoxin or the trianatoxin has provoked a proliferative process which was accompanied(or which conditioned) by an intensive synthesis of the ribonucleic acid in the cytoplasm and in the nucleoli of the lymphoid and of the reticulum cells, and by its further maturation to lymphocytes and to plasma cells.

The second immunizing shot after 30 days has provoked a similar process which was however diffuse over a large area since at the time of the second immunization the lymph node itself was still considerably enlarged and its cells, including even the small lymphocytes, were enriched with ribonucleic acid.

The obtained data permit to raise the question:- what is the significance of the indicated processes in the genesis of immunity?

In recent times it has been established that the synthesis of the protein substances which are required for the growth and for the division of the cells as well as the secretion of the protein substances by the cells are achieved with the participation of ribonucleic acid. For the synthesis of the proteins a preliminary multiplication of the ribonucleic acid is required(BELOZERSKII, 1948; BRACHET, 1942, 1950, 1955; CASPERSON, 1941; LEVINSON & PAVLOVA, 1949; BRACHET, 1942, 1950, 1955; ROSKIN, 1951; MAKAROV, 1956, and others).

From the data quoted in this work it is evident that the introduction of the polyanatoxins has produced an intensive reproduction of the ribonucleic acid in the cells of the lymph nodes. EHRICH, DRASKIN and FORMAN(1949) as well as HARRIS and HARRIS(1949), by using biochemical and histochemical methods, have established that, after the immunization with different antigens, the amount of the ribonucleic acid will increase in the regional lymph nodes.

Our researches have indicated that the immunization with pentaanatoxin and with trianatoxin, side by side with the reproduction of ribonucleic acid, also provoked a hyperplastic process, with the new formation of lymphoblasts and of plas-

moblasts, with their subsequent maturation into lymphocytes and plasma cells. (p.25) - The electron microscopic researches(BRAUNSTEINER & PAKESCH,1955), the researches with the phase-contrast microscope(MOESCHLIN,1951), and the researches with fluorescent antibodies(COONS,LEDUC & KONNOLY,1957) indicated that the plasma cells, particularly their young forms(FAHRAEUS,1948) are producing the antibodies. The comparison of these data with the results of our investigations permits the conclusion that the ribonucleic acid which was newly formed after the immunization took part in at least two processes:- in the hyperplastic process(multiplication of the cellular elements of the lymph nodes) and in the synthesis of the antitoxins. GINDIN and FORSHTER(1958) drew a similar conclusion on the basis of their study of another model- the formation of agglutinins in mice by the soluble antigens derived from cultures of the Breslau bacillus.

The titration of the blood serum of the immunized rabbits showed that in 2-5 days after a single-shot immunization, antitoxins could not be detected in the peripheral blood; the histochemical research has also showed that by this time the synthesis of ribonucleic acid was already going on at an energetic manner. In other researches, with another antigen(diphtheria anatoxin) and on other objects of experimentation(on horses), it was shown that, after the second immunization(revaccination), the process of intensive synthesis of the ribonucleic acid could be seen not only in the lymphoid organs but also in the lymphocytes of the peripheral blood to such a degree as the antitoxin titre was being increased in the blood(GINDIN & OGIENKO,1959).

In the present investigation, it was established that the antitoxins appeared roughly on the tenth day in titrable amounts in the blood; that they continued to increase, and they reached their peak on the 24th to 30th days.

Five days after the second immunization(revaccination) the titre of the antitoxins has grown several times (see Tables 1 and 2).

The comparison of the morphological and histochemical findings with the immunological data proves that on the day of the second immunization the regional nodes were still hyperplastic and rich in cells which were saturated with ribonucleic acid. The second immunizing shot leads to the synthesis of ribonucleic acid at a larger scale, to the reinforcement of the hyperplastic process and, evidently as a sequela to this, to a more intensified formation of the antitoxins. It can be

surmised that this also includes the cause of the increase of immunogenesis after the second shot of immunization.

The introduction of pure aluminium hydroxide into the control STAT animals provoked a weak proliferative process and a weak multiplication of ribonucleic acid. It is possible that these processes were conditioned by the absorption of the products of cellular disintegration and of the products(p.26) (cont.on p.27 of original). . .

(p.26)

TABLE 1

ACCUMULATION OF ANTITOXIN IN THE BLOOD OF RABBITS AFTER IMMUNIZATION WITH PENTA-ANATOXIN.

Day of examination	Titres of antitoxin in A.U. perfringens	edematiens	tetanus	botulinus A	botulinus B
A. Single-shot immunization					
2	< 0.1	0.05	<0.001	<0.1	<0.1
5	<0.1	0.0	<0.001	<0.1	<0.1
10	<0.1	>0.1<1.05	>0.01<0.1	<0.1	<0.1
24	>0.1<6.25	>1.0< 2	1	<0.1	<0.1
30	>0.1<0.25	>1 < 2	<1<5	<0.1	<0.1
35	0.1	1	1	<0.1	<0.1
B. Two-shot immunization					
2	>0.25<0.5	2	>1<5	> 0.1<1	0.1
5	>0.5< 1.0	>2<5	3	>0.2<0.5	>0.1<0.25
15	1.0	>5<10	>3< 5	>0.5<1	>0.25<0.5
20	>1.0<2	>5<10	>3<5	>0.5<1	>0.25<0.5

(for Table 2 see next page)

(cont.p.26)

TABLE 2

ACCUMULATION OF ANTIBODIES IN THE BLOOD OF RABBITS AFTER IMMUNIZATION WITH TRIANATOXIN

Day of examination	Titres of antitoxin in A.U. perfringens	edematiens	tetanus
	A. Single-shot immunization		
2	< 0.1	< 0.5	> 0.001
5	< 0.1	< 0.5	> 0.001
10	= 0.1	> 0.5 A.U. < 1.0	> 0.01 < 0.1
24	> 0.1 < 0.25	> 1.0 < 2.0	1.0
30	> 0.1 < 0.25	> 2 < 5	> 1.0 < 5
35	0.1	2	1.0
	B. Two-shot immunization		
2	> 0.25 < 0.5	> 2 < 5	> 1 < 5
5	> 0.5 < 1	> 2 < 5	> 1 < 5
10	> 1.0 < 2	> 10 < 20	> 5 < 10
21	> 1.0 < 2	> 10 < 20	> 5 < 10

(p.27...cont.)..of denaturation of the proteins of the edema fluid from the focus of inflammation. This process requires further study.

As it is seen from the above introduced findings, in the lymph nodes no pathological processes were found--such as for instance suppuration, necrosis-- which could have served as an obstacle for the use of the preparation for human injections. The discovered hyperplastic process, which is gradually regressing, is inevitable in any immunization because it is connected with the immunogenesis itself as numerous researches have shown.

CONCLUSIONS

1) The subcutaneous injection of penta-anatoxin and of trianatoxin provokes in the regional lymph nodes an intensive multiplication of ribonucleic acid and a proliferation of the lymphoblastic and plasmoblastic elements, with their subsequent maturation. These processes are connected with the immunogenesis.

2) The maintenance of the indicated processes for a months(period of the time of observation) proves the prolonged immunizatory stimulation which arises from the

(p.27 cont.)

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site of inoculation of the antigen.

3) The second shot of immunization, 30 days after the first, provokes a similar but more diffuse process which apparently conditions a more intensive production of the antitoxins.

4) Pathological processes which could be taken as an objection to the human use of the indicated preparations had not turned up in our material.

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(p.29)

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INTERACTION OF ANTIGENS AT COMBINED IMMUNIZATION*(p.29-35)

(* First Communication: Study of the effectiveness of combined immunization with staphylococcus anatoxin and with perfringens anatoxin in experiments on rabbits).

At the study of the immunological effectiveness of a combined preparation which is composed of purified sorbed anatoxins, a weaker antigenic effect was revealed to us with the use of a combination of the staphylococcic and the tetanus anatoxins, and with the use of a combination of the anatoxins of perfringens and edematiens and staphylococcus than in case of immunizing with them separately(VOSKRESENSKII & LEBEDEVA, 1957).

At the investigation of the need for the inclusion of the staphylococcus anatoxin in the composition of the combined preparations, we set the task before us to find out which of the ingredients of the mentioned combined preparations show an oppressing, inhibitory effect upon the immunological effectiveness of the staphylococcic anatoxin so that this inhibitory phenomenon could be removed.

In the present work we studied the effectiveness of the immunization with the staphylococcus anatoxin in a mixture with the perfringens anatoxin. The staphylococcic anatoxin was obtained from the toxin of Staphylococcus Vud 46. The stock was cultivated in bouillon of the acid hydrolysate of caseine with yeast extract. The toxin was neutralized by the addition of a 0.4% formalin, and by keeping it at 37°C for ten days.

The purified concentrated anatoxin of the perfringens(Stock No.28, series No. 649), containing 50 binding units in one ml, was produced by the Gas Gangrene Laboratory of our Department(S.A.ZELEVINSKAYA).

The purified concentrated staphylococcus anatoxin contained 80 binding units in one ml. The precipitation was made with a 1/N hydrochloric acid at the isoelectric point. The preparations were separately sorbed to aluminium hydroxide.

(p.30) Before the immunization of the animals, the sorbed anatoxins were mixed in different proportions.

For the experiment, rabbits were taken which weighed an average of 2.5 to 3 Kg and possessed natural staphylococcic antitoxin of < 0.125 A.U.

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1. The immunization was done subcutaneously in two shots, at a 20-day interval between the injections. The doses of the preparation were the same at the first and at the second injection. The combined preparation was inoculated in an amount not exceeding 1.5 ml.

2. In the first experiment with the combined immunization, six groups of rabbits were taken, each group consisting of 5 animals.

3. Group I received , at each injection(1st and 2nd), 25 binding units of the staph. anatoxin plus 12.5 binding units(B.U.) of the perfringens anatoxin.

Group II anatoxins..25 B.U. of staph. and 25 B.U. of perfringens anatoxins

Group III " ..25 B.U. of staph. and 50 B.U. of " "

Group IV " ..50 B.U. " " & 12.5 B.U. of " "

Group V " ..50 B.U. " " & 25 B.U. of " "

Group VI " ..50 B.U. " " & 50 B.U. of " "

For the control, according to the same schedule, 3-3 rabbits were immunized with each of two doses of staph. anatoxin and each of 3 doses of perfringens anatoxin.

The content of the staph. antitoxin in the blood sera was determined by the method of neutralization of the hemolytic properties of the standard staph. toxin.

The revaccination was carried out 3 months after the second inoculation, with a single shot, with a dose identical for all groups of rabbits, i.e., with 25 B.U. of staph. anatoxin and 25 B.U. of the perfringens anatoxin.

The control rabbits obtained the same doses of the corresponding anatoxin.

The blood sera of the rabbits were examined before the immunization, then on the 18th day after the first injection, on the 5th, 10th, 20th and 30th days after the second injection of the antigen before the revaccination, and on the 5th, 10th 20th and 35th day after the revaccination. 43 days after the revaccination the rabbits were examined for resistance to the intracutaneous introduction of the staphylococcic toxin and of the staphylococcic culture(Stock 0-15).

In Table 1, the average arithmetic titres of the staph. antitoxin are shown in the blood sera of the rabbits at different periods of time after the immunization and revaccination with the combined(p.31) preparation containing 25 B.U. of the staph. anatoxin. As it can be seen from the data of this Table, the peak titres of

(p.31 cont.)
the staph. antitoxin were lower on the 5th to 10th days after the second inoculation, than in the control animals with all three doses of the perfringens anatoxin.

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This was especially remarkable after the revaccinations:- on the 10th day, in the rabbits revaccinated with the combined preparation, the titres of the staph. antitoxin were correspondingly 2.0 A.U., 1.0 A.U., 0.62 A.U., and in the control it was 7 A.U. Almost the same ratios were also shown on the 20th day (Table 1).

In this way, the dose of 25 B.U. of the staph. anatoxin, in combination with each of the three doses of perfringens anatoxin, caused a weak formation of the staphylococcic antitoxin in the blood of the immunized animals. This was observed both under the conditions of the initial immunization and under the conditions of the revaccination.

The resistance of these animals to the intracutaneous introduction of the staphyloccus toxin and of the cultures on the 43rd day after revaccination was also twice as low as in the control animals. The control rabbits have tolerated 20 skin-necrotic doses of the staph. toxin, and 8 to 16 skin-necrotic doses of the staph. culture No. 0-15, and the experimental rabbits tolerated 10 and less than 10 skin-necrotic doses of the toxin, and 8 and less than 4 skin-necrotic doses of the culture.

In the same Table, the average titres of the staph. antitoxin were also given which were obtained after an immunization with a dose of 50 B.U. of the staph. anatoxin in the composition of the combined preparation. The level of the staph. antitoxin after the first immunization with a dose of 50 B.U., in combination with the same three doses of the perfringens anatoxin, was almost identical with the control. On the 10th day of revaccination, in the two first groups of rabbits, the titres of the blood serum proved to be lower than in the control animals, and in the third group (Staph. anatoxin 50 B.U. plus perfringens anatoxin 50 B.U.), no differences were seen from the control.

In the subsequent experiment, higher doses of the staph. anatoxin were investigated:- 75 B.U., 100 B.U., and 150 B.U. The perfringens anatoxin was taken always in the same dose of 25 B.U. The methodology of the experiment was similar to the previous one.

The revaccination was made three months later with a preparation containing 100 B.U. of the staph. anatoxin, plus 25 B.U. of the perfringens anatoxin in the sorbed form in the inoculated dose.

(p.32)

TABLE 1.

DYNAMICS OF THE FORMATION OF STAPHYLOCOCCUS ANTITOXIN AT IMMUNIZATION WITH A MIXTURE OF PURIFIED SORBED ANATOXINS OF PERFRINGENS AND STAPHYLOCOCCUS

STAT

a	b	Time of examination of serum, in days: after 1st inoculat. 18 d.	after second inoculation: 5	10	20	30	90	after the revaccination: 5	10	20	35
a...purified sorbed staph. anatoxin, in binding units (B.U.)											
b...purified sorbed perfringens anatoxin, in binding units (B.U.)											
Titres of antitoxin given in A.U.											
25	-	0.25	3.0	2.6	0.8	0.5	0.41	3.3	7.0	7.0	1.0
25	12.5	0.22	2.2	1.4	0.37	0.56	0.25	1.1	2.0	1.0	0.58
25	25	0.16	0.65	0.41	0.16	0.2	0.21	0.75	1.0	0.83	0.25
25	50	0.18	6.78	1.5	0.54	0.37	0.41	1.25	0.62	0.75	0.25
50	-	0.29	3.3	2.0	0.5	0.5	0.25	6.0	5.0	2.5	0.75
50	12.5	0.42	4.4	2.0	0.77	0.45	1.55	3.5	2.0	1.0	1.0
50	25	0.22	3.4	1.8	0.65	0.35	0.45	1.3	2.0	1.0	0.33
50	50	0.44	3.4	1.75	0.46	0.45	0.33	3.0	4.7	2.75	0.87
75	-	-	10.3	5.5	1.4	1.5	0.29	5.3	8.3	3.1	3.0
75	25	-	4.0	3.0	1.0	3.0	0.37	3.0	6.0	2.0	1.5
100	-	-	12.6	8.6	3.0	2.0	0.4	6.6	11.3	4.6	4.0
100	25	-	6.0	3.6	1.3	1.0	0.25	5.0	6.3	3.6	3.3
150	-	-	9.3	6.6	1.6	2.3	0.3	5.0	9.3	4.0	4.0
150	25	-	5.3	4.3	1.0	1.3	0.29	2.0*	4.6	1.2	1.0

(* in original, erroneously written: 210)

As it can be seen from Table 1, the dose of 75 B.U. and 100 B.U. of the staph. anatoxin in the combined preparation which contained 25 B.U. of perfringens anatoxin has assured a high level of staph. anatoxin; yet, even with this, the titres were by ½ a ½ to 2 times lower than after the immunization with a mono-preparation. The dose of the staphylo. anatoxin raised to 150 B.U. had not resulted in a subsequent increase in the titres.

The dynamism of the formation of the perfringens antitoxin was determined by us by means of the lecitho-vitelline reaction at the same time as the staphylococcus antitoxin. (See Table 2)

(see next page).

(p.33)

TABLE 2

DYNAMICS OF THE FORMATION OF THE PERFRINGENS ANTITOXIN AT THE COMBINED IMMUNIZATION WITH STAPHYLOCOCCIC ANATOXIN.

ANATOXINS in B.U.		Antitoxin titres given in A.U. Days after 2nd inoculation				Before revac.	Days after revaccination			
perfringens	staphylo.	5	10	20	30	90	5	10	20	30
12.5 B.U.	-	3.6	0.28	0.33	0.1	0.17	4	5	5.25	2
12.5	25 B.U.	4.6	1.5	0.45	0.2	0.1	2.6	6.6	20	3.3
12.5	50	4.2	0.8	0.35	0.1	0.15	3.5	12	2.3	1.0
25 B.U.	-	4.0	1.5	0.75	0.2	0.15	2.3	15	5.3	3.0
25	25 BU.	4.6	3.3	0.66	0.4	0.2	6.6	16	13	7.0
25	50	4.8	1.4	0.45	0.18	0.33	4.0	13	16	2.6
25	100	-	-	0.3	0.2	-	-	16	-	-
50	-	3.3	1.0	0.33	0.1	0.25	3.0	28	6.0	2.0
50	25	4.2	1.5	0.5	0.23	0.17	1.0	5.5	4.5	1.25
50	50	4.3	2.6	0.41	0.06	0.25	3.3	18.6	14.6	1.5

(p.34 cont.)

above all it should be mentioned that the staph. anatoxin had not shown any inhibitory effect upon the perfringens antitoxin. After the revaccination with this, even some increase was noted in the titres and a more prolonged maintenance of the high level of the perfringens antitoxin almost in all groups of rabbits. However, we do not think it possible to talk of a stimulating influence of the staph. anatoxin upon the formation of the perfringens antitoxin, i.e., this question needs a special study.

The obtained results prove that, with the combined immunization, the perfringens anatoxin, in doses of 12.5 and 25 and 50 B.U., inhibits the immunogenic activity of the staph. anatoxin at all the examined doses of the latter in the mixture(from 25 B.U. to 150 B.U.). The researches have also proved that the dose 50, 75 and 100 B.U. of the staph. anatoxin in mixture with 25 and 50 B.U. of the perfringens anatoxin assures a sufficiently high level of the staph. antitoxin, although even in this case, its titres were by ½ to 2 times lower than in the control group of animals.

The findings which are presented in Table 2 indicate that the staph. anatoxin does not show any substantial influence upon the antigenic action of the perfringens anatoxin. STAT

It should be remarked that the increase in the dose of the staph. anatoxin in the range from 25 to 100 B.U. causes a parallel increase in the titres of the staph. antitoxins, at the same time when the dose of 150 B.U. did not produce any further increase in the titres. It can be surmised that this dose is already provoking some immunological overstimulation.

CONCLUSIONS

1) In case of immunization with a mixture of purified sorbed anatoxins of the perfringens and the staphylococcus, an inhibition of the antigenic activity of the latter has been observed.

2) In case of immunization with the staph. purified sorbed anatoxin (in doses from 25 to 150 B.U.) in a mixture with the same perfringens anatoxin (doses from 12.5 B.U. to 50 B.U.), no unfavorable influence is observed by the staph. anatoxin upon the antigenic effect of the perfringens anatoxin.

(p.35) 3) In case of an immunization with a mixture of purified, sorbed anatoxins of the perfringens and the staphylococcus, a satisfactory immunological effect is achieved, in respect to the formation of the staph. antitoxin, if the doses of each component are 50 B.U. However, better results are reached with the combination of 75 to 100 B.U. of the staph. anatoxin with 25 B.U. of the perfringens anatoxin.

(No literature quoted)

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(p.37)

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INTERACTION OF ANTIGENS AT COMBINED IMMUNIZATION. (p.37 - 41)

(SECOND COMMUNICATION: Effectiveness of the combined immunization with a mixture of purified sorbed staphylococcic and edematiens anatoxins in experiments on rabbits).

In the previous work(VOSKRESENSKII & LEBEDEVA) it was proved that, in case of immunization of the rabbits with a mixture of purified concentrated adsorbed anatoxins of the perfringens and the staphylococcus, an inhibition of the antigenic action of the latter can be observed.

In the present work, the interaction of the antigens was studied in case of the immunization with a mixture of purified concentrated sorbed staphylococcic and edematiens anatoxins. For this, the corresponding anatoxins were utilized which were produced on caseim media, and precipitated with a 1/N hydrochloric acid.

The purified concentrated staph.anatoxin contained 100 B.U. in one ml. The edematiens anatoxin was diluted from dry anatoxin to a content of 100 B.U. in one ml. The sorption of each anatoxin was separately made on aluminium hydroxide. The ready preparation of the staph.anatoxin contained 5 mg of Al_2O_3 in 1 ml.

In one ml of the edematiens anatoxin, there was 3 mg of Al_2O_3. The sorption was complete. In the experiments, rabbits of 2.5 - 3 Kg weight were used which had a natural staph.antitoxin of $\leqslant$ 0.125 A.U. in their sera.

The inoculations were made under the skin of the side in two shots at 30-day intervals. At the immunization with the combined preparation, three doses of the staph.anatoxin were used, 25 - 50 - and 100 B.U., and the dose of the edematiens anatoxin in both mixtures was equal to 40 B.U., the dose of the latter was accepted in the Gamalei Institute of Epidemiology and Microbiology for the composition of the combined preparations. The mixture (p.38) of the antigens was introduced in a volume of 1 to 1.5 ml. Six months after the second inoculation, the rabbits were revaccinated with one shot of the same doses which they had received at the initial immunization. The sera of the blood were investigated on the 10th, 20th, and 30th days after the second inoculation, at the 6-month before the revaccination, and

on the 5th, 10th, 20th and 30th days after the revaccination.

In Table 1, the average arithmetic titres of the staph. antitoxin are contained for each group of the immunized rabbits(5 animals in each group). From Table 1 it is evident that the dose of 25 B.U. of the staphylo. anatoxin in the combined preparations provoked considerably less formation of antitoxin than in the case when the immunization was done with separate anatoxins. (The average titres on the 10th day after the second inoculation were correspondingly 1,8 A.U. and 7,5 A.U.). A dose of 50 B.U. of the staph. anatoxin proved to be considerably effective:- 8 A.U. with the combined immunization, and 12 A.U. in case of the separate inoculation. The increase in the amount of the staph. anatoxin to 100 B.U. did not lead to any substantial increase in the amount of the antitoxin under the conditions of either the combined or the separate immunization.

The revaccination of the rabbits, which have been immunized with the combined preparation has also shown a weak effectiveness of the 25 B.U. dose of the staph. anatoxin. The average peak titre of the serum was always 2,8 A.U., at the same time when the average peak titre, after the revaccination of the animals which had been immunized with staph. anatoxin alone, was equal to 6 A.U.

The rabbits immunized with a dose of 25 B.U. of the staph. anatoxin, in a mixture with the edematiens anatoxin, in distinction from the control rabbits which were inoculated with staphylo. anatoxin alone, have not shown either any resistance to the intracutaneous introduction of 5 M.L.D. of staphylococcic toxin at the time when they were examined two months after the revaccination.

The rabbits which at the initial immunization were given doses of 50 B.U. and 100 B.U. of the staph. anatoxin, after the revaccination with the same dosages, showed an increase in the formation of the staph. antitoxin parallel with the increase in the anatoxin dosage.

The highest peak of the titres in the blood sera was observed in case of a revaccination with a dose of 100 B.U. of anatoxin(9 A.U. of antitoxin). However, the twice smaller dose of 50 B.U. has also given good result(peak titre- 6,5 A.U.)

In this manner, the dose of 50 B.U. of the purified concentrated adsorbed staph. anatoxin was proved to be sufficiently effective in combined immunization with the edematiens anatoxin, when the latter's dosage was 40 B.U., under the conditions of both the initial immunization and the revaccination. The investigation of the re-

sistance of the rabbits which were immunized with the combined preparation, to the intracutaneous introduction of the staphylococcic toxin and of the staphylococcus culture two months after the revaccination has proved that they as well as the control animals tolerated 5 to 10 M.L.D. of the staph. toxin and 6 M.L.D. of the staph. culture.

STAT

(p.39) TABLE 1

DYNAMISM OF THE FORMATION OF STAPHYLOCOCCUS ANTITOXIN AT THE IMMUNIZATION WITH A MIXTURE OF PURIFIED SORBED EDEMATIENS AND STAPHYLOCOCCIC ANATOXINS

ANATOXINS		Time of examination of serum in days							
		AFTER SECOND INOCULATION				AFTER REVACCINATION			
staphyl. in binding units	edematiens B.U.	10	20	30	6 months	5	10	20	30
		Titres given in antitoxic units A.U.							
25 BU.	-	7,3	3,3	1,5	0,25	6	4	3,5	1,6
25	40	1,8	1,0	0,35	0,25	2,8	1,7	0,7	0,45
50	-	12,0	4,0	1,4	0,33	5,6	3,3	2,5	1,0
50	40	8,0	3,0	1,2	0,25	4,2	6,5	3,2	1,8
100	-	13,3	6,0	2,0	0,25	7,3	8,0 9,0	3,0 5,5	2,6
100	40	9,6	3,6	1,3	0,25	9,0	[illegible]	[illegible]	3,0

(p.40) TABLE 2

DYNAMISM OF THE FORMATION OF EDEMATIENS ANTITOXIN AT IMMUNIZATION WITH A MIXTURE OF PURIFIED SORBED EDEMATIENS AND STAPHYLOCCUS ANATOXINS

ANATOXINS		Time of examination of sera in days								
		AFTER SECOND INOCULATION					AFTER REVACCINATION			
edematiens	staphy.	10	20	30	3m.	6m.	5	10	20	30	60
40 B.U	25 BU.	29 A.U.	17,5	10	3,3	0,6	17,5	29	27	22,5	4,8
40	50	29,0	15,5	8,5	2,7	0,5	19,5	32,0	24,5	21,5	5,7
40	100	35,0	13,5	7,0	2,3	0,7	14,4	38,7	36,0	26,9	8,6
40	-	20,0	12,5	7,5	2,1	0,5	11,3	11,3	20,0	15,0	-

(p.40 cont.)

From the data included in Table 2 it is evident that the purified sorbed staph. anatoxin does not cause any inhibition of the antigenic activity of the edematiens anatoxin.

The titres of the edematiens anti-toxin, in case of an immunization with the combined preparation, proved to be higher than in case of introducing the same doses

of the edematiens anatoxin alone.

However, it is difficult to speak of a stimulation if the effect of the edematiens anatoxin, in case of an immunization with it in mixture with the staph. anatoxin, on account of the considerable individual variations of the titres in the control group of animals as well as in the group of the experimental animals. STAT

On the basis of the present work we are free to draw the following conclusions:

1) In case of an immunization of the rabbits with a mixture which contains 40 B.U. of purified sorbed edematiens anatoxin and 25 B.U. of a similarly produced staphylococcic anatoxin, an inhibitory effect is observed upon the immunological action of the latter. The increase of the dosage of the purified sorbed staph. anatoxin in the mixture to 50 and to 100 B.U. assures a sufficiently high level of staphylococcic antitoxin in the blood.

2) In case of similar ratios of dosage of both purified sorbed anatoxins, the edematiens anatoxin does not show any inhibitory effect.

(No/literature/quoted)

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(p.43) B.V.VOSKRESENSKII & Z.I.LEBEDEVA.

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EFFECTIVENESS OF IMMUNIZATION WITH CONCENTRATED PURIFIED ALUMINUM-HYDROXIDE ADSORBED STAPHYLOCOCCIC ANATOXIN IN CASE OF USING IT IN THE COMPOSITION OF A COMBINED PREPARATION FOR ACTIVE IMMUNIZATION AGAINST WOUND INFECTIONS (p.43 - 59)

* * *

The study of the effectiveness of immunization with concentrated purified Aluminium-hydroxide- adsorbed staph.anatoxin, with introducing it into a mixture with other antigens, had been undertaken in connection with the basic topic of the scientific research work of the Anaerobic Laboratory of the Department of Wound Infections of the N.F.GAMALEI Institute of Epidemiology and Microbiology of the Academy of Medical Sciences of the U.S.S.R., directed to the creation of a combined preparation for active immunization against anaerobic woumd infections. The inclusion of the staphylococcic anatoxin in the composition of these preparations was dictated by the circumstance that a staphylococcic infection is very frequently the cause of the provocation of purulent complications in the injured persons, and in the postoperative period, and it considerably aggravates the course of sickness. Moreover, it is well known that the inflammatory processes caused by staphylococci in the wound that is infected with the spores of the tetanus bacillus is a factor which enables the development of the tetanus infection.

The effectiveness of the immunization with concentrated purified aluminium-hydroxide adsorbed staphylococcic anatoxin was studied with introducing it into the composition of two series(Nos.1 and 2) of combined anatoxin "SPES" and into the composition of four series of polyanatoxin. In addition to the staphylococcic anatoxin, concentrated purified aluminium-hydroxide adsorbed anatoxins of tetanus, of perfringens, and of edematiens have entered into the composition of the preparation "SPES", and, in addition to the enumerated anatoxins, similarly treated anatoxins of the septic vibrio amd of the Vibrio histolyticus were also added to the composition of the polyanatoxin.

(p.44) The native staph.anatoxin was produced by us, the concentration and the purification of it was made by the scientific collaborator of the Department of

Biochemistry of Microbes ,L.I.MARMALEVSKAYA, under the direction of the Chief of this Department, V.A.BLAGOVESHCHENSKII. The sorption of the anatoxins was made by the scientific collaborators from the Anaerobic Laboratory, Z.M.VOLKOVA and S.A. ZELEVINSKAYA, for the prepared mixture. STAT

The composition of the tetra-anatoxins and of the polyanatoxins and the doses of each ingredient in them are given in Table 1.

TABLE 1

COMPOSITION OF THE COMBINED ANATOXINS,AND SUMMARIZED DOSAGE OF EACH INGREDIENT IN THE EXPERIMENTAL IMMUNIZATION OF RABBITS(2 injections at 20-day interval)

NAME OF PREPARATION	INGREDIENTS AND THEIR SUMMARIZED DOSAGE					
	Tetanus anatoxin B.U.	Perfringens anatoxin B.U.	Edematiens anatoxin B.U.	Septic Vibr. anatoxin B.U.	Histolyticus anat. B.U.	Staphylo. anatoxin B.U.
Polyanatoxin Ser.1.a	400	20	20	10	10	25
Polyanatoxin Ser.1 b	600	40	50	20	20	50
Polyanatoxin Ser. 1 v.	800	80	50	30	30	50
Polyanatoxin Ser.2	300	25	20	15	10	35
Tetraanatoxin Ser.2 SPES	600	50	100	-	-	50
The same	600	50	100	-	-	70
Tetraanatoxin Ser.1 a	800	80	62.5	-	-	100

In the experiments two series of staph.anatoxin were utilized— Nos.25 and 43. The anatoxin of series No.25 was prepared from a toxin which was produced on bouillon of fish hydrolysate, and contained 25 B.U. in one ml. The anatoxin of Ser.No.43 was produced from a toxin which was obtained on a fluid medium of casein hydrolysate; in one ml of this anatoxin, 20 B.U(binding units) were contained.

(p.45) We bring forward the data of L.I.MARMALEVSKAYA which refer to the methodology of treatment and to the chemical characteristics of these two anatoxins. The amount of the total nitrogen in the native anatoxin of Ser.No.25 amounted to 263.8 mg %. The purified preparation was made with the fractional salting our by ammonium sulfate. The preparation was concentrated 17 times, and it contained 389 mg% of total nitrogen. The purification of the preparation and the removal of the inert

substances was 24% ,with a loss of activity of approximately 40% at this. The so purified concentrated staphylococcic anatoxin of Ser.No.25 contained STAT 250 B.U. in one ml, maintaining this number of binding units even at further storage under refrigeration conditions.

The concentration and purificiation of the staph.anatoxin of Ser.43 was done with HCl at the isoelectric point which was determined in advance by means of titration. For the characteristics of this anatoxin we include the following data:

	Native anatoxin	Purified concentrated anatoxin
Total nitrogen	323.4 mg %	139 mg %
Protein nitrogen	33 mg %	74.6 mg %
B.U. in one ml	20	280
Loss at treatment	-	none
Charge of B.U. per 1 mg of protein nitrogen	66.6	375.3

It should be remarked that the B.U. content in the given anatoxin becomes diminished shortly after its preparation, and it was found 140 B.U. in one ml.

The sorption of the anatoxins was made to Al hydroxide, prepared by the Department of Biochemistry. The adsorbent contained 10.5 - 11.0 mg Al_2O_3 in one ml. By studying that the adsorption of the proteins depends upon their concentration and upon the salt, the pH, the temperature and upon the nature of the substrate— the amount of the sorbent which is required for the full adsorption of the antigen was determined for each preparation in a preliminary experiment. For the full sorption of the purified concentrated staph.anatoxin of Ser.No.25, it was required to add 50% Al hydroxide, containing 10.5 mg in one ml. For the full sorption of the native caseine-anatoxin of Ser.No.64(total nitrogen= 247.8 mg %, protein N = 12.6 mg %), 54 % of Al hydroxide was needed,with a content of 11 mg of Al_2O_3 in 1.0 ml of the sorbent, and in the anatoxin---5.9 mg per 1.0 ml.

All experiments were done on rabbits of the chinchilla species,with an average weight of 2 - 2.5 Kg. The content of the natural staphylococcic anatoxin was not more than 0.125 A.U. in the blood serum of the rabbits.

INITIAL IMMUNIZATION.

Before going over to the exposition of the results of the basic experiments, we briefly mention the data obtained at the first initial immunization with a single dose of purified concentrated Al-hydroxide adsorbed staph.anatoxin, since this

question has been also insufficiently studied, and it has a direct relation to the tasks which we set for ourselves.

STAT

TABLE 2

EFFECTIVENESS OF THE INITIAL IMMUNIZATION WITH NATIVE AND ALUMINIUM HYDROXIDE ADSORBED STAPHYLOCOCCUS ANATOXIN

PREPARATION	Dosage and repetition of inocul. B.U.	Interval days betw. injections	Number of rabbits	Average max. titres of sera A.U.	Average titres 3.5 months later A.U.	Average titres of sera after 5 months, in A.U.
Native staph. anatoxin	30 x 3= 90	20 & 10	12	4,7	0,19	0,29
Native staph. anatoxin Al hydr. ads.	30 x 2= 60	20	17	9,8	0,33	0,27
The same	30 x 2= 60	5	5	2,0	-	-
Native staph. anatoxin	30 x 3= 90	5	12	3,7	0,175	0,17
Native staph. anatoxin Al hydrox. adsorbed	30 x 3= 90	5	12	6,2	0,4	0,5

(p.47) As it can be seen from Table 2, the two-shot inoculations with sorbed staph. anatoxin, at doses of 20 plus 30 B.U. (total---60 B.U.), and with a prolonged interval between the injections(20 days) has shown twice as good effect as the three-shot inoculation with the same doses of native anatoxin, i.e., with a totally larger dose(90 B.U.), with intervals at 20 and 10 days. It should be remarked that the titres of the sera in the rabbits which were immunized with native anatoxin, were 0.175 A.U. as an average after 3.5 months, and 0.17 A.U. after 5 months, at the same time when in the animals which were given the same doses of sorbed anatoxin, at the indicated time there were kept respectively 0,4 and 0,5 A.U., which also proves the great advantages of the immunization with sorbed anatoxins. The great importance of the intervals between the injections was also proved:- after a two-shot inoculation with the sorbed staph anatoxin at a 20-day interval, the average arithmetical titre of the sera in the rabbits was 9.8 A.U., and after the introduction of the same doses of anatoxin at short intervals (5 days) the average peak titre of the sera ahs always been only 2 A.U. Evidently, twenty days after the

first inoculation with adsorbed anatoxin, the reactivity of the organism has been already reconstructed to sufficient degree, and the 5-day time interval is not sufficient for this, which also explains the difference in the response to the second injection.

The importance of the dosage of the sorbed staph. anatoxin at the initial immunization has been also revealed: with the enlargement of the total dosage of the antigen in the range from 30 to 150 B.U., the titres of the sera are increased; it should be noted that, at this, in case of the total dosages of the preparation from 50 to 100 B.U., a noticeable parallel increase has not been observed in the formation of the antitoxin. However, with a total dosage of 150 B.U., it has been sharply marked (about 6 A.U. at the average with doses of 50 to 100 B.U., and 15.3 A.U. after a two-shot inoculation with ~~in~~ 75 B.U.). Complete correspondence was also observed between the total dosage of the sorbed anatoxin and the prolongation of the maintenance of anatoxin level. Thus, 2.5 months after the initial immunization in the rabbits which had received 30-50 B.U. the average titre of the serum was 0.5 A.U.; after the introduction of 100 B.U. it was equal to 0.75 A.U., and after 150 B.U. — 1.3 A.U. In this manner, the results of all these experiments indicate without doubt the great advantage of the initial immunization with sorbed staph. anatoxin and the appropriateness of the wide use of this preparation in the practice.

The results of the initial immunization with the purified concentrated, Al hydroxide adsorbed staphylococcic anatoxin, in case of its subcutaneous injection, —in the composition of the combined preparations of tetraanatoxin SPES and of the polyanatoxin— are given in Table 3.

At the inspection of the obtained data the attention is attracted to the considerably marked lag of the titres of the staphylococcic ~~XXX~~ anti toxin in the rabbits which were immunized with the combined preparations, in comparison with the animals which received the same or even smaller doses of a single adsorbed staphylococcic anatoxin. About this, the following examples are testifying from Table 3.

1) Rabbits which were immunized with a total dosage of 70 B.U. of the staph. anatoxin in the mixture of the tetraanatoxin SPES, Ser. 2, showed a maximum titre of 1.8 and 2.0 A.U. in the sera as an average while the average maximum titres of the sera after immunization with the same total dosage of a single shot of adsorbed staph. anatoxin, given even at a smaller dosage (60 B.U.), were 4.4 and 3.6 A.U., and with doses of the concentr. staph. anatoxin — 5.7 and 4.0 A.U. respectively.

(p.48)

TABLE 3

EFFECTIVENESS OF THE TWO-SHOT INOCULATION WITH ADSORBED STAPHYLOCOCCIC ANATOXIN UNDER VARIOUS CONDITIONS OF IMMUNIZATION

PREPARATION/	Total dose of staph. anatoxin in B.U.	No. of rabbits	TIME OF EXAMINATION OF SERA AFTER IMMUNIZATION, days, and their titres, in A.U.							
			Before	10	15	20	30	60-75	90-120	150-180
N. Ads. St. A	30	2	0,125	3,0	-	0,87	0,5	0,5	-	-
Polyanatoxin Ser.1 a	25	5	0,175	-	0,75	-	-	0,65	-	0,125
Polyanatoxin Ser.2	35	2	-	0,75	-	0,75	-	-	-	0,125
K St.A	50	2	0,125	5,0	4,5	4,0	1,25	-	-	1,0
K Ads. St.A	50	3	0,125	6,7	-	1,7	-	0,5	-	-
SPES Ser.2	50	5	0,125	2,7	-	1,55	0,54	0,17	0,21	-
Polyanatoxin Ser.1 b	50	5	0,175	-	2,2	-	-	0,85	-	0,29
N Ads. St.A	60	8	0,125	4,4	-	3,6	2,7	-	0,29	0,28
K St.A	70	3	0,125	5,7	5,3	4,0	1,4	-	-	0,37
SPES Ser.2	70	3	0,125	1,8	1,5	2,0	0,4	-	-	0,125
K. Ad. St.A	100	5	0,125	6,0	-	2,7	-	0,75	-	-
Tetraanatoxin Ser.1 a	100	5	0,125	4,4	-	2,8	1,4	-	-	0,125
Polyanatoxin Ser.1 v	125	5	0,15	0	2,8	-	-	1,45	-	0,33
K Ad. St.A	150	6	0,15	13,3	-	7,2	-	74days 1,3	-	1,3

ANNOTATION: K St.A . . . Concentrated staphylococcic anatoxin

N Ad.St.A. . .Native adsorbed staphylococcic anatoxin

K Ad.St.A. . . Concentrated purified adsorbed staph.anatoxin.

(p.49, cont.) 2) In the rabbits immunized with polyanatoxin of Ser.2, and each of which received with this polyanatoxin 35 B.U. of staph.anatoxin, the maximum titres of the sera were 0.75 and 0.75 A.U. as an average in 1.0 ml of the serum while the animals which were given even a somewhat smaller dose, 30 B.U., but with a single shot of the sorbed staph.anatoxin, had 3.0 and 0.87 A.U.

(p.49 cont. ...)

3) In the groups of rabbits which received each a total of 50 B.U. of the sorbed staph.anatoxin in a mixture of the tetraanatoxin SPES, the maximum titres of the sera were 2.7 and 1.55 A.U., while the sera of the animals which were immunized with the same total dosage but of a single shot of adsorbed staph.anatoxin, contained 6.7 and 1.7 A.U. as an average, and those which received the same total dosage of the concentrated non-adsorbed staph.anatoxin-- 5.0 and 4.0 A.U. Weak titres of the sera(on the 15th day-- 2.2 A.U.) were also shown in the rabbits which were immunized with the same total dosage of staph.anatoxin included in the polyanatoxin of Ser.1-b.

4. After the two-shot inoculations with the tetraanatoxin SPES of Ser. 1-a, with a total dose of the staph.anatoxin equallling 100 B.U., fully satisfactory results have been obtained:- the titres of the staph.antitoxin were 4.4 and 2.8 A.U. on the 10th and on the 20th days as an average. However, they proved to be somewhat lower always than in the animals which received the same total dosage of a single shot of the adsorbed staph.anatoxin(6.0 and 2.8 A.U.) and a smaller total dosage of the same preparation---60 B.U.(4.4 and 3.6 A.U.).

(p.50) 5. The effectiveness of the immunization, even with such a large dose of the staph.anatoxin as 125 B.U., which was introduced into the composition of the polyanatoxin, Ser.1-a, proved to be considerably lower(2.8 A.U.) than after the inoculation with a smaller dosage of a single shot of the concentrated staph.anatoxin(50 B.U. at 15 days---4.5 A.U.; 70 B.U. after the same period of time---5.3 A.U.).

In this way, the lowering of the action of the staph.anatoxin at the initial immunization, when it was in the composition of the tetra- and of the polyanatoxins-- proved to be clearly marked almost at all the investigated variants of both combined preparations, with the exclusion of tetraanatoxin SPES Ser.1-a. However, even in this instance, the titres of the staph.antitoxin were somewhat lower than at the initial immunization with the same dosage, or even with smaller dosage of a single shot of the staph.anatoxin.

It should be supposed that the basic cause of the weakness of efficiency of immunization with the staph.anatoxin in the composition of the investigated variants of the tetra- and poly-anatoxins is the inhibition of the effect of this an-

atoxin by the other components, or preferably by anyone of them, which may be connected with both the activity of each ingredient and with the circumstance that the dosage of the individual components proved to be imbalanced.

STAT

We will not delay longer at this question, since the review of the literature on the interaction of antigens at combined immunizations has been published in the Journal for M.E.I., No.12, 1958.

EFFECTIVENESS OF THE REVACCINATION WITH SORBED STAPHYLOCOCCIC ANATOXIN---ALONE AND IN COMPOSITION WITHIN A COMBINED PREPARATION.

At the study of the effectiveness of revaccination after the initial immunisation with sorbed preparations, first of all the question arises which type of anatoxin will do it most remuneratively, the sorbed one or the non-sorbed one? At the answer to this question we studied not only the indices of the antitoxin titres but also their dependence upon the intensity of the initial immunization, the duration of the maintenance of a sufficiently high level of antitoxin in the blood and the dynamism of the increase of antitoxin level in the near time after the revaccination.

As it can be seen from Table 4, the best indices of the production of the staph.anatoxin (sic!) are observed in rabbits which were revaccinated with concentrated non-adsorbed anatoxin(average arithmetic titre 13.7 A.U.).Considerably smaller titre of the sera was seen in the animals which were revaccinated with the same anatoxin in the adsorbed condition(average arithmetic titre 7.7 A.U.), and by the following month, even with a larger(p.51) dose, proved to be the native anatoxin(average arithmetic titre 6.2 A.U.). Similar relationships were also observed in regard to the duration of the maintenance of the antitoxin level in the blood; thus, upon the examination of the sera 5 months after the revaccination, the average arithmetic titres of the sera were equal respectively to 0.56 - 0.34 and 0.22 A.U.

In the same Table the results of the revaccination with polyanatoxin are also shown after an initial immunization with the same preparation; the non-sorbed preparation, even under the given conditions, showed better effect than the sorbed preparation in regard to the height of the antitoxic titres and in regard to the duration of the maintenance of their level.

(p.51) TABLE 4

EFFECTIVENESS OF THE REVACCINATION WITH ADSORBED AND NON-SORBED STAPHYLOCOCCIC ANATOXINS.

STAT

PREPARATION/	Dose in B.U.	No.of rabbits	TIME OF THE EXAMINATION OF THE SERA titres of sera in A.U. before revaccination	10 days after	20 days after	30 days after	5 months after
			A.Group.				
N St.A	75	4	0,5	6,2	3,1	3,9	0,22
K St.A	50	5	1,12	13,7	8,3	8,0	0,56
K Ad.St A	50	5	0,75	7,7	6,0	6,7	0,34
			B.Group.				
			before revaccination	7days after	14 days after	30 days after	2 months after
Polyanatoxin Ser.2	17,5	3	0,25	10,0	7,7	8,3	2,3
Polyanatoxin Ser.2 (sorbed)	17,5	5	0,25	5,7	5,9	4,6	1,3

ANNOTATION: N.St.A. . .Native staphylococcic anatoxin

K St.A . . .concentrated Staphylococcic anatoxin

K Ad.St.A. . . concentrated adsorbed(Al hydroxide) staphylococcic anatoxin

(p.52) The results of the initial immunization and of the revaccination with the tetraanatoxin SPES are included in Table 5.

The revaccination with the combined preparation was done as a rule with half a dose of the one used at the initial immunization,with the same antigen.

As it has been already told, after the initial immunization of the rabbits with the staph.anatoxin in the composition of the tetraanatoxin SPES and in the composition of the polyanatoxin, the titres of the sera proved to be considerably lower than with the use of the same doses of a single shot of the staph.anatoxin. Similar results were also obtained at the revaccination with tetraanatoxin SPES. From Table 6 it is seen that, even with the for the action of the staphylococcic anatoxin most successful(most fortunate) combination of the ingredients of the te-

traanatoxin, the revaccination with an adsorbed preparation did not give the usual result-- the rise of the titres higher than the peak level after the initial immunization-- and it gave rather bas results in comparison with the single shot of staphylo.anatoxin(the average arithmetic titres were correspondingly, 3.8 and 7.0 A.U. ten days after the revaccination, and 1.4 and 8.0 A.U.-- 30 days after the revaccination). The unfavorable effect of the remaining components of the tetraanatoxin SPES upon the effectiveness of the inoculations with a single shot of the sorbed staph.anatoxin:- the titres of the sera in the rabbits which were initially immunized with tetraanatoxin were shown to be considerably less than in the rabbits which received the same dosage of the staph.anatoxin in a single shot at the initial immunization(70 B.U.)(the average values of the arithmetic titres were correspondingly 8.7 and 12.7 A.U. after ten days, and 3.3 and 6.0 A.U.--after 30 days of the revaccination).We also remark that,in the supplementary experiments (on 16 rabbits) of the study of the effectiveness of immunization with sorbed staph. anatoxin,in the composition of the tetraanatoxin SPES, the doses of the remaining (other)ingredients of which have been somewhat reduced, with a content of 24.5 B.U. of sorbed staph.anatoxin in one ml, and 200 B.U. of tetanus anatoxin, 25 B.U. of the perfringens anatoxin, and 40 B.U. of the edematiens anatoxin, ---the revaccination with the same combined anatoxin has also shown weaker results than the revaccination with a single XXXXXX sorbed staphylo.anatoxin alone(the average arithmetic titres were,correspondingly, 3.4 and 4.3 A.U. on the tenth day, and 1.5 and 3.6 A.U. on the 20th day after the revaccination).

As it has been already mentioned, after the first immunization with the polyanatoxin, the rabbits showed a weak production of the staph.antitoxin. The revaccination of all these roups of rabbits was made with single doses of the polyanatoxin of Series 2, containing a moderate amount of all ingredients in comparison with other investigated combined preparations.

As it can be seen from Table 6, in almost all cases the revaccination with this polyanatoxin showed a fully satisfactory result. A weak production of the staph antitoxin was observed only in those rabbits that at the XXXXX initial immunization had received the largest of the used doses of the complex preparation (polyanatoxin Series 1-v). In this case,the effect of the revaccination was found so weak that the titres of the staph.antitoxin had not even reached the peak values

(p.53)

TABLE 5.

EFFECTIVENESS OF THE INITIAL IMMUNIZATION AND OF THE REVACCINATION WITH THE ALUMINIUM HYDROXIDE ADSORBED STAPHYLOCOCCIC ANTITOXIN ALONE AND IN COMPOSITION OF THE TETRA-ANATOXIN "SPES"

STAT

PREPARATION/	Dose in B.U.	Interval betw. injections days	No. of rabbits	AVERAGE TITRES OF THE SERA IN A.U. before imm.	10 days after	30 days after	2 months later	Months after init. immun.
A. INITIAL IMMUNIZATIONS.								
Tetraanatoxin SPES Ser.2	25x 2=50	20	5	0,125	2,7	0,54	0,17	4
The same	35x 2=70	20	3	0,125	1,8	0,4	-	6
K.Ad.Sta.A	35x 2=70	20	3	0,125	5,7	1,42	-	6
Tetraanatoxin SPES Ser.2	50x 2=100	20	5	0,125	4,4	1,4	-	5,5
K.Ad.St.A	50x 2= 100	20	5	0,125	6,0	0,65	0,71	2,5

B. FIRST REVACCINATION.

			AVERAGE TITRES OF SERA IN A.U. before revacc.	10 days after	30 days after
SPES Ser.2	25	4	0,21	0 -	0,87
K Ad.Sta.A	35	3	0,125	8,7	3,3
K Ad.Sta.A	35	2	0.37	12,7	5,0
SPES Ser.1-a	50	5	0,125	3,8	1,4
K.Ad Sta A	50	2	0,75	7,0	8,0

ANNOTATION: (As on Table 4)

Table 6(on next page)

(p.54)

TABLE 6

EFFECTIVENESS OF THE INITIAL IMMUNIZATION AND OF THE REVACCINATION WITH ALUMINUM-HYDROXIDE SORBED STAPHYLOCOCCIC ANATOXIN IN THE COMPOSITION OF THE TETRAANATOXIN "SPES"

STAT

A. INITIAL IMMUNIZATION.

PREPARATION	Dose BU	Interval in days between inject.	No. of rabbits	AVERAGE TITRES OF THE SERA IN A.U. before	10 days after	20 days after	30 days after
Tetraanatoxin SPES	24.5 B.U. x 2 = 49	20	16	0.125	2.8	1.14	0.66

B. REVACCINATION TWO MONTHS AFTER THE INITIAL IMMUNIZATION

PREPARATION	Dose B.U.	No. of rabbits	AVERAGE TITRES OF THE SERA IN B.U. before	3d. after	5d. after	10d. after	15 d. after	20d. aft.	30d. aft
Tetraanatox. SPES	24.5	1) 4/3/20	0.34	-	-	-	-	-	-
		1) 6/6/10	0.31	-	3.0	-	-	-	-
		1) 10/10/10	0.24	-	-	3.4	2.2	1.5	-
Adsorbed Staph. anatoxin	24.5	6	0.3	-	4.2	4.3	3.8	3.6	-

1) 4 . . . number of examined rabbits

3 . . . number of increased titres in the sera

20 . . . total number of rabbits in the experiment.

(p.55) (cont.) . . . which were observed after the initial immunization. It should be remarked that a similar effect of the revaccination was also observed after the initial immunization with tetra-anatoxin, series 1-a, which in respect of the tetanus, perfringens and edematiens anatoxins, had almost no difference from the polyanatoxin, series 1-a; this, in its turn, confirms the relationship of the effectiveness of the Staph. anatoxin with the dosages of the other ingredients in the combined preparation, and it suggests the idea about the fact that, after the use of

excessive doses of it, the inhibition of the action of the Staph. anatoxin may continue for the length of a longer segment of time(in our experiments STAT for 6 months).

TABLE 7.

DYNAMISM OF THE FORMATION OF ANTITOXIN IN THE BLOOD OF RABBITS AFTER REVACCINATION WITH NON-ADSORBED AND ALUMINUM HYDROXIDE ADSORBED STAPHYLOCOCCIC ANATOXINS.

P R E P A R A T I O N S	Non-adsorbed staph. anatoxin	Aluminum-hydroxide adsorbed staphylococcic anatoxin.
Number of rabbits	33	35
Average titres of sera before revaccination	0.7	0.49
TIME OF EXAMINATION OF SERA AFTER REVACCINATION, In Days;		
3 — Number of rabbits	11	16
3 — No. of animals with increased titres	5	8
3 — Average titre	0.96	0.88
5 — No. of rabbits	22	26
5 — Those with increased titres	22	26
5 — Average titre	6.5	4.7
10 — No. of rabbits	33	35
10 — Those with increased titres		
10 — Average titres	10.6	9.8
20 — No. of rabbits	33	35
20 — Average titres	5.9	7.2
30 — No. of rabbits	33	35
30 — Average titres	3.9	4.7

(see Table 8 over)

(p.56)

TABLE 8

EFFECTIVENESS OF THE INITIAL IMMUNIZATION AND OF THE REVACCINATION WITH ALUMINUM HYDROXIDE ADSORBED STAPHYLOCOCCIC ANATOXIN ALONE AND IN THE COMPOSITION OF THE POLYANATOXIN

STAT

A. INITIAL IMMUNIZATION

* * * * * * * * * * * * *

PREPARATION	TOTAL DOSE B.U.	NO. OF RABBITS	TIME OF EXAMINATION OF SERA IN DAYS titres of sera in A.U. before	10	15	20	30	2½ months	5½-6 months
K Ad. Sta. A	30	2	0,125	3,0	-	0,87	0,5	-	-
The same	50	3	0,125	6,7	-	1,7	-	0,5	-
The same	100	5	0,125	6,0	-	2,75	-	0,71	-
The same	150	6	0,15	15,3	-	7,2	-	1,3	-
Polyanatox. Ser.2	35	5	-	0,75	-	0,75	-	-	0,125
Polyanatox. Ser.1-a	25	5	0,175	-	0,75	-	-	2mo. 0,65	6 mo. 0,125
Polyanatox. Ser.1-b	50	5	0,175	-	2,2	-	-	0,85	0,29
Polyanatox.	125	5	0,125	-	2,8	-	-	1,45	0,33

B. REVACCINATION

* * * * * * * * *

			TIME OF EXAMINATION OF SERA IN DAYS titres of sera in A.U. before	7	10	14	20	30	60	5mo.
K Staph. A	50	1	0,5	-	8,0	-	10,0	10,0	-	-
K Ad. Staph. A	50	1	0,5	-	8,0	-	5,0	6,0	-	0,25
K. Sta. A	50	2	0,87	-	13,0	-	5	6,0	-	0,37
K Ad. St. A	50	2	0,75	-	7,0	-	6,5	8,0	-	0,5
K St. A	50	2	2,0	-	20,0	-	8,3	8,0	-	0,75
K Ad. Sta. A	50	2	1,0	-	8,0	-	6,0	6,0	-	0,37
Polyanat. ser.1-2 non-sorbed	17,5	1	0,125	16,0	-	10,0	-	10,0	2,0	-
Same, sorbed	17,5	1	0,125	8,0	-	10,0	-	8,0	2,0	-
Polyanatox. ser.1-b non sorbed	17,5	1	0,125	6,0	-	7,0	-	7,0	2,0	-
Same, sorbed	17,5	2	0,37	7,0	-	6,0	-	4,5	1,75	-
Polyanatox. non sorbed	17,5	1	0,5	6,0	-	6,0	-	8,0	3,0	-
Same sorbed	17,5	2	0,25	2,1	-	1,75	-	1,25	0,25	

ANNOTATIONS: as for Table 4; Polyanatoxin...the Series 2 of this.

(p.58) The results of the study of the dynamism of the increase in the staph. antitoxin in the next days after the revaccination are given in Table 7. These had STAT not shown any particular differences between the effect of the non-sorbed and of the sorbed anatoxins. However, it can be said that, with an identical speed of accumulation of the antitoxin in the blood during the length of the first ten days, the titres of the sera in the rabbits which were revaccinated with the sorbed anatoxin were somewhat lower than with the use of the non-sorbed antigen, but in the subsequent time, between the 20th and the 30th days, side by side with the lowering of the amount of anatoxin in both cases, these ratios happened to become reversed.

At the study of the given question in regard to the rabbits which have gotten a two-shot inoculation of the tetraanatoxin SPES at the first immunization, and two months after the second injection, at the revaccination with the same preparation, it became known that, three days after this, the titres of the sera were already increased in the majority of the animals, and on the fifth day -- in all revaccinated animals. It should be remarked, nevertheless, that the effectiveness of revaccination with the staph. anatoxin, when in the composition, the combined preparation proved to be somewhat weaker than when the single sorbed staph. anatoxin was used under similar circumstances(the arithmetic average of the maximum titres was correspondingly 3.4 and 4.3 A.U.)

In this way, the results of the study of the efficiency of revaccination with the staph. anatoxin under different conditions of its use showed the advantage of the utilization of the concentrated non-adsorbed preparations for this purpose.

In some cases, we have investigated the effect of a second revaccination which was done 5 months after the first revaccination. Satisfactory results were obtained only at the use of the concentrated non-adsorbed staph. anatoxin in the dose of 50 B.U.(the average arithmetic of the maximum titres in four rabbits was equal to 7.5 A.U.). The effectiveness of the sorbed staph. anatoxin proved to be weaker than the effect of the native anatoxin, and noticeably lower than at the first revaccination(average maximum titre of the sera of 5 rabbits was 1.2 A.U.).

We have also carried out investigations of the immunity in rabbits after the first immunization and after the revaccination with staph. anatoxin(36 rabbits) by means of intracutaneous introduction of the culture and of the toxin of Staphylococcus. The best results(40 M.L.D. of toxin and 3 M.L.D. of the culture) were ob-

tained in those animals that were immunized with the largest dose(a total of 150 B.U.) of the concentrated adsorbed staphylococcic anatoxin.

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(p.59) The weakest results (2.5 M.L.D. of toxin and 1.7 M.L.D. of culture) were obtained one months after the revaccination with the SPES tetra-anatoxin.

CONCLUSIONS.

1) At the immunization with sorbed staphyloco cic anatoxin in a mixture with sorbed anatoxins of the agents of the anaerobic wound infections(with tetanus, perfringens, edematiens, histolyticus and of Septic Vibrio) considerable inhibition may occur in the activity of the staph.anatoxin. This phenomenon, evidently, depends upon the interaction of the antigen at their imbalanced dosage(ratio) in the combined preparation.

2) The inhibition of the effect of the staphylococcic anatoxin, apparently due to the same reason, may be also observed at revaccination with the indicated complex preparations.

3) For the assurance of the proper effectiveness of each of the ingredients of the combined preparation, a study of the interaction is necessary which exists between these ingredients, and a corresponding selection of the antigen dosages is needed at the composition of their mixture.

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(no literature references)

(p.60: Blank).

(p.61)

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COMPARATIVE STUDY OF THE ANTIGENIC AND IMMUNOGENIC SUBSTANCES OF THE STAPHYLOCOCCIC ANATOXIN PURIFIED BY VARIOUS METHODS AND SORBED ON THE HYDROXIDE AND THE PHOSPHATE OF ALUMINIUM (p.61-71)

- * -

Numerous communications of recent years indicated the considerable increase of the cases of serious sicknesses(septicopyemia in children, pneumonia, osteomyelitis, mastitis, skin diseases,etc) which are produced by pathogenic antibiotic-fast staphylococci(WALSH & McDERMOT,1956; HOWE,1956; I.G.RUFANOV,1957; A.M.DOLGOPOL'SKAYA,1958,and many others). By studying this,it must be supposed that the active immunization of certain contingents of the people with staphylococcic anatoxin and the inclusion of this anatoxin in the composition of the widely used combined preparations is entirely appropriate(B.V.VOSKRESENSKII,1958).

At the present time, for the immunization of people, concentrated purified sorbed antigens are generally used, which permitted to considerably increase the effectiveness of inoculations and to reduce the reactogenicity of the preparations.

To the problem of purification of the staphylococcic anatoxin the works of a number of investigators were devoted, and various methods were suggested for the production of purified staph.preparations(HOLT,1936; TASMAN and others, 1952; TURPIN,RAYNAUD & RELYVELD,1954; TURPIN,RELYVELD,PILLET & RAYNAUD,1956 and others). However, it is well known that the immunogenic properties of highly purified antigens are very slight if they are used without deposit-producing substances, therefore all purified antigens are supposed to be used in a deposited status.

In the last year,for the sorption of the purified anatoxins, the hydroxide of aluminium($Al(OH)_3$ and the phosphate of aluminium $AlPO_4$ have been chiefly used. We were not successful to find any foreign works about the use and the (p.62) study of the effectiveness of the purified sorbed staph.anatoxin-- alone or in composition with other antigens in a combined preparation. Individual works about this problem was carried out in the Department of Wound Infection of the N.F.GAMALEI Institute of Epidemiology and Microbiology by B.V.VOSKRESENSKII and by Z.I.LEBE-

DEVA(1956). The authors have proved the high effectiveness of the purified sorbed staphylococcic anatoxin.

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The objective of our work has been the experimental study of the influence of the method of purification and of the type of the sorbent upon the immunogenic properties of the purified sorbed staph. anatoxin.

For the purification of the staph. anatoxin three methods have been used:

1) precipitation by hydrochloric acid(HCl) at the isoelectric point with additional purification by means of sorption to $Al(OH)_3$, with subsequent elution(method suggested by BLAGOVESHCHENSKII, of the GAMALEI Institute of Epidemiology and Microbiology, Academy of Medical Sciences, U.S.S.R);

2) precipitation with metaphosphoric acid HPO_3 in the cold, with subsequent fractionation of the mixture of phosphates(the method of TURPIN, RELYVELD, PILLET & RAYNAUD, 1954, modified by us);

3) complete salting out by ammonium sulfate $(NH)_4SO_4$(according to the method of HOLT, 1936), with subsequent fractionation with ammonium sulfate.

Two series of stapgylococcus anatoxin, No. 61 and No. 68, were purified with these methods; the anatoxins contained 14 B.U. in one ml, each of them.

The biochemical indices of the purified staph. anatoxins are given in Table 1.

Each of the purified anatoxins was sorbed to $Al(OH)_3$ and to $AlPO_4$(after purification, with precipitation by means of hydrochloric acid, and, after additional purification, the sorption of the anatoxin was done only to $AlPO_4$). The aluminum hydroxide was given to us by the Department of Biochemistry of the Microbes, of the GAMALEI Institute of Epidemiology and Microbiology; and the $AlPO_4$ suspension was prepared by ourselves according to the method of HOLT(1947).

The schedule of the preparation of the sorbed preparations for the immunization of rabbits is given in Table 2.

In all preparations, the sorption of the antigen was complete(full), and in a volume of 1.5 ml each of the preparations contained 20 B.U. of the staph. anatoxin, and 4 mg of Al_2O_3, or 10 mg of $AlPO_4$.

The preparations were inoculated at two times(each preparation of the series No. 61 was given to three rabbits, and the preparations of the series No. 65 to five rabbits) subcutaneously, in the amount of 1.5 ml, at 20-day intervals. The revaccination of the animals was done two months after the second initial inocula-

tion, with native non-sorbed anatoxin(20 B.U.). The blood was examined for its anti-toxin content 20 days after the first injection, on the 5th, 10th, 20th, 30th, and 60th day after the second injection, and on the 5rd, 5th, 7th, 10th, 15th, 20th, 30th, and 72nd day after the revaccination.

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(p. 63)

TABLE 1

PURIFICATION OF THE STAPHYLOCOCCIC ANATOXINS WITH VARIOUS METHODS

No. of series	Anatoxin	Method of additional purification & precipitation	INDICES (see explanation below) a	b	c	d	e	f	g	h	i	Output in %
		a...concentration by volume; b...B.U. in one ml; c...amount of nitrogen in mg% TOTal; d...amount of protein nitrogen in mg%; e...B.U. per mg% of protein nitrogen; f...total nitrogen removed in %; g...protein N removed in %; h...coefficient of purity by total nitrogen; i...coefficient of purity by protein nitrogen.									
61	native	-	-	-	14	304.4	41.0	34.2	-	-	-	-	-
	con- A)	HCl, no added purif.	10	58	121.4	68.4	34.7	96.1	83.4	10.4	2.4	41.4
	centr- B)	HCl, sorp. $AlOH$, elu.	10	12	12.3	6.1	196.7	99.6	98.6	21.2	5.7	8.5
	ated C)	HPO_3, no purif.	10	88	150.0	81.4	108.1	95.1	80.2	12.7	3.1	62.8
	and D)	HPO_3, frac. by phosp.	10	50	32.6	23.0	217.3	99.0	94.4	33.3	6.3	35.8
	puri- E)	$(NH_4)_2SO_4$ no other	9	100	190.6	130.5	76.1	93.1	64.7	11.4	2.2	79.3
	fied F)	$(NH_4)_2SO_4$ fraction.	9	74	74.4	54.0	137.0	97.3	85.4	21.6	4.0	58.7
68	native	-	-	14	296.4	37.4	37.4	-	-	-	-	-
	con-	as A) above	14	110	128.6	71.8	153.2	96.1	86.3	18.1	4.0	56.1
	centr-	as C) above	14	70	94.1	59.8	117.0	97.8	88.6	15.8	3.1	35.7
	ated	as D) above	14	25	19.7	10.9	229.3	99.6	98.0	27.0	6.1	12.7
	and	as E) above	9	90	74.7	41.9	214.7	97.2	92.0	25.5	5.7	71.4
	puri-	as F) above	9	40	26.7	9.9	404.0	99.0	97.1	31.9	10.8	31.7
	fied											

(p. 65)

The sera were titrated separately for each group, the geometric mean of the titres was computed, also its logarithm, and the standard deviation(STEARMAN, 1955).

(For Table 2 see next page)

(p.65 cont.)

TABLE 2

PRODUCTION OF SORBED PREPARATIONS(ON THE BASIS OF 7-8 INJECTIONS;ANATOXIN OF STAT SERIES No.61)

METHODS OF PURIFICATION of the anatoxins	a	b	c	d	e	f	g	h
HEADINGS OF COLUMNS: a....conditional name of the preparation; b... B_cU_c per one ml; c....anatoxin volume per ml; d.... $AlPO_4$ volume per ml; e....$Al(OH)_3$ vol.per milliliter; f...volume of physiol.solution in ml; g...TOTAL VOLUME in ml; h...removed N/precipit.fluid in ml.								
HCl precipitation	II	58	2,8	6,0	-	3,2	12,0	-
HCl precipitation plus purification sorption to $Al(OH)_3$ with elution	I-A	12	13,3	6,0	-	-	19,3	7,3
Precipitation with HPO_3	II	88	1,6	-	4,0	4,9	10,5	-
The same	II	88	1,8	6,0	-	4,2	12,0	-
Precipitation with HPO_3 plus fractional precipitation with phosphates	II-A	50	2,8	-	4,0	3,7	10,5	-
The same	II-A	50	3,2	6,0	-	2,8	12,0	-
Salting out with $(NH_4)_2SO_4$	III	100	1,4	-	4,0	5,1	10,5	-
The same	III	100	1,6	6,0	-	4,4	12,0	-
Salting out with $(NH_4)_2SO_4$ plus fractionation with $(NH_4)_2SO_4$	III-A	74	1,9	-	4,0	4,6	10,5	-
The same	III-A	74	2,2	6,0	-	3,8	12,0	-

ANNOTATION: At the further discussion of the matter, we shall give the anatoxins which have been purified by the different methods by their conditional denomination(I,I-A and so on) which are contained in the present table.

(p.66) The comparison of the immunological effectiveness of the preparations by the values of the geometrical means of their titres in the different groups of the inoculated animals was carried out by us with the aid of the t-test. The value t which was obtained by the comparison of the two groups of animals was compared with the t value as given in the Tables of FISCHER and YATES(1943), for the corresponding

number of the degrees of freedom, and of the selected level of significance of the experiment/ We have worked with a 0.05 level of significance. STAT

On FIG.1, the results are given of the comparison of the antitoxic titres of the various groups of animals by the t-test; the animals were immunized with preparations sorbed to aluminium phosphate.

As it can be seen from the following, the level of the antitoxin in the blood of the different groups of animals, both after the initial immunization and after the revaccination, had no real differences basically. However, it should be remarked that, five days after the second injection, the animals which were immunized with Preparation I-A, in regard to the height of the antitoxic titres(4.1 A.U.) (per one ml), have given real differences in comparison with the animals which were given the Preparations II (1.2 A.U.), Preparation III (1.1 A.U. per ml), and Preparation III-A (1.5 A.U. per ml), but similar difference was not seen in relation to Preparation I and II-A. In the following period, the antigenic properties of Preparation I-A have been equal to the others.

Seven days after revaccination, the best antitoxic titres were noticed(6.9 A.U. per ml) in the animals which received sorbed anatoxin III-A. Thirty days after revaccination, the best geometric average of the titres was reached in the group of animals which were given Preparation I-A(3.3 A.U. per ml), which had a true difference from the groups of animals that had been immunized with Preparation II-A (1.1 A.U. per ml). This difference in the titres lasted for 72 days.

However, it does not follow from this that Preparations I, I-A, or III-A have a substantial superiority above the others, since their advantage was manifested at only/ separate times, and only in respect to one or to a few preparations. Therefore, it can be reckoned that the staph. anatoxins, purified by the various methods and sorbed to aluminium phosphate, possess identical antigenic properties at the two-shot immunization of rabbits, with a subsequent revaccination.

The strenght of immunity is determined by the intracutaneous inoculation of different amounts of M.N.D.(minimum necrotic dosis) of the staphylococcus toxin (Series No.27) and of the culture(Stock 0-15) 72 days after the revaccination.

Different dilutions of the toxin(1:2,5; 1:5; 1:10, and 1:20) and of the staphylococcus cultures(1,2,4,8,and 16 billion microbic cells in one ml, by the optical standard) were inoculated intracutaneously into the lateral surface of the body in the amount of 0.1 ml.

(p.67) FIG.1: EFFECTIVENESS of the immunization of rabbits with the purified $AlPO_4$-sorbed staphylococcic anatoxin.

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B. Statistical treatment---comparions of the antigenic properties of the preparations purified by the various methods.

On the ordinata: Log of the titres; titres in A.U.

On the abscissa: Number of days (0 to 152)

On the diagram: II. injection ...Revaccination.

Different preparations(II.II-A, III.III-A) marked by different lines.
(also I.I-A

p.68 cont.) We considered the results after 48 hours, and counted the amount of the M.N.D.(minimum necrotic dose) of the toxin and of the culture transmitted to each rabbit. The experiments showed that there is a high resistance in all groups of the rabbits to the infection which gave from 4 to 32 and more M.N.D. of the toxin and from 2.5 to 11 M.N.D. of the Staphylococcus culture.

The antigenic and the immunogenic properties of the same preparations were simultaneously investigated, but only of those which were sorbed to $Al(OH)_3$. The schedule of the immunization, the doses and the time of the blood sampling in the rabbits were the same as in the previous experiment.

On FIG.2, the results of the comparison of the average(geometric) of the titres of the antitoxin are represented in the blood of the different groups of animals dynamically, according to the $\underline{t}$ test.

Twenty days after the first inoculation the titres of the antitoxin did not show a true difference, and as an average they were 1.4 A.U. per one ml. Five days after the second injection, the best titres were found in the group of animals which were immunized with the ~~XXXXX~~ Preparation III-A...-11.3 A.U per one ml.. and the average titre of the antitoxin of all the other groups was equal to 4.5 A.U. per one ml. This superiority of the preparation kept on until the 20th day, when it has already provoked a truly excellent production of the antitoxin in comparison with Preparation III, and after 30 days--- also in relation to anatoxin II. Sixty days after the second injection, the titres of the antitoxin have considerably declined in all groups of animals, and they has slight differences(from 0.4 to 0.7 A.U. per ml).

Beginning with 10-15 days, a rapid decline of the antitoxin was observed in the blood of the animals, and on the 72nd day the average titre of it had been 0.5

A.U. per ml. In this time, there was already no real difference in the content of the antitoxin in the blood of the different groups of animals. STAT

After revaccination, a quick accumulation of the antibodies had been produced in the blood of the animals. On the 7th, 10th, 20th and 30th days, the group of animals which had received anatoxin III-A at the first immunization, possessed larger antitoxic titres than the groups of rabbits immunized with preparations II and III. (NOTE: the previous paragrapg should precede the one before it).

The strength of immunity in the rabbits, as it has been indicated above, was shown by means of intracutaneous inoculation of different amounts of the minimal necrotic dosage(M.N.D.) of the staphylococcic toxin and of the staphylococcus culture. The individual resistance of the animal varied within wide ranges and it was roughly identical in all groups(from 4 to 32 M.N.D. and more of the toxin, and from 3 to 11 M.N.D. or more of the culture).

We have also made a comparison by means of the t test of the antigenic properies(by the geometric average of the titres of the staphylococcus antitoxin)(cont.) (p.69) FIG.2: EFFECTIVENESS of the immunization of the rabbits with purified, and $Al(OH)_3$ adsorbed staphylococcic anatoxin.

B. Statistical treatment...comparison of the antigenic properties of the preparations purified by various methods.

On the ordinata: Log of the titres; titres in A.U.

On the abscissa: number of days(0 - 152)

On the diagram: II injection...revaccination

Different preparations(II, II-A, III, III-A)are marked by different lines.

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(cont.from above)
(p.70) ...of each staph.anatoxin purified by this or another method, and sorbed either to $AlPO_4$ or to $Al(OH)_3$. We provide the obtained results in Table 3(see next page).

The obtained data prove that the antigenic properties of one and the same purified anatoxin, sorbed to $AlPO_4$ and to $Al(OH)_3$ are of equal value. However, it should be remarked that Preparation III-A, sorbed to $Al(OH)_3$ had provoked , on the 30th day after the second injection, a higher formation of antitoxin in the animals than the same anatoxin when it was sorbed to $AlPO_4$. In the subsequent period, this difference disappeared, and therefore there is no sufficient ground to talk

of any definite advantage of this preparation.

TABLE 3

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COMPARISON OF THE t-TEST OF THE ANTIGENIC PROPERTIES OF THE PURIFIED STAPHYLOCOCCIC ANATOXINS SORBED TO $AlPO_4$ and to $Al(OH)_3$

Provisional name of the purified anatoxins	Before the second injection	DAYS AFTER SECOND INJECTION					DAYS AFTER REVACCINATION						
		5.	10.	20.	30.	60.	3..5	7	10	15	20	30	72
II	N	D	N	N	N	N	N	N	N	N	N	N	N
II-A	N	D		N	N	N	N	N	N	N	N	N	D
III	D	D	N	N	N	N	N	N	N	N	N	N	N
III-A	D	D	D	D	N	N	N	N	N	N	N	N	N

ANNOTATION: D.... statistically significant difference

N.... " non-significant "

In this manner, from the experiments which were done by us it results that the antigenic and immunogenic properties of the purified sorbed anatoxins do not depend upon the type of the sorbent used/$AlPO_4$ or $Al(OH)_3$/ and upon the methods employed for the purifications.

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STAT

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EXPERIMENTAL STUDY OF THE REACTOGENICITY OF THE $Al(OH)_3$ AND $AlPO_4$ SORBED PURIFIED STAPHYLOCOCCIC ANATOXIN (p. 73- 80)

* * *

At the present time, for the immunological prevention of infectious diseases in human beings, the depot antigens are being widely used. Among the various depot making substances, at the production of the depot anatoxins, practical use comes only to the hydroxide of aluminum and to the phosphate of aluminum.

All purified anatoxins issued by the I.V.S.-s and the Gamaleï Institute of Epidemiology and Microbiology of the Academy of Medical Sciences of the U.S.S.R. were sorbed to $Al(OH)_3$. This adsorbent is widely used also in many foreign countries.

The aluminium phosphate is also widely used abroad(England, Denmark, Switzerland, U.S.A. and others), but its use was almost entirely neglected in our country. Each of these adsorbents has its advantages and its disadvantages(in regard to the complexity of its preparation, its optimum pH for sorption, and in regard to the adsorptive power).

Great significance comes to the reactogenicity of the sorbed preparations when it is the question of an evaluation of the quality of the adsorbent. This question was studied by many investigators, separately for the preparations deposited with aluminium hydroxide or phosphate(A.A.VOROB'EV, and A.V.MARKOVICH, 1953; L.G.KOVTUNOVICH, 1953; A.P.GINDIN & B.V.ZHIV, 1955; HOLT, 1950, and others), however, we did not find any work of comparative studies of the local tissue reactions after the inoculation of one and the same anatoxin, whose separate portions were sorbed on different adsorbents. In relation with this, we have made our project to be a comparative study of the reactive power of the purified staphylococcic anatoxin which was sorbed either to $Al(OH)_3$ or to $AlPO_4$.

(p.74) Identical doses (20 B.U.) of the purified staph. anatoxin Series No.61 were sorbed on two doses of each adsorbent:

$Al(OH)_3$. . . . . 4 and 10 mg Al_2O_3

$Al(PO_4)$. . . . . 4 and 10 mg $AlPO_4$.

(p.74 cont.) FIG. 1:DYNAMISM of the hyperemia of the skin at the site of the inoculation of the staphylococcic preparations.

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Ordinata: sizes in cm^2(areas)

Abscissa: 24 hours and days(3 to 30)

On diagram: _____ $Al(OH)_3$ / 10 mg Al_2O_3/

– – – – – 10 mg $AlPO_4$

———— $Al(OH)_3$ /4 mg Al_2O_3/

– – – – – 4 mg $AlPO_4$

} + plus 20 B.U. of staph. anatoxin.

– – – – – – – – – – –

FIG.2: DYNAMISM OF THE FORMATION AND OF THE RESORPTION OF THE INFILTRATIONS AT THE SITE OF THE INOCULATION OF THE STAPHYLOCOCCIC PREPARATIONS.

Ordinata: diameters of the areas in cm.

Abscissa: time from 24 hours to 3 to 30 days).

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The investigations were done on four groups of rabbits(each group contained 6 rabbits). They had an average weight of 2.0 to 2.5 Kg. The hair on the lateral surface of the body was thoroughly cut off, and the studied preparations were injected subcutaneously in amounts of 1.0 to 1.2 ml. Twentyfour hours, five, ten, fifteen , twenty and thirty days(and in one group also 96 days) after the inoculation a rabbit was killed of each group. The site of the inoculation(p.75) of the preparation was cut out, together with the tissues surrounding it; it was fixed, and preparations were made according to the ordinary histological method, with staining of the sections by hematoxylin and eosin.

At the same time, we produced a recording of the extents of hyperemia(congested area) of the skin and of the infiltrations at the site of the inoculation of the preparations(Fig.1 & 2).

The dimensions of the hyperemic area of the skin reached their maximum already on the first day, independently from the amount and from the type of the adsorbent. In the following day, the hyperemia quickly subsided, and after the inoculation of the preparations which contained 4 mg of $AlPO_4$ or $Al(OH)_3$(4 mg Al_2O_3) it disappeared on the tenth day. However, it should be mentioned that, in this period, the hyperemia of the skin was somewhat more marked after the inoculation of the anatoxin that was sorbed to $Al(OH)_3$.

– MICROPHOTO No.1 –

(p.75 cont.) The maximum dimensions of the area of hyperemia of the skin which was caused by preparations containing 10 mg of $AlPO_4$ or $Al(OH)_3$(10 mg Al_2O_3) have been approximately identical(2.9 to 3.2 cm^2). but, with the use of the Al hydroxide, the hyperemia of the skin kept on for 25 days, while at the site of the injection of the anatoxin which was sorbed to Alphosphate, it had already disappeared on the twelfth day.

(p.76) The study of the dynamisms of the formation of the infiltrations at the site of the inoculation of the sorbed anatoxins showed that in all cases their dimensions were growing until the tenth to the fifteenth day; thereupon they were gradually reduced, and compact subcutaneous formations remained at their sites. On the 30th day, the infiltrations which were formed after the inoculation of the anatoxin that was sorbed to Al hydroxide, independently from its content in the preparation(4 or 10 mg of Al_2O_3), had had larger dimensions in diameter(0.85 cm for the 4 mg and 1.2 cm for the 10 mg of Al_2O_3) than after the inoculation of the anatoxins sorbed to aluminum phosphate(0.1 cm for 4 mg, and 0.5 cm for 10 mg of $AlPO_4$).

= MICROPHOTO No. 2=

(No legend)

In no single case did we observe the formation of abscesses or of necrosis at the site of the inoculation of the preparations.

The microscopic changes in the tissues which were observed after 24 hours were roughly identical for all four preparations. The site of the inoculation of the sorbed preparations(Microphoto 1) represented a focus of inflammation with markedly expressed edema of the derma above it, and inflammation of the connective tissue with edema in the skin of the mice. The injected preparation was revealed in a type of basophil, partly amphophil mass with copious and diffuse and focal infiltration by the polymorphonuclear leukocytes,...(cont. below)

(p.77) Microphoto 3 and 4(without legends)

p.78) Microphoto 5 and 6(no legends)

(text cont.)...more intensively expressed at the periphery of the focus. Around the mass of the inoculated preparation the vessels were dilated and filled with red blood cells and with leukocytes. Some muscle fibers, directly adjacent to the mass of the inoculated preparation, suffered some dystrophic changes.

(p.79) At the comparison of the morphological picture of the histological pre-

parations which were obtained on the fifth day it was impossible to detect any sort of substantial qualitative differences in the nature of the tissue reaction which would depend upon the type of and the dosage of the sorbent.

(p. 79) Tent to fifteen days after the inoculation(Microphoto 2), around the mass of the inoculated preparations a capsule had formed of dense connective tissue, consisting basically of mature elements of connective tissue(fibrocytes, collagenous fibres) and of vessels. Between the clusters of collagen fibers, of the capsule and between the capsule and the focus of inflammation which was infiltrated by leukocytes there was arranged a wide layer of macrophages and of giant cells of the type of "foreign body cells". At certain sites, connective tissue has grown into the peripheral layer of the inflammatory focus. The muscles had normal construction.

From the peculiarity of the tissue reaction at the inoculation of the preparations in this period it should be said that the great portion of the macrophages which were surrounding and embedding themselves into the mass of the injected preparations that contained the aluminum hydroxide ,was found in different phases of disintegration, which has not been observed at the use of the aluminum phosphate (Microphoto 3 and 4).

In a later period of time, on the 20th and 30th days, the proliferation of the granulation tissue, and its maturation have become stronger. At this time also, in the same was as on the 10th and to the 15th day, the macrophagic reaction with the anatoxin that is sorbed to $Al(OH)_3$ was manifested comparatively weaker, and the macrophages which are arranged in the direct neighborhood of the inoculated preparations, had been in different phases of decay which was the most markedly shown (Microphoto 5) after the inoculation of anatoxins with large content of aluminium hydroxide(10 mg of Al_2O_3).

We have carried out a morphological study of the site of injection of the anatoxin which was sorbed to 10 mg of aluminium phosphate, 96 days after the injection. The whole mass of the preparation was permeated by cords from the connective tissue,and it was surrounded by a large amount of macrophages, beyond which the dense connective tissue capsule followed, between the clusters of which plural accumulations of macrophages and polymorphonuclear leukocytes were arranged (Microphoto 6).

According to the data of numerous investigators, the formation of the leukocytic "bulwark" around the injected deposited preparation(I.A.CHALISOV & K.N.SHEVCHENKO*CHALISOVA,1936; L.G.KOVTUNOVICH,1953; A.P.GINDIN & B.V.ZHIV, 1955; YU.B. VOLGIN,1955; HAVENS,1931; GARRISON,1935; EISLEI & EIBL,1949,and others) has a great importance for the resorption of the injected preparation and for the development of immunity.

Thus, A.P.GINDIN and B.V.ZHIV indicate in their work (p.80) that"the resorption of the precipitated vaccins against intestinal infections goes on by means of phagocytosis in the following sequence:

1) phagocytosis of the microscopic and submicroscopic particles of the vaccine by the leukocytes(granulocytes);

2) phagocytosis of the coarser particles by macrophages;

3) phagocytosis of the coarsest particles by giant cells."

EISLER & EIBL also attach a special significance to the accumulation of the phagocytizing cells around the "depot" for the process of immunogenesis, and they think that the smaller the diameters of the particles of the combination of "adsorbent-antigen" are, the easier the phagocytosis will run,and the larger the immunizing effect will be.

In our experiments with the inoculation of the anatoxin sorbed to aluminum hydroxide, into the animals, at various times a disintegration of the macrophages was observed at the site of the inoculation of these preparations. This fact shows that the aluminium hydroxide causes a toxic effect upon the phagocytes,and, in connection with this, the resorption of the antigen which is sorbed to $Al(OH)_3$ will occur under unfavorable conditions.

In this way, our investigations indicate that the purified staph.anatoxin which is sorbed to Al hydroxide, xompared with the same anatoxin sorbed to an equal amount of aluminium phosphate, possesses a great tendency to cause reactions(reactogenicity). This finds corroboration in the large size of the hyperemia of the skin and of the infiltrations, in the duration of their existence, and in an expressed toxic action upon the phagocytizing cells.

The above explanation permits to draw the conclusion that the purified staphylococcic anatoxin which is sorbed to $AlPO_4$ will proveke a slighter reaction, reactive change in the tissue at the site of its inoculation,and therefore, in

this regard, the aluminium phosphate as an adsorbent is superior to the aluminium hydroxide.

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(p.81)

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PRELIMINARY DATA FROM THE STUDY OF THE PERTUSSIS-DIPHTHERIA-TETANUS VACCINE.
(p.81 - 90)

* * *

One of the basic objectives of the Soviet public health service in our times is the prevention of the infections of childhood. The findings of the past few years are also pointing to the need of an active immunization of children against tetanus.

If we pay any attention to the fact that the single-shot inoculations against pertussis, diphtheria and tetanus are not sufficient, the appropriateness of preparing combined preparations for the prevention of these infections becomes entirely obvious.

Since the Gamalei Institute, in accordance with the plans, will issue purified adsorbed diphtheria anatoxin and pertussis vaccine, and since the production of the purified sorbed tetanus anatoxin has been mastered by the Institute, it seems timely to us as well as necessary to arrive at the production of a triple combined preparation which consists of the tetanus and the diphtheria anatoxins, with addition to them of the pertussis vaccine.

By setting out for the work, we set for ourselves the goal to get a combined preparation, to study it tentatively in experiments, and also, as it will be possible, to elaborate methods for its control.

For the production of the combined vaccine we have utilized native as well as concentrated purified tetanus and diphtheria anatoxins and formalinized suspension of the pertussis bacteria. For an adsorbent, we have used aluminium hydroxide which was added to one ml of the preparation at the rate of 2.5 mg. Merthiolate(1:10,000) has been used as a preservative.

The tetanus anatoxin was produced on meatless casein medium; the diphtheria anatoxin was made on a medium of tryptic digestion.

(p.82) The concentration and the purification of these preparations was carried out in compliance with an operating instruction for the purification and concentration of the tetanus and of the diphtheria anatoxins which were elaborated in

the N.F.GAMALEI Institute.

The purified tetanus anatoxin had 1500 B.U. in one ml, with a charge of 2500 B.U. to one mg of protein nitrogen.

In one ml of the diphtheria anatoxin, 280 L.f. was contained with a charge of 550 L.f. on 1 mg of total nitrogen.

The suspension of the pertussis bacteria had 50 billion microbic bodies per one ml. The microbic bodies were cultivated on a thick polysynthetic medium, without the addition of blood. Formalin has been added to the suspension at the rate of 0.02%.

The preparation of the non-adsorbed combined preparations was done by means of mixing the corresponding volumes of the tetanus and diphtheria anatoxins(starting from their concentration), after which the required amount of pertussis vaccine was added.

Samples of the adsorbed combined preparations have been made in the following manner. To the mixture of the purified anatoxins which were taken in the appropriate amounts, the required amount of aluminium hydroxide was added, after which we checked the fullness of the sorption of the preparations. We washed the sorbed antigens three times with physiological saline; after the third washing of the antigens, we removed the supernatant fluid, and to the precipitate we added the required amount of formalinized pertussis bacteria.

We checked the obtained samples for sterility in compliance with the existing instructions for the production of these preparations.

At the selection of the concentration of antigens, we started out from the assumption that, at the immunization of children, into an amount of 0.5 - 1 ml such a quantity of antigen be introduced which would be required for the creation of immunity of a sufficient intensity. At this, we were guided by the available data in the literature as well as by our own preliminary findings during the experiments on animals.

The concentration of the antigens in the prepared samples is illustrated in Table 1.

By approaching the study of the harmlessness of the by us produced combined vaccines and of the components which enter into them, we set for ourselves the task to work out the most suitable method of checking the harmlessness of the pertussis-diphtheria vaccine.

Table 2 illustrates the findings at checking the harmlessness of the individual components which went into the combined preparation, as well as the data at checking the non-adsorbed and adsorbed combined vaccines.

(p.83)

TABLE 1

CONCENTRATION OF ANTIGENS IN THE PREPARED SAMPLES

PREPARATION	/ CONCENTRATION OF ANTIGEN IN ONE ML.
(KDS)= PDT No.6 native anatoxins	/ P 20 billion; D 30 L.f.; T 50 B.U.
PDT No.5 purified anatoxins	/ P 20 billion; D 30 L.f.; T 50 B.U.
APDT No.8 purified sorbed anatoxins	/ P 20 billion; D 30 L.f.; T 50 B.U.; Al_2O_3 2.5mg
PDT purified anatoxins	/ P 40 billion; D 60 L.f.; T 200 B.U.
APDT purified sorbed anatoxins	/ P 40 billion; D 60 L.f.; T 200 B.U.
ANNOTATION:	/ (K):P...pertussis component
	/ D...diphtheria component
	/ (S)T...tetanus component.

- - - - - - - - - - - - - - - -

On the basis of the conducted investigations we have arrived at the conclusion that the sorbed and non-sorbed tetanus and diphtheria anatoxins do not give a reaction in the guinea pigs at the site of the inoculation of the preparation, and that the formalinized pertussis vaccine will give a severe reaction upon its subcutaneous inoculation into guinea pigs, and that it will result in their death.

This may be explained so that the quintuple or the decuple doses of the vaccine for man which we introduced have been too large, or that this method is unsuitable for the examination of the harmlessness(p.86) of the combined preparations which contain the corpuscular antigen.

On the basis of the experiments it was also made clear that the addition of the pertussis component to the diphtheria-tetanus anatoxin has considerably strengthened the reaction in the guinea pigs at the site of the inoculation of the preparation.

The pertussis vaccine, in regard to its harmlessness, as well as the samples of these combined preparations where the pertussis component has entered have been checked on white mice, in accordance with the Instruction for checking the harmlessness of the pertussis vaccine. All experimental animals remained without any visible manifestations of sickness, and all remained alive. It is well known that

the vaccine N I I S I is tested by the same method.

TABLE 2. STAT

HARMLESSNESS OF SUBCUTANEOUS INOCULATION INTO GUINEA PIGS

PREPARATION	NUMBER OF ANIMALS	AMOUNT OF INJECTED PREP. IN ML	SITE OF INOCUL.	REACTION AT THE SITE OF INOCULATION AFTER 15 d.	After 30 d.
a)		b)	c)	d)	e)

(Table taken apart into text)

DIPHTHERIA ANATOXIN, Purified.- a) 2.- b) 5.- c) in the two flanks.- d) uneventful; e) uneventful.

Purified TETANUS ANATOXIN.- a) 2.- b) 5.- c) in the two flanks.- d) uneventful.; e) uneventful.

PERTUSSIS VACCINE.- a) 2.- b) 5.- c) in the two flanks.; d) necrosis.- e) the guinea pigs died.

DT(Diphtheria plus tetanus purified anatoxins).- a) 2.- b) 5.- c) in the two flanks.- d) uneventful; e) uneventful.

ADT.- a) 7.- b) 5.- c) in the two flanks; d) depot of the size of a pea. e) depot of pea size.

ADT.- a) 5.- b) 3.- c) in the flanks and leg, each one ml; d) depot of the size of a millet grain; e) depot of the size of a millet grain.

PDT(non-adsorbed).- a) 2.- b) 5.- c) in the two flanks.-d) 1. necrosis on both sides, 2. infiltration of 3-4 cm diameter.- e) 1. scars on both sides, 2. cold abscess.

PDT(non adsorbed).- a) 2.- b) 3 .- c) in the two flanks; d) 1. with flank depots the size of a pea; 2. necrosis of both flanks; e) 1. cords on both sides, 2. necrosis left, scar on right side.

THE SAME.- a) 2.- b) 3.- c) in the two flanks and leg. each one ml.; d) 1. with flank depots the size of a pea, infiltration under the legs, 2. uneventful on flanks, infiltration on legs; e) 1. uneventful, 2. uneventful.

APDT(adsorbed).- a) 2.- b) 5.- c) in the two flanks; d) 1. open abscess.- dense infiltration of the size of a hazelnut, 2. on both sides, open depot the size of a hazelnut; e) 1. open depot, 2. slight scar at site of injection.

THE SAME.- a) 2.- b) 3.- c) in the two flanks; d(1. depot on both sides the size of a hazelnut, 2. the same.- e) 1. open depot, 2. on both sides, open depot. the size of a pea. (over).

(TABLE 2 cont.)

APDT.- a) 1.- b) 3.- c) in the two flanks and legs, each one ml. d) with STAT flank depots the size of a hazelnut, under the leg infiltration; e) with depots at the flank and under the leg of a diameter of a large bean.

* * * * * - - - - - - - -

(p.86 cont.) On the basis of the above exposed facts, we have come to the conclusion that the checking of harmlessness of the PDT and APDT, in regard to their pertussis component, is properly done on mice(white mice) as this is made at the verification of the harmlessness of the pertussis vaccine. The harmlessness of the diphtheria and tetanus anatoxins is checked as usual.

TABLE # 3

IMMUNIZATION OF GUINEA PIGS WITH PDT & APDT - - - -

a	b	c	d	e	f	g	h	i
PDT No.6 native anatoxins	P 20 bill.	-	-	-	-	30	6	-
	D 30 L.f.	-	1	11	0.04	100	5	2
	T 50 B.U.	1	-	6	0.1	100	5	-
	(binding units)							
PDT No.5 Purified anatoxins	P 20 bill.	-	-	-	-	-	-	-
	D 30 L.f.	-	0.2	8	0.02	100	4	4
	T 50 B.U.	1	-	2	0.001	100	5	-
APDT Purified sorbed anatoxin	P 20 billion	-	-	-	-	-	-	-
	D 30 L.f.	-	0.2	7	1.14	100	7	-
	T 50 B.U.	1	-	7	>0.1<1	500	7	-

HEADINGS: a...Preparation; b...concentration of antigens in one ml; c...amount of inoculated vaccine, ml, for checking the tetanus component; d...amount of inoculated vaccine, ml, for checking the diphtheria component; e...number of guinea pigs; f...amount of antitoxin in A.U.; g...toxin in M.L.D. after 30 days; h...survived guinea pigs; i...dead guinea pigs.

ANNOTATION: (K): P...pertussis component

D....diphtheria component

(S): T...tetanus component

- -

(p.87) Nothwithstanding the fact that the inoculation of the combined vaccine into the guinea pigs was accompanied by inflammatory symptoms, and that the inoculation of this preparation into white mice does not result in such symptoms, nevertheless, in regard to the tetanus and the diphtheria anatoxins, we propose to carry out the checking of the harmlessness of the vaccine on guinea pigs in view of the considerable sensitiveness of these animals to the toxins of the tetanus and diphtheria bacilli.

The experiment for the study of the immunological effectiveness of the combined vaccine is shown in Table 3.

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Three samples were taken for the experiment:

1. PDT No. 6, prepared from native anatoxins, with addition of the pertussis vaccine to them;

2. PDT No. 5, into which went the purified tetanus and diphtheria anatoxins, with subsequent addition to them of the pertussis component;

3. APDT No. 8, whose component parts are purified anatoxins sorbed on aluminium hydroxide, with addition of a formalinized suspension of pertussis bacilli to it.

The concentration of the antigens is shown in the Table.

If, at the immunization with 0.2 ml of Preparation No. 5, which was prepared from purified anatoxins, 0.02 A.U.* of diphtheria antitoxin has been obtained (*FOOTNOTE: with titration by the method of JENSEN, modified by K.T. CHALYAPIN), then, at the immunization of the animals with the same amount of the sorbed preparation we had already received 1.14 A.U.

The same is also true in regard to the tetanus component:- at the immunization with the purified preparation it was 1/1000 A.U., and at the immunization with the purified sorbed anatoxin it reached $>0.1<1$ A.U.

The complex preparation composed of native anatoxins, by its immunogenicity, has yielded to the combined preparation into which the purified sorbed anatoxins were mixed, nevertheless the accumulation of the antitoxin in the animals was satisfactory. None the less, we do not consider it adequate that the combined native preparations can be produced in view of the possible sensitization of the organisms with their employment.

The quoted experiments showed that the best antigen which has the greatest immunological effectiveness is the APDT which includes the purified aluminium-hydroxide adsorbed anatoxins, which is completely in accordance with the findings available in the literature on this subject.

The determination of the immunological propertees of the combined preparations, as to the pertussis component, is carried out (p. 88) by the method of KENDRICK (U.S.A.) by means of a single-shot intraabdominal immunization of mice with subsequent intracerebral infection (Stock of Kendrick, No. 18323).

The pertussis vaccine was checked before its addition, and mixture with the

complex preparations as well as the immunogenicity of the combined vaccines PDT and APDT has been studied among whose composing parts this series of pertussis vaccine was also present.

(p.88) TABLE 4

IMMUNIZATION OF GUINEA PIGS WITH PDT AND APDT

PREPARATION	CONCENTRATION OF ANTIGEN IN ONE ML	a	b	c	d	e	f	g
PDT	P suspension 40 bill. D anat. 60 L.f. T anat. 200 BU	0.5	-	17	0.03	D500	4	2
					-0.95	T500	5	-
					Simultaneously			
						T500 D500	5	3
APDT	P 40 bill. D 60 L.f. T 200 B.U.	0.5		19	0.25	D500	4	-
						D1000	1	1
			0.5	19	1.5	T500	4	-
						T500	2	-
					Simultaneously			
						T500 D500	5	2
Tetanus anatoxin	Purif. sorbed 100 B.U.		1	10	2	1000	10	-
	Purified 100 B.U		1	10	0.1	100	10	-
Diphtheria anatoxin	Purified sorbed 30 L.f.	1		10	0.25 -0.5	500	5	2
	Purified 30 L.f.	1		10	0.015 -0.03	30	3	-

* * * * * -

The experience showed that the immunogenicity of the pertussis component in the PDT and the APDT has been the same as in the initial series of the pertussis vaccine. On the other hand, it should be remarked that at the present time there is no reliable method for the determination of the immunogenic properties of the pertussis vaccine and only by the findings of the observation can authentic data be obtained on the effectiveness of the pertussis antigen.

In the preliminary experiments we wished to find whether an inhibition of any of the components would exist which components were added to the samples of our combined preparations. For this purpose, an experiment was arranged which is shown in Table 4(above).

We immunized the animals with the compex vaccine preparation made from purified anatoxins, with the addition of the pertussis vaccine to it(PDT) as well as STAT immunization was made on guinea pigs with a combined vaccine composed of the purified sorbed anatoxins, with the addition of the formalinized pertussis microbes to it(APDT).

As controls we have used guinea pigs which had been separately immunized with the purified and with the purified sorbed tetanus anatoxin, and we also used animals which were given injections of both the native and the purified sorbed diphtheria anatoxin.

The conducted experiments did not permit to notice a marked competition of the anatoxins which were put into the combined preparation.

This problem, in regard to the PDT and the APDT was left open by us since it requires serious elaboration which could not be done during the preliminary experiments. It should be remarked that the competition will be necessarily visible and noticeable if the quantitative ratios of the antigens will not match. To correctly balance the antigens in the combined samples is not an easy matter; however, we endeavour to do this in our further work.

CONCLUSIONS

1) the pertussis-diphtheria-tetanus vaccine(PDT) can be produced from native and purified anatoxins, with the additon of the pertussis vaccine. The adsorbed (p.90)pertussis-diphtheria-tetanus vaccine(APDT) is produced from purified sorbed anatoxins with the subsequent addition to it of the pertussis vaccine.

2) The harmlessness of the PDT and of the APDT, in regard to the pertussis component, is checked on white mice in compliance with the Instruction for the testing of the harmlessness of the pertussis vaccine. The harmlessness of the PDT and of the APDT, in regard to the tetanus and the diphtheria components, is checked on guinea pigs by means of inoculation of 3 ml of the vaccine under the skin of the flank(in two times, each with 1.5 ml).

3). In comparison with the PDT, the APDT possesses a great immunogenic effectiveness in regard to the tetanus and the diphtheria components.

4) The immunogenicity of the pertussis component in the PDT and in the APDT has been the same which is also in the initial series of the pertussis vaccine.

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(over)

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(p.91)

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STAT

PREVENTIVE PROPERTIES OF THE ALPHA-ANTITOXIN OF THE TYPE-A PERFRINGENS AT THE PASSIVE AND ACTIVE IMMUNITY IN RELATION TO THE VARIOUS MEANS OF INFECTION
(p.91 - 99)

* * *

The data published in the literature on the serum prophylaxis and the serum therapy of gas gangrene during the period of the Second World War are chiefly talking of the fact that the positive results which were obtained in the trials on experimental animals have not been sufficient corroboration in themselves for a wide use of the sera under the conditions of war.

According to the data of McCLENNAN, in 1917-1918 the morbidity of gas gangrene had been 3.6 cases among one thousand wounded. In the Second World War, according to the data of various authors, the morbidity has been in the range of from 4 to 45 cases per 1000 of injured. (ODOM--4.5; NIL & KOLLE--7; LANGLEY & WINKELSTEIN --9.5; D'EP---30; ROSS & RAIEN---45 cases).

The introduction of massive doses of specific antitoxic sera have comparatively slightly reduced the mortality among the sick from gas gangrene. Meanwhile, the experimental data indicate quite clearly that the alpha-antitoxin not only protects the animals from infection, but it also possesses therapeutic properties. The cause of the noted non-concordance between the experimental material and the data of their epidemiological verification is left at the present time not satisfactorily explained.

In recent times, it was noted that the lethal dose at the infection with perfringens bacillus is considerably decreased (reduced) (smaller) in those cases in which the culture is introduced into the wound together with little pieces of gauze or with dirt onto the injured muscles. It is supposed that the malignant course of gas gangrene depends not only upon the action of the bacterial toxins but also upon other factors which are taking place at the septic degeneration of the devitalized tissues. Therefore, particular importance is attributed to the decision of the question about the protective role of the antitoxin at passive and

(p.92 cont.) particularly at active immunization, under conditions of infections which come near to a natural infection.

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With this purpose, we have arranged a series of experiments of infecting the passively and actively immunized animals(guinea pigs and rabbits) by two methods: 1) by inoculation of cultures in a mixture with chlorinated calcium into the undamaged muscles, and 2) by putting into the wounds on crushed muscles small pieces of gauze which XXXcontained the cultures of the perfringens bacillus.

METHODOLOGY OF INFECTION AND RESULTS

A day's culture of B.perfringens, Stock SR_{12} was washed out from the toxin by means of thrice centrifuging it in physiological saline, and it was brought up by the optical standard to one billion microbes(microbic bodies) in one ml. From the basic dilution, suspensions of microbic bodies were prepared containing 200 - 100 - 50 - 20 - 10 million microbic bodies in one ml. Guinea pigs which weighed from 300 to 350 g were infected with one ml culture of each dilution. In the experiment forty guinea pigs were taken. To 20 guinea pigs of the first group, the cultures were inoculated into the undamaged muscles in a mixture with 0.1 ml of a 30% solution of calcium chloride. Each dose of the culture was inoculated into each of 4 guinea pigs.

Twenty guinea pigs of the second group were infected by bringing the culture into the wound with injury of the integrity of the muscular tissue. The infection of these pigs was done in the following manner:- from the surface of the rear thigh the fur was cut off, the skin was rubbed with alcohol and smeared with iodine. Along the adductor muscles of the thigh, the skin was incised, the muscle was elevated, and crushed with a Kocher forceps. Under the damaged muscle, pieces of gauze were laid, containing a culture of the perfringens bacillus, after which the ends of the wound were sewed up. Four guinea pigs were thus inoculated, each with a dose of the culture.

The observation of the inoculated animals was carried out for the period of fourteen days. The obtained results are represented in Table 1.

The results given in Table 1 indicate that, after the inoculation of the B. perfringens culture into the undamaged muscles, the death of the animals comes from considerably larged doses of the culture than in the cases of infection of the wounds with presence of injured muscles. The guinea pigs inoculated intramus-

cularly by the introduction of 200 million microbic bodies died with developed gas gangrene 72 to 96 hours after the infection. Of four pigs inoculated with 100 million microbic bodies, one died; in the surviving three pigs, a gangrene of moderate severity has developed, manifested by edema(p.93) of the hind extremity, with subsequent necrosis, but the general condition of the animals was satisfactory, the wounds healed with cicatrization, and the animals recovered their health.

TABLE 1

INDEX MORTALITY OF THE B. PERFRINGENS DOSE AT DIFFERENT METHODS OF INOCULATION OF THE CULTURE

MODE OF INOCULATION	Infecting dose of culture (millions)	Obtained results*	Time of death of the animals, hours
Inoculation of culture into undamaged muscles	200	4 / 0*	48 - 96
Same	100	4 / 3	72
Same	50	4 / 4	-
Same	25	4 / 4	-
Same	20	4 / 4	-
Infection of wound with injury of muscles	200	4 / 0	24 - 48
Same	100	4 / 0	24 - 48
Same	50	4 / 0	48 - 96
Same	25	4 / 0	24 - 96
Same	10	4 / 1	72 - 96

* Numerator...number of animals dying in the experiment; denominator...number of animals left alive.

In the guinea pigs infected with 50 - 25 - 10 million microbic bodies, only a local symptom was noticed in the form of dense infiltrations, resorbed in 15 to 20 days.

Different results have been obtained after putting the microbic bodies into the wound with additional injury to the muscular tissue. All animals infected with 200 - 100 - 50 - 25 million microbic bodies, became seriously sick, and they died within 24 to 96 hours after the infection. From four guinea pigs, which were

infected with 10 million microbic bodies, three guinea pigs died.

These experiments had corroborated the available findings that the lethal dose of the B.perfringens culture is considerably reduced at the inoculation which follows after the wounding, in presence of a damaged tissue in the wound. It may be thought that, after m i l i t a r y i n j u r i e s , in the presence of considerably deeper(more profound) injury of the tissues, with fractures of the bones, by the damage of the large blood vessels considerably less insemination of the wound with bacteria is sufficient for the development of a lethal infection than this is observed under the conditions of our experiment.

(p.94) STRENGTH OF IMMUNITY, IN PASSIVELY IMMUNIZED GUINEA PIGS WITH DIFFERENT METHODS OF INFECTION.

For the purpose of determining the relationship between the preventive properties of the Alpha-anatoxin of perfringens at the passive immunization, and with the various methods of its inoculation, we have given various doses of the anti-perfringens serum to guinea pigs 24 hours after their infection. The serum was injected at one ml amount which contained 50 - 100 - 200 - 250 A.units. Each dose of antitoxin was given to ten guinea pigs. The titre of the antitoxin in the blood of the inoculated animals was tested 6 hours, and 24 hours after the injection of the serum. The mixture of blood sera of each group of guinea pigs was titrated. In Table 2, the results of the titration are included.

TABLE 2

TITRE OF ANTITOXIN IN THE BLOOD OF GUINEA PIGS AFTER INJECTION OF VARIOUS DOSES OF THE SERUM

NUMBER OF PIGS	AMOUNT OF ANTITOXIN, A.U., GIVEN TO EACH PIG	TITRE OF ANTITOXIN A.U. after 6 hours		A.U. after 24 hours	
10	50	0.05	0.1	0.1	0.5
10	100	0.5	1.0	1.0	2.0
10	200	0.5	1.0	2.0	3.0
10	250	1.0	2.0	2.0	3.0

Twentyfour hours after the injection of the serum into forty guinea pigs, they were infected with the culture of B.perfringens which was washed from the toxin.

In twenty pigs, the inoculation was made into undamaged muscles with the culture which contained 200 million microbic bodies in one ml, in the same wasy as in the preliminary experiments, in a mixture with 0.1 ml of a 30% solution of calcium chloride.

The remaining 20 guinea pigs were inoculated by putting the culture on pieces of gauze into the crushed muscles. From them, each of ten pigs received 100 million microbic bodies, and each of ten others received 10 million.

The results, given in Table 3, indicate that 0.1 A.U. of antitoxin of the perfringens protected the animals from infection with 200 million microbic bodies in cases when the infection was without an injury to the muscles. Out of five guinea pigs which received 50 A.U. and had 0.1 A.U. in their blood, only one guinea pig died nine days after the infection. In the four other guinea pigs, in the region of the inoculation of the culture, edemas and subsequently necrosis developed. The guinea pigs remained alive.

In the pigs which were given 100 - 200 and 250 A.U. and whose antitoxin content in the blood has reached 1 - 2 A.U., only an insignificant infiltration was noticed. Two control guinea pigs, inoculated with 100 million microbic bodies, died 72 hours after the infection.

Wholly different data have been obtained as a result of the inoculation of the guinea pigs with B. perfringens culture under the condition of an injury of the muscular tissue. The inoculation of 100 million microbic bodies led to the death of all guinea pigs, notwithstanding the high content in one ml of the blood in antitoxin(1 - 2 A.U.). At inoculation of 10 million microbic bodies, three pigs which received 50 A.U. were also killed; of three pigs which received 200 A.U. one remained alive; all the pigs which received 200 - 250 A.U. remained alive.

(p.96) In this manner, these experiments clearly indicated that the strength of the passive immunity is not identical in case of the different methods of infecting the animals. If at the infection without an injury of the muscles the content of 0.1 A.U. is sufficient for the end that the animal be protected from death, then, at injury of the muscles and a disturbance of the oxidative processes in the tissues even 1-2 A.U. in a ml of blood is not sufficient for the prevention of the death of the animals, due to a quickly developing infection.

(over Table 3)

TABLE 3

RESISTANCE OF THE PASSIVELY IMMUNIZED GUINEA PIGS TO INFECTION WITH B. PERFRINGENS CULTURE

METHOD OF INFECTION	AMOUNT (A.U.) OF PASSIVELY INJECTED ANTITOXIN	AVERAGE TITRE OF ANTITOXIN BEFORE INFECTION in A.U.	DOSE OF CULTURE AT INFECTION (in millions)	RESULTS
Intramuscular without injury of muscle	50	0.1	200	5 / 4*
Same	100	1.0	200	5 / 5
Same	200	2.0	200	5 / 5
Same	250	2.0	200	5 / 5
	CONTROL	-	100	2 / 0
INFECTION of wound with injury of muscle	50	0.1	100	2 / 0
Same	100	1.0	100	2 / 0
Same	200	2.0	100	2 / 0
Same	250	2.0	100	2 / 0
Same	50	0.1	10	3 / 0
Same	100	1.0	10	3 / 1
Same	200	2.0(2.0	10	3 / 3
Same	250	2.0	10	3 / 3

* Numerator... number of animals in the experiment;
denominator...number of animals remaining alive.

RESISTANCE OF THE ACTIVELY IMMUNIZED RABBITS TO INOCULATION WITH THE B. PERFRINGENS CULTURE.

The second series of experiments was arranged on rabbits for the clarification(under different conditions of infection) of the preventive action of the perfringens antitoxin which has formed in the organism of the animal as a result of an active immunization.

In advance, in a number of experiments the value of the lethal dose of the B. perfringens culture(washed from toxin) was determined for rabbits under various conditions of infection(Table 4).

TABLE 4

MINIMUM LETHALITY OF THE B. PERFRINGENS STAT DOSE

for rabbits in case of different ways of inoculating the culture

MODE OF INFECTION	INFECTING DOSE OF CULTURE, billions	RESULTS	TIME OF DEATH IN HOURS
Intramuscular inoculation of culture without injury to muscle	1.0	2 / 2*	-
Same	1.5	2 / 2	-
Same	2.0	2 / 1	96
Same	2.5	2 / 0	48 - 72
Infection of wound with injury to muscle	1.0	2 / 1	120
Same	1.5	2 / 0	96
Same	2.0	2 / 0	72 - 96
Same	2.5	2 / 0	-

* NUMERATOR. . . number of animals in the experiment;

DENOMINATOR. . . number of animals remaining alive.

The data given in Table 4 indicate that the size of the lethal dose of the culture for rabbits is also changing in relationship with the way of inoculation which has been also confirmed by the data obtained in the experiment with guinea-pigs.

For the active immunization, rabbits were used which weighed 2 Kg; there were a total of 28 rabbits. They were immunized with two shots under the skin, each shot being one and 2 ml of perfringens anatoxin containing 26(25) B.U. in one ml; the injections were given at 20-day intervals. Fifteen days after the injection the titre of the antitoxin in the blood was determined(the titration was done intravenously on mice), and the animals were subjected to the inoculation of the infecting agent. The methods were the same two which had been used in the previous experiments. The observation on the animals was done for fourteen days.

The obtained results are given in Table 5.

(see next page)/

TABLE 5

RESISTANCE OF THE ACTIVELY IMMUNIZED RABBITS TO THE INFECTION WITH B. PERFRINGENS, RELATED TO THE VARIOUS METHODS OF INFECTION

METHOD OF INFECTION	TITRE OF ANTITOXIN BEFORE INFECTION in B.U.	IMMUNIZING DOSAGE OF CULTURE in billions	RESULTS
Intramuscular infection with culture into uninjured muscle	>0.25<0.5	2.5	2 / 2*
Same	>0.5 <1.0	2.5	3 / 2
Same	>1.0 <2.0	2.5	5 / 5
	>2.0 <3.0	2.5	8 / 8
	CONTROL -0	2.5	3 / 0
Infection of wound with injury of muscle	>0.25<0.5	2.5	2 / 2
	>0.5 <1.0	2.5	3 / 2
	>1.0 <2.0	2.5	3 / 2
	> 2.0 <5.0	2.5	2 / 2
	CONTROL -0	2.5	2 / 0

* NUMERATOR. . . number of animals used in the experiment;

DENOMINATOR. . . number of animals remaining alive.

As it can be seen from the given table, in 17 animals which were intramuscularly infected without an injury of the muscle, within 24 hours after the introduction of the culture only one bad reaction was noticed which(p.98) was manifested in the consolidation and edema of the tissues and soreness and tenderness at touch. In 48-96 hours, the local manifestations have almost completely disappeared, and the general condition of the animals became good.

One rabbit in whose blood there was 0.5 0.1 A.U. died from gas gangrene 4 days after the inoculation.

Three control rabbits died 48-72 hours after the infection. All the other animals remained healthy.

Out of ten rabbits which were subjected to the inoculation with injury of the

muscles, 4-5 days after the infection two died. In one of them the antitoxin titre has been >0.5<1.0 A.U. ; in the other >1.0<2.0 A.U. The rabbits in whose blood there was >0.25<0.5 A.U., remained alive. Two of the control rabbits infected with 2.5 billion microbic bodies died 40 hours after the inoculation. In two rabbits suppuration appeared in the region of the wound, in some time the wounds cleared up and healed with cicatrization.

STAT

In this manner, it can be said that, under the condition of an active immunization, the intensity of the immunity against gas gangrene that was incited by inoculation of a culture of B. prefringens into the wound had been considerably higher than in the animals which had received passive immunization. It is evident that the resistance to infection is considerably increased in the actively immunized organism in comparison with the passively immunized ones. In case of a passive immunization a rather large concentration of antitoxin is required in the blood for the protection of the animal from death. However, it must be remarked that in one case the actively immunized rabbit died, notwithstanding the presence of a considerable amount of antitoxin in its blood. It is possible that this depends upon the quickly onsetting disintegration of the tissues and, as a result, upon the accumulation of its non-specific toxic products which cannot be neutralized by the antitoxin of the perfringens bacillus.

CONCLUSIONS.

1) The minimum lethal dose of the B. perfringens culture is not a fixed value and it depends upon those conditions which are created in the organism in presence of the infection. With the inoculation of the culture in a mixture with calcium chloride, without damage of the muscles, a considerably larger amount of microbic bodies is required in order to cause the death of the animal than at the infection accompanied by crushing of the muscular tissue.

2) In case of passive immunity, the resistance of the infected animals is not identical, and it depends upon the way of the infection. In case of an infection of the culture into the healthy muscle, 0.1 A.U. in one ml of blood is enough to protect the animal from death. The disturbance of the oxidative processes in the muscles, which ensue as a (p. 99) result of their injury, lowers the intensity of the immunity. The presence of 1 (one) A.U. in one ml of blood, in the majority of cases, will not protect the animals from a lethal dose of the culture. The con-

(p.99 cont.)tent of 2 A.U. in one ml of blood has protected all animals from death.

3) In case of the same conditions, in the actively immunized anis[redacted], the power of immunity is considerably higher than in case of passive immunity. In case of infection of the wound which also has a damage of the muscles, 0.25 A.U. is enough to protect the animals from an infection with a massive dose of the culture. However, under some conditions, the presence of 1 (one) A.U. had not protected the animals from death.

(No literature is quoted)

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(p.101)

L.YA.MARMALEVSKAYA

STAT

(Biochemical Department; Chief:- V.A.Blagoveshchenskii)

METHODS OF CONCENTRATION AND PURIFICATION OF THE PERFRINGENS ANATOXINS PRODUCED ON CASEIN MEDIA* (p.101 - 108)

(FOOTNOTE:This material was reported at the scientific council of the Gamalei Institute of Epidemiology and Microbiology, in 1957).

* * *

The basic condition of the solution of the problem of active immunization against gas gangrene is the obtaining of highly antigenic and immunogenic anatoxins,which is particularly important in respect to the chief agent of the gas gangrene-- the B.perfringens.

The use of native perfringens anatoxins for the purpose of active immunization is extremely hindered on account of the considerable pollution of them with ballast(inert) materials of the nutrient medium as well as with the products of the metabolism and of the disintegration(lysis) of the bacterial cell.

The purpose of the present work was the elaboration of an industrial method for the concentration and the purification of the perfringens anatoxins to the end of their use for the active immunization of people against one of the principal agents of gas gangrene.

Notwithstanding the weight of the problem of a specific prophylaxis of gas gangrene,extremely limited is the number of the works in the literature which refer to the purification and concentration of toxins and of anatoxins for the active immunization against this infection.

For the purification of the perfringens toxins, a number of authors--HENRY and LACEY(5), ADAMS & HENDEE(4), VAN HEYNINGEN(9)-- have used the fractional precipitation with ammonium sulfate.

For the purification of anatoxins, S.A.ZELEVINSKAYA and co-authors(2), T.P. CHERKAS(3) have used combined methods uniting the $(NH_4)_2SO_4$ fractionation with the adsorption and the elution of the preparations.

Satisfactory results(p.102) have been obtained with the precipitation of a number of toxins with various acids/PENFOLD(6),TURPIN (8),V.A.BLAGOVESHCHENSKII(1)/

The object of the numerous researches of PILLEMER and of his collaborators(7) has been the fractional precipitation of toxins and anatoxins in a methanol -

aqueous mixture with regard to pH, ionic power and temperature.

In the present work we used those methods of purification and STATconcentration of the anatoxins which, according to the data in the literature, had given the best results.

EXPERIMENTAL PART

The native perfringens anatoxins were the culture medium itself of the B. perfringens which had been cleaned of the microbic bodies and was made harmless (detoxicated) by formalin.

The content in total nitrogen varied in the different series of anatoxins from 2.6 to 6.8 mg in one ml.

The antitoxin-binding capacity of the preparations has been from 0.5 to 2 B.U. in one ml.

The native anatoxins were submitted to concentration and purification by precipitation with one Normal hydrochloric acid at the isolectric point. For this purpose, to the native anatoxins 25 wt% of table salt was added, after the solution of which the isoelectric point was determined in a separate test. The isoelectric point for the various series of anatoxin usually varied in the range of the pH values from 3.5 to 4.0.

The required amount of hydrochloric acid was added after the evaluation of all precipitable amount of anatoxin. The precipitate was removed by separating, it was dissolved in physiological solution in 1/20 part of the initial volume, with addition(drop-wise) of a 30% NaOH until the pH of 6.4 to 6.7 was reached.

As it is seen from the data presented in Table 1, the purification as to the protein nitrogen has been up to 85%, with an output of biological activity from 50 to 100%.

The concentrates were subjected to second re-precipitation with hydrochloric acid, acetic acid, trichloracetic acid, and metaphosphoric acids under identical conditions.

As the experiments proved, after the second precipitations with the listed acids, the concentrates still contained a considerable amount of ballast(inert) substances and they were markedly pigmented, which hindered their use for active immunization.

(over)

PRECIPITATION WITH NEUTRAL SUBSTANCES:

ETHYL ALCOHOL and ACETONE.

STAT

For getting highly purified preparations of the perfringens anatoxin, the conditions of precipitation were studied with ethyl alcohol and acetone, with strict regard to the pH, ionic power and temperature of the anatoxin concentrates which were obtained after precipitation of the native anatoxins at the isoelectric point of unit-normal hydrochloric acid.

On the basis of the large number of the experiments, it was established that, for the precipitation of the anatoxin with ethyl alcohol or with acetone, the value of the ionic power should not exceed 0.1. The optimum value of pH for the precipitation, with the given ionic powder, was equal to 6.4 - 6.7.

The concentrate, with an ionic strength of 0.1 - 0.07, and with a pH of 6.4 to 6.7, was cooled in advance to the temperature of -3°C to -4°C; to it, an equal amount of acetone or 1½ volume of 96° ethyl alcohol was added, precipitated at -10 to -15°C. In 5 to 10 minutes, a flaky precipitate fell out which was separated by centrifugation. The obtained precipitate was easily dissolved in physiological saline and it was also concentrated 4-5 times in relation to the initial concentrate. The findings about the concentration and about the purification with ethyl alcohol and acetone are represented in Tables 2 and 3 of which it is evident that with these methods high-purity anatoxins were produced(from 67 to 97.7 % by the protein nitrogen content) with an output of anatoxin as to B.U. of an average of 60 to 80% of that of the native anatoxin.

By these methods, we succeeded to a considerable degree to get rid of the pigment matters accompanying the active protein.

With the given methods we have concentrated and purified 5000 liters of native perfringens anatoxin for industrial purposes.

ADSORPTION AND ELUTION.

For the purification of the perfringens anatoxins we have also utilized the method of adsorption and elution.

As an adsorbent, aluminium hydroxide was used which was prepared according to the altered prescription(formula) of WILLSTETTER, accepted for the production of the adsorbed diphtheria anatoxin.

On the basis of the conducted investigations of the conditions of adsorption

and elution of the perfringens anatoxin, it is possible to recommend the following methodology for the purification of this antigen:- the concentrate of the STAT anatoxin is diluted to its double with M/60 buffer phosphate at pH = 4.6(text continues after the Tables...)

(p.104)

TABLE 1

CONCENTRATION OF THE NATIVE PERFRINGENS ANATOXINS AT THE ISOELECTRIC POINT

a	b	c	d	e	f	g	h	i	j	k	l	/#/

HEADINGS: a...Series of anatoxin; b...Native:activity by B.U. in one ml; c...Native:total N mg%; d...protein N mg% native; e...Native: charge of B.U. per 1 mg of total N; f...Native: charge of B.U. per 1 mg of protein N; g...CONCENTRATION 20 times by volume: activity by B.U. in one ml; h... Concentrate: total N in mg%; i...Concentrate:protein N in mg%; j...Concentrate: charge of B.U. per 1 mg of protein N; k...% of purification by protein nitrogen; l...percentage of output in B.U.

608	1	357.0	10.0	0.3	10	10 = 20	204.5	173.0	8.6	13.5	75	
623	0.5	378.0	8.4	0.1	6	5	183.3	134.1	4.0	21	50	
609	1	380.0	16.7	0.3	6	20	71.4	73.2	30	78.7	100	
627	0.5	504.2	24.0	0.1	2	10	82.1	70.0	14	85.5	100	

TABLE 2.

CONCENTRATION AND PURIFICATION OF PERFRINGENS ANATOXIN WITH ETHYL-ALCOHOL

(Table turned into text: HEADINGS: a...Series of anatoxin;
NATIVE ANATOXIN: b...activity in B.U. per one ml; c...Total N mg%; d...protein N mg%; e...charge of B.U. per 1 mg% of total N; f...charge of B.U. per one mg% of protein N.
CONCENTRATE 20 times by volume,1 N-HCl: g...activity in B.U. in on mg; h...protein N in mg%; i...charge of B.U. per 1 mg% of protein N.
CONCENTRATE WITH ETHYL ALCOHOL: j...activity in B.U. in one mg; k...protein N in mg%; l...charge of B.U. per one mg of protein N; m...concentration by volume.
*** n...% of purification by total N; ~~X~~ o...% of purification by protein N; p...% of output in B.U. of the native anatoxin.

* ANATOXIN 640 plus 639; b...1; c...649.6; d...121.4; e...0.21; f...1.2; g...20; h...203; i...9.8; j...80; k...67.2; l...120; m...4; ~~X~~ n...99.9; o...99.3; p...80.

* *ANATOXIN 644: b...1; c...574.0; d...23.0; e...0.17; f...4.3; g...20; h...120; i...16.6; j...60; k...50.4; l...120; m...4; n...99.9; o...97.0; p...75.

(cont.over)

(table 2 cont.)

*ANATOXIN 644 plus 643: b...1; c...580.0; d...25.0; e...0.17; f...4.0; g...20; h...134.4; i...11.0; j...60; k...46.2; l...130.4; m...4; n...99.9; o...99.1; p...75.

*ANATOXIN 644: b...; c...299.6; d...95.0; e...0.47; f...1.5; g...20; h...97.8; i...20.4; j...80; k...42.0; l...190; m...4; n...99.9; o...94.5; p...66.

*ANATOXIN 649: b...1; c...326.2; d...12.6; e...0.3; f...8.3; g...20; h...102.2; i...10.0; j...40 to 60; k...25.2; l...200; m...4; n...99.9; o...97.6; p...62.

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(p.105)

TABLE 3

CONCENTRATION AND PURIFICATION OF PERFRINGENS ANATOXIN WITH ACETONE

(Table transformed into text; Headings same as for Table 2, but columns "j,k,l,m" are for CONCENTRATE WITH ACETONE)

* ANATOXIN 611: b...1; c...392.0; d...34.0; e...0.2; f...3.0; g...20; h...20.0; i...71.0; j...60; k...61.6; l...100; m...3; n...99.6; o...97.0; p...100.

* *ANATOXIN 620 :b...0.5; c...411.6; d...280.0; e...0.1; f...0.2; g...10; h...124.6; i...8; j...40; k...78.4; l...64; m...5; n...99.9; o...99.7; p...80.

*ANATOXIN 608: b...1; c...357.0; d...10.0; e...0.3; f...10; g...20; h...240.0; i...9; j...60; k...184.0; l...38; m...4; n...99.4; o...77.0; p...75.

*ANATOXIN 623: b...0.5; c...378.0; d...8.4; e...0.1; f...6; g...10; h...104.0; i...10; j...50; k...275.8; l...21; m...5; n...99.3; o...67.2; p...100.

* *ANATOXIN 609: b...1; c...390.0; d...16.7; e...0.9; f...6; g...20; h...71.4; i...28; j...40; k...114.8; l...44; m...4; n...99.8; o...91.4; p...50.

*ANATOXIN 626: b...0.5; c...553.0; d...99.4; e...0.09; f...0.5; g...10; h...233.8; i...4.3; j...50; k...30.8; l...183; m...5; n...99.9; o...99.7; p...100.

*ANATOXIN 627: b...0.5; c...504.2; d...24.0; e...0.09; f...2; g...10; h...70.0; i...14; j...40; k...84.0; l...47; m...4; n...99.9; o...96.4; p...100.

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TABLE 4.

(see next page)

(p.106) TABLE 4 (turned here sidewise)

PURIFICATION OF THE PERFRINGENS ANATOXIN BY ELUTION STAT

SERIES OF ANATOXINS →	533	546	537	533	548
	-		-	-	-
	645		37,40	50	50
CONCENTRATE 80 times by volume					
1. activity by BU in one ml	160	40	100	125	100
2: protein N in mg%	400.0	154.6	292.2	525.0	216.7
3: B.U. charge per one mg of protein N	40	26	34	23	46
ELUATE I:					
1. (as above)	70	20	100	90	100
2. (as above)	53.3	14.0	54.6	44.8	45.0
3. (as above)	134	143	183	222	220
ELUATE II.					
1.	40	20	100	90	100
2.	61.6	77.4	51.8	35.0	36.6
3.	82	26	193	300	280
ELUATE III:					
1.	40	20	100	60	100
2.	43.8	48.8	47.6	14.0	23.4
3.	116	41	210	430	344
ELUATE IV:					
1.	20	10	100	20	100
2.	X 22.2	15.4	30.8	3.0	50.4
3.	91	70	324	700	200
ELUATE V:					
1.	-	10	-	20	-
2.	-	25.2	-	7.0	-
3.	-	40	-	286	-
Percentage of output of the concentrate in B.U.	75	76	100	100	96
Percentage of purification by protein N.	95	90	86	95	83

(Text continues from page 93 of translation)...Depending upon the purity of the concentrate, 60% to 80%(by volume) of $Al(OH)_3$ suspension is added. STAT

After 10-15 minutes of stirring the mixture at room temperature, the precipitate of the adsorbate is separated by centrifugation and it is twice washed with M/60 buffer phosphate at a pH = 4.6.- In the first supernatant fluid, for the determination of the degree of adsorption by means of the lecithinase reaction, the amount of the non-adsorbed antigen is titrated off. To the washed precipitate, M/15 buffer phosphate at pH 7.8 is added in the amount of half to one third of the volume of the concentrate taken for the adsorption.

After a thorough stirring, the mixture is left for two hours at room temperature, after which the supernatant fluid is removed by centrifugation.

Since it had been shown that a single elution does not liberate the whole antigen from the adsorbed condition, the elution is repeated again under somewhat different conditions. After the separation of the I. Eluate, the precipitate is stirred up anew in M/15 buffer phosphate taken in the amount of 1/3 to 1/4 of the volume of the initial concentrate, and the mixture is left for two hours in the thermostat at 37°C. After the separation of the II. Eluate by centrifugation, the precipitate is again treated 2-3 times with buffer phosphate, at which the time of keeping it in the thermostat is successively increased to two days, and the amount of the eluent is selected with such a consideration that the total volume of all 4-5 Eluates should be equal to the volume of the original concentrate which was taken for the adsorption.

The data about the purifications of the seven industrial series of perfringens anatoxins, with the method of adsorption and elution, are given in Table 5(sic).

The obtained data prove that with these methods, an 80% purity could be achieved by the protein nitrogen, with an output of biologically active protein up to 75% of the concentrate and 50% of the native perfringens anatoxin.

CONCLUSIONS

1) The precipitation of the native perfringens anatoxins by hydrochloric acid at the isoelectric point gives a possibility to produce primary concentrates purified for protein nitrogen up to 85%, with an antigen output of from 50% to 100%.

2) The precipitations of the perfringens concentrated anatoxins with the aid of ethyl alcohol or with acetone in the cold, under the strict control in regard

to ionic strength and pH and temperature, permits the maximum amount of liberating (cleaning) it from ballast(inert) proteins and pigments, with a practically full output of the antigen. The obtained preparation contains from 90 to 200 B.U. per one mg of protein nitrogen in case of (p.108) purification with ethyl alcohol, and from 21 to 100 B.U. per one mg of protein nitrogen in case of purification with acetone.

3) The method of aluminium hydroxide adsorption ,with subsequent elution by means of twice replaced sodium phosphate, may be successfully used for further purification of the original concentrates ,and it permits,with a 50% yield of the antigen, to clean it from the inert ballast nitrogenous substances up to a purity of 80%.

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(p.109)

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STUDY OF THE CHEMICAL AND AMINO-ACID COMPOSITION OF THE PERFRINGENS ANATOXIN*

(p.109 - 113)

(*FOOTNOTE: The material has been reported at the Conference of the Biochemical Department of the Gamalei Institute, in 1959).

* * *

The purified preparations of the perfringens anatoxin were obtained by the precipiation of the native antigens at the isoelectric point with successive trans-precipitations with ethyl alcohol. These preparations did not contain ballast(inert) substances,and they had a considerable charge of activity per one mg of protein nitrogen, which proved the high degree of purity of these antigens.

Then,subsequently, it was possible to approach the determination of the chemical and amino acid composition of the antigens obtained by us. Certainly, the determination of the amino acid composition would have been considerably interesting to be done in the separate isolated electrophoretic homogenous fractions of the highly purified anatoxin, but the purpose of the present work was the determination of some chemical indices of the industrial purified preparations of anatoxin which could be released without further fractionation.

For the elucidation of the chemical composition of the antigen, in the antigen determination was made of the total and of the protein nitrogen according to the micro-Kjeldahl method, of the total phosphorus according to the Fiske-Subbarou method, of the reducing substances according to the Hagedorn-Jensen method, of the moisture and of the ash contents by the gravimetric methods(1).

The results of the chemical analysis,expressed in percentage of the dry organic substance, are represented in Table 1.

From the data presented in Table 1, it is evident that the perfringens anatoxin,purified by alcohol, is a protein with traces of phosphorus,and with an insignificant amount of reducing substances.

The amino acid composition of the purified protein preparations of the perfringens anatoxin as well/ as the amino acids which are present as an average were determined by the method of the partition chromatography on paper .

(p.110)

TABLE 1

CHEMICAL COMPOSITION OF THE PERFRINGENS ANATOXINS PURIFIED BY ETHYL ALCOHOL(in % of dry organic material)

SERIES OF ANATOXIN	Ash content	Moisture	Reducing substances	Phosphorus	PROTEIN
No.651	43.5	6.6	6.1	0.17	97.7
Mixture of 5 series	42.7	4.5	3.6	0.18	89.0
No.649	40.0	2.1	5.9	0.27	98.2
Nos.629 plus 666	45.2	3.7	4.0	0.1	102.1

The lyophillically dried,purified preparation was hydrolyzed with 6-N hydrochloric acid for 36 hours.

The determination of the qualitative amino acid composition was done on mono-metric descending chromatograms.

As a solvent, a mixture of N-butyl alcohol, acetic acid and water in the ratio of 40:15:5 and 40:10:50 was used.

The developing of the chromatogram was done with a 0.2% solution of ninhydrine in acetone.

For the exposition of a few amino acids the highly sensitive specific reactions have been used.

The presence of arginine was determined by the reaction with sodium hypobromite; of the histidine--by the reaction with diazo-benzol sulfonic acid(1); of the phenyl alanine and of tyrosine-- by the reaction with 0.1% NaOH(3); of the proline and oxy-proline--by the reaction with isatine; of the methionine-- by the platinum iodide(2).

The free amino acids were determined in the protein-free filtrates of the native and of the purified perfringens anatoxins.

As a result of the chromatographic study of the amino acid composition in the purified anatoxin the following amino acids have been discovered(Fig.1):-- cystine, cysteine, lysine, histidine, arginine, aspargic acid, serine, glycocoll, oxy-proline, glutaminic acid, treonine, alanine, proline, tirosine, alpha-amino butyric acid, methionine, valine, phenyl-alanine, the group of leucines.

At the study of the free amino acids of the anatoxin preparations, in the

process of their purification and concentration, in the native anatoxins the presence of the following amino acids has been established:- cystine, cysteine, lysine, histidine, arginine, aspargic acid, serine, glycocoll, oxyproline, glutamic acid, treonine, non-identified amino acids, alanine ...

(p.111) (full-page illustration)

FIG.1: AMINO ACID COMPOSITION of the perfringens anatoxin purified by ethyl alcohol. ~~EXTRY~~ FIRST dot:...evidences; SECOND dot: ...hydrolysate of the anatoxin.--1...cystine, cysteine; 2...lysine; 3...histidine; 4...arginine; 5...aspargic acid; 6...serine, glycocoll; 7...oxyproline; 8...glutamic acid, treonine; 9... alanine; 10...proline; 11...tirosine; 12...alpha-amino butyric acid; 13...methionine, valine; 14...phenylalanine; 15...group of leucines.

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p.112) (full-page illustration)

FIG.2: FREE AMINO ACIDS of the native, concentrated and alcohol-purified perfringens anatoxin.--1, 2...cystine, cysteine; 3...lysine; 19...histidine; 5... arginine; 6...asparagonic acid; serine; 7...glycocoll; 8...oxyproline,...20...glutaminic acid, treonine; 10...non-identified amino acid; 11...alanine; 12...proline; 13...tirosine, alpha-amino butyric acid; 14...tryptophane; 16...methionine, valine; 20...phenylalanine; 17, 18...group of leucines.

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(p.113) In the anatoxins, after concentration with HCl at the isoelectric point, as well as in the anatoxin which is purified with ethyl alcohol, no free amino acids had been detected(Fig.2).

In this manner, the conducted research showed that the qualitative amino acid composition of the purified perfringens anatoxin was closely related to the amino acid composition of the nutrient medium.

CONCLUSIONS.

1) By the chemical analysis of the purified perfringens anatoxin its protein nature was established.

2) By the method of chromatography on paper the presence of 19 amino acids has been demonstrated in the composition of the protein of the anatoxin.

3) In the filtrates of the native anatoxins, 20 free amino acids have been identified.

4) The purification by means of hydrochloric acid and ethyl alcohol has given

a possibility to completely get rid of the free amino acids of the nutritive medium.

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PRODUCTION OF EDEMATIENS ANATOXIN ON NUTRIENT MEDIUM FROM FISH-BONE MEAL; ITS ANTIGENIC AND IMMUNOGENIC PROPERTIES(p.115- 121)

* * * *

Different meat media are usually utilized for the production of the various toxins and anatoxins which are employed for the hyper-immunization of the horses for the purpose of obtaining therapeutic sera. Apart from their expensiveness, the making of the meat media is connected with a whole series of invonveniences which are tied up with the need for using fresh meat and animal enzymes, which does not permit the creation of reserves for the raw material. These inconveniences are stopped if, as raw material for the preparation of the media, casein is used, and fish meal and the like. The different media whose base is the hydrolysates of the above listed products have already reached a sufficiently wide spread in the production of the bacteriological preparations and at the preparation of the diagnostic media.

In the literature, there are informations for the possibility of getting diphtheria, tetanus and botulinus toxins on media of the hydrolysates of gelatin and casein. From the gangrenous toxins, the toxins of the Vibrio septicus and of the perfringens were obtained on a medium of caseine hydrolysate(BERNHEIMER, 1944; ADAMS at alii, 1947); in respect to the edematiens toxin, we were unsuccessful in finding such an information.

In the Department of Nutrient Media of the N.F.Gamalei Institute for Epidemiology and Microbiology, a method has been worked out for the preparation of a medium in the form of an acid hydrolysate of fish-bone meal. The fish-acid bouillon was composed of fish-acid hydrolysate and a decoction of liver. At the preparation of the fish-acid hydrolysate we were guided by the indices of the content in amino nitrogen and of pepton in the so-called bouillon on pepsin peptone(peptic digestion of meat).

(p.116) The variation of the amount of amino nitrogen and of pepton in the dif-

ferent series of both media has been identical: the content in amino nitrogen varied from 110 to 190 mg%, and the content in the pepton varied from 1.5 to 3 g%. The variations of these indices depend, evidently, upon the quality and the properties of the initial raw material.

The medium from the acid hydrolyzate of fish meal has been used by us for the cultivation of B.edematiens, and for obtaining toxins, and thereafter even anatoxins. In the following year, for the production of toxins and anatoxins of the edematiens, prepared for the immunization of horses, we have used a medium of peptic digestion of the meat. This medium has served as a control. The experiments were made with three stocks of the B.edematiens.: Nos.4,277 and 794 (*FOOTNOTE: The stock of Cl.edematiens No.794 was prepared by the Department of Anaerobic Infection of the Gamalei Institute of Epidemiology and Microbiology, in January 1952). The stocks were kept in soldered test tubes on a medium of Pope's bouillon, with small pieces of fresh meat, 0.1 % of agar, 0.4% of gelatine, and 0.5% of glucose. The medium was poured over with vaseline oil. The test tube of a two-day culture of the edematiens, cultivated upon the above indicated medium, was seeded on one quarter with fish or meat medium. Before the seeding, to one quarter, 0.5% glucose has been added. The seedings were put into thermostat at 37°C for various periods of time. On the meat medium of peptic digestion, the growth of the microbes has started as a rule 18 to 24 hours after the seeding; in rare cases, a delay of the start of growth was observed up to 40 and 48 hours.

On the medium made from hydrolysate of fish meal, in the majority of the cases the beginning of the growth was retarded; the growth started 40 to 48 hours after seeding, and sometimes even 72 hours after. In some cases the growth of the microbe began already 18 to 24 hours after the seeding.

We could not observe any correlation between the strength of the obtained toxins and the time of the start of the culture's growth.

The nature of the growth on both media was identical. First, the medium had proportionately become turbid. With the stirring of the culture, gas production was observed. In the smears made at this time, large(prominent) bacilli were visible which were arranged in chains. The lysis of the microbes was still absent. In a few hours after the start of the growth, the microbes already settled down at the bottom, and the surface layer of the fluid became clear; the gas production was

stronger. In the following day the gas formation became weaker; at this time, in the smears, side by side with the usual bacilli, much detritus was seen, i.e., STAT signs of a lysis of the culture were observed. At the 3rd to 5th day, the gas production almost completely stopped, in the smears only individual undamaged bacilli and much detritus was seen since the basic mass of the bacilli had already undergone the lysis.

The study of the dynamism of toxin production on the fish medium has indicated that the maximum accumulation of toxin occurs XX (p.117) at the same time as on the pepsin pepton, too, i.e., on the 3rd to 5th day after the start of the growth.

In some experiments, the dynamism of the toxin production was folloed up during a longer period of time. The results of the experiments are given in Fig. 1, 2, and 3.

As it can be seen from these data, in principle there were no differences in the dynamism of the toxin formation of Cl. edematiens Nos. 277 and 764 on the medium of pepsin pepton and on the medium from the hydrolysate of the fish meal.

FIG. 1: DYNAMISM of the toxin formation V. (No. 4, 277, and 794 on the medium of pepsin pepton in a medium from hydrolysate of fish meal).

On the 3 diagramsL(one for Stock 4, for Stock 794 and for Stock 277):

ORDINATAS: Strength of toxin in M.L.D. in cm^2

ABSCISSA: Days(2 to 20)

(Annotation for lines)Conventional signs: _____pepsin-pepton medium, Experiment No. 11; - - - hydrolysate of fish meal medium; experiment No. 11; _._._. hydrolysate of fish meal medium, experiment No. 6.

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In correspondence with the obtained data, for the preparation of the anatoxin we have taken the culture from the thermostat 3-5 days after the start of the growth, i.e., in the period of the maximum accumulation of the toxin in the medium.

In the below given Tables 1 and 2, the figures of the peak strengths of the toxin are represented. In a part of the experiments, the seedings into both media have been carried out simultaneously by which the possibility of variation of the results due to the diversity in the initial culture was excluded.

The results of these experiments are given in Table 1.

As the given data indicate, on the medium made from fish meal hydrolysate toxins were produced which were not inferior in power, and in some instances they

were even superior to those produced on the pepsin pepton medium. In a further experiment, the seedings were often done non-simultaneously:- occasionally only on the pepsi-pepton medium, (p.118) and occasionally on the fish meal hydrolysate medium. In case of comparing the strength of the produced toxins, it must be taken into account that the difference may depend upon not only the quality of the media but also the quality of the initial cultures. Nevertheless, in case of a sufficiently large number of seedings, the variation which is made on this account is ~~XX~~ levelled off, and the comparison of the strength of toxins may allow a presentation and manifestation of the quality of the medium.

In Table 2, we included the comparative data of the strength of the edematiens toxins produced on pepsin pepton medium and on medium made form hydrolysate of fish meal, in all experiments arranged by us (in the course of the years 1952-1953). With the comparison of the toxin formation of the used three Stocks, we see that the No.4 Cl. edematiens is not inferior to the others either when it is cultivated on the pepsin pepton medium or, particularly, when cultivated on the madeium made from the hydrolysate of the fish meal.

The strengths of the toxins of the No.277 and 794 Cl. edematiens, which were produced on fish meal hydrolysates, in the majority of cases have been the same or even higher than on the pepsin pepton medium.

From the Table it can be concluded that on the medium from fish meal, a larger amount of strong toxins was produced, but, together with thism also larger amount of weak toxins. In some case, though rare cases, the growth of the microbes was absent on the fish medium.

In this manner, at the cultivation of Cl. edematiens on the medium made from fish meal, great variations of the strength of toxins and less stable results have been obtained. These variations may be explained, as it seems to us, by the considerable variations in the quality of the initial fish meal.

The provided data undoubtedly prove the possibility of cultivating the Cl. edematiens on the above mentioned nutritive medium and the possibility of getting toxins which, in respect to strength, are not inferior to those produced on the meat media.

The obtained toxins were transformed into anatoxins by means of adding to ~~XX~~ them 0.4% of formalin and by keeping them in the thermostat at 37°C for 48 hours.

The antigenic properties were determined with the aid of the reaction of antitoxin combining(binding) in experiments on white mice. The antigenic properties were investigated in 41 series of anatoxins; out of these, in 19 series which were produced on meat media, and in 22 series which were produced on fish media. The results are presented in Table 3.

In this Table, the relationships are shown between the number of units of binding of anatoxin, and the strength of the initial toxin. From the presented data it follows that there exists a definite parallelism between the number of binding units of the anatoxin and the strength of the initial toxin. The indicated regularities were observed for anatoxins prepared on both meat(meat) and fish media.

(p.119)

TABLE 1

TOXIN FORMATION OF No.4, 277 and 794 Cl. EDEMATIENS ON PEPSIN PEPTON(:PP) MEDIUM AND ON MEDIUM MADE FROM FISH MEAL HYDROLYSATE(:FH)(summarized data)

(Table turned into text)(HEADINGS: a...name of medium; b...total number of seedings; c...OF THESE, GIVING STRENGTH IN M.L.D. PER ONE ML(all -subsequent headings beneath this one); c...no growth; d...≤100; e...>100≤200; f... total(NOTE: c,d,e,f have been found unsuitable for immunization); g... >400≤1000; h...>2000≤4000; i...>4000; j...total(NOTE: g,h,i,j, were headed:"suitable for immunization".

NO.4 STOCK PEPSIN PEPTON: b...21; c...-; d...1; e...5; f...6; g...13; h...2; i...-; j...15.

FISH HYDROLYSATE: b...15; c...2; d...5; e...2; f...9; g...3; h...3; i...-; j...6.

NO.277 STOCK PEPSIN PEPTON: b...16; c...-; d...-; e...2; f...2; g...11; h...3; i...-; j...14.

FISH HYDROLYZATE: b...18; c...2; d...-; e...1; f...3; g...6; h...8; i...(one) 20,000; j...15.

NO.794 STOCK PEPSIN PEPTON: b...17; c...-; d...-; e...1; f...1; g...9; h...7; i...-; j...16.

FISH HYDROLYZATE: b...21; c...1; d...1; e...2; f...4; g...7; h...9; i...(one) 10,000; j...17.

No.277 STOCK PEPSIN PEPTON: b...54; c...-; d...1; e...8; f...9; g...33; h...12; i...-; j...45.

NO.794 STOCK fish hydrolyzate: b...54; c...5; d...6; e...5; f...16; g...16; h...20; i...2; j...38.

(over)

(Table 1 cont.)

No.277 STOCK PEPSIN PEPTON: b...33; c...=; d...=; e...3; f...3; g...30; h...10; i...=; j...30.

No.794 STOCK FISH HYDROLYZATE: b...39; c...3; d...1; e...3; f...7; g...13; h...17; i...2; j...32.

TABLE 2.

TOXIN FORMATION OF NO.4,277,& 794 Cl.EDEMATIENS ON PEPSIN PEPTON MEDIUM AND ON MEDIUM MADE FROM HYDROLYZATE OF FISH MEAL(at simultaneous seeding of both media)

(HEADINGS: exactly as for Table 1 see previous page)

NO.OF STOCK

NO.4 PEPSIN PEPTON: b...9; c...=; d...2; e...3; f...5; g...3; h...1; i...=; j...4.

FISH HYDROLYZATE: b...9; c...=; d...4; e...1; f...5; g...2; h...2; i...=; j...4.

NO.277 PEPSIN PEPTON: b...9; c...=; d...=; e...2; f...2; g...6; h...1; i...=; j...7.

FISH HYDROLYZATE: b...9; c...=; d...2; e...=; f...2; g...4; h...2; i...1; j...7.

NO.794 PEPSIN PEPTON: b...7; c...=; d...1; e...=; f...1; g...3; h...3; i...XX =; j...6.

FISH HYDROLYZATE: b...7; c...=; d...1; e...1; f...2; g...1; h...3; i...1; j...5.

NO.4 PEPSIN PEPTON: b...25; c...=; d...3; e...5; f...8; g...12; h...5; i...=; j...17.

NO.277 (nothing listed)

NO.794 FISH HYDROLYZATE: b...25; c...=; d...7; e...2; f...9; g...7; h...2; i...2; j...16.

NO.277 PEPSIN PEPTON: b...17; c...=; d...1; e...2; f...3; g...9; h...4; i...=; j...13.

NO.794 FISH HYDROLYZATE: b...16; c...=; d...3; e...1; f...4; g...5; h...5; i...2; j...12.

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(p.120)

TABLE 3

CORRELATION BETWEEN THE STRENGTH OF EDEMATIENS TOXINS AND THE ANTITOXIN COMBINING POWER OF THE ANATOXIN OBTAINED FROM THEM

STRENGTH OF TOXIN	MEDIUM	NUMBER OF SERIES OF ANATOXIN	AMONG THEM HAVING BINDING UNITS > 2 <4	≥4 <10	>10 <20	>20 <40	> 40 <60
400	Pepsin pept.	2	1	1	-	-	-
1000	Fish hydrolyz.	-	-	-	-	-	-
1000	Pepsin. pept.	11	2	7	2	-	-
2000	Fish hydrolyz.	5	1	4	-	-	-
2000	Pepsin pept.	5	-	-	4	1	-
4000	Fish hydrolyz.	7	-	-	5	2	-
4000	Pepsin pepton	1	-	-	-	1	-
10000	Fish hydrolyz.	8	-	-	6	2	-
10000	Pepsin pepton	-	-	-	-	-	-
20000	Fish hydrol.	2	-	-	-	-	2
TOTAL	Pepsin pepton	19	3	8	6	2	-
	Fish Hydrolyz.	22	1	4	11	4	2

TABLE 4.

IMMUNOGENIC PROPERTIES OF THE EDEMATIENS ANATOXIN IN THE EXPERIMENTS ON GUINEA PIGS

No. of anatoxin series	NUTRIENT MEDIUM	Number B.U. per ml	No. of animals	AMONG THEM GIVING ANTITOXIN TITRE in A.U. >0.1	>0.1 <1	>1 <5	>5 <10	>10
			SINGLE-SHOT IMMUNIZATION					
2	Meat medium	10	23	2	1	16	3	1
2	Fish medium	10	20	2	2	13	3	-
			Two-Shot IMMUNIZATION					
6	Meat medium	4-10	35	-	8	17	5	5
6	Fish medium	4-10	32	-	3	13	10	6

ANNOTATIONS.

In the Table, the titres are presented 30 days after the single shot immunization, and 15 days after the two-shot immunization.

(p.121) The immunogenic properties of the anatoxins were investigated in experiments on guinea pigs. The immunohenic properties of the anatoxins which were produced on fish media were compared with the anatoxins which were produced on meat media. All together 14 series of anatoxins(7 anatoxins for each type) were examined. The examination of the immunogenic properties was done by means of single-shot and two-shot immunizations of the guinea pigs with the edematiens anatoxin to which 0.5% potassium alum has been added. The single shot immunization was done with one ml of the anatoxin, the two-shot immunization with one and 2 ml.

The experiments with the single-shot immunization were conducted on 43 guinea pigs; of them, 23 were immunized with anatoxins prepared on meat medium, and twenty on fish medium.

The experiments with the two-shot immunization were conducted on 67 guinea pigs; of them, 35 were immunized with anatoxins prepared on meat media, and 20 ---on fish media. The results of the experiments are presented in Table 4.

As it can be seen from the Tables, the majority of the guinea pigs immunized either with one shot or even with two shots,had an antitoxin titre greater than one A.U. In the experiments with the one-shot immunization only individual animals had had a titre greater than 5 A.U., in the experiments with the two-shot immunization the number of such animals was greater.

As it can be seen from the presented data,the immunogenic properties of the anatoxins produced on a meat medium do not differ from those prepared on a fish medium. The edematiens anatoxins produced an a nutrient medium made from fish-bone meal were used by O.A.KOMKOVA for immunization of horses which were productors of antiedematiens serum. In case of immunization of the horses with this anatoxin, no kind of collateral phenomena have been observed which would have been different from those observed earlier when the horses were immunized with anatoxins which were prepared on meat nutrient media.The titres in the horses remained approximately at the same levels as previously.

CONCLUSIONS.

1) The nutrient medium from a hydrolysate of fish-bone meal is fully suitable for the production of edematiens toxins and antitoxins under industrial conditions.

2) There were no differences of principle observed in the dynamisms of the

toxin formation of the Cl.edematiens on the indicated nutrient media.

3) The edematiens anatoxins, produced on a nutrient medium from hydrolyzate of fish-bone meal, in regard to its antigenic and immunogenic properties, is not inferior to those anatoxins which were prepared on meat media of peptic digestion. And they may be used for the immunization of horses.

(No literature quoted)

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TO THE PROBLEM OF THE MECHANISM OF THE TOXIN FORMATION AT CULTIVATION OF AN EDEMATIENS CULTURE IN CELLOPHANE BAGS(p.123- 131)

* * *

The works of ZELEVENSKAYA, AKIMOVA, GIL'GUT,VLASOVA(1955) showed the possibility of getting highly active toxins of the agents of gas gangrene(perfringens,edematiens, septic Vibrio) at cultivation of the clostridia in cellophane bags immersed into the nutritive media.

The peak of toxin production in these cultures followed considerably later than in case of a cultivation of the culture directly in the nutritive medium. At this time, the standard of microbic bodies in the bags increased many times. We have assumed that the strengthening of the toxicity of the filtrates which were made from the cultures that were grown in cellophane bags was due to the lysis of a huge amount of microbic bodies which have accumulated in a rather small volume of the bag.

By observing the cultures in the process of incubation for morphology, we have noticed that, by the grade of ageing, the microbic cells have lost their capacity of being stained. At the end of the incubation period, they appear as a stainless mass on the ground of which a slight number of intensively stained, large burnt bacilli are found.

TWORT BORDERT(BORDET,1910) ,D'HERELLE(D8Herelle, 1926) have established that, in young bouillon cultures, which were obtained by means of a subculture from old bouillon cultures, bacteriophages are frequently formed.

The bacteriophages in anaerobic cultures, according to the data of ZAEVA (1945), have been discovered in 1940 by ZAEVA, ZHURAVSKAYA and POGOSOVA. Thus, for instance, edematiens bacteriophage was produced from a ten-year old culture. (p.116) ELFORD(Elford,1938), GUELIN(Guelin,1952) described the morphology and the reproduction of two perfringens bacteriphages in the presence of the B.perfringens culture. The authors found that the multiplication of the phage bodies

was completed during the growth of the culture. 15 minutes after the inoculation of the phage. Under the effect of the phage in the culture, hollow (STAT ty) long preserved bacterial shells have appeared untouched. The presence of these shells made false the calculation of the number of live bacteria when it was counted with the chamber.

D'HERELLE, at studying the development of the bacteriphage in aerobic cultures, came to the conclusion that at the start of the young culture, only a few of the bacterial corpuscles are seen; later, their reproduction occurredd gradually, during definite intervals:- the phage penetrated into the microbic cells, by forming colonies of a certain number of elements within the cells. Thereafter, the bacteria suddenly became disrupted, by letting free into the environment the younf corpuscles of the bacteriophage. This escape occurred during the entire growth of the bacteria, with different latent periods, depending upon the phage and the bacteria. These data were subsequently evolved by many authors. However, some of them thought that the reproduction of the bacteriophage occurred after the growth of the bacteria, independently from the destruction of the microbic cell. The increase in the number of bacteriphage ceased at the commencement of the lysis of the culture. The lysis of the microbic cells was a secondary phenomenon, and it happened then when the ratio between phage and bacteria has reached a definite value.

The electronic microscope permitted us to detect the presence of the edematiens bacteriophage both in the young cultures during the growth process of the bacteria and in the old museum stocks which were preserved in the museum of cultures for 4 to 6 years. In the present work, we are presenting a number of photographs made with the electronic microscope from the Bac. edematiens cultures. The reproduction of the Bac. edematiens, in the investigated media, occurred at a slower rate than the reproduction of the Bac. perfringens. Often, the opalescence of the medium came only in 18 to 24 hours, and has bubbles appeared on its surface. At this time, the culture was composed of large bacilli which intensively stained with methylene blue. Long flagella were arranged on the sides of the bacilli.

For the electronic microscopy, beginning from the moment of the appearance of the macroscopically noticeable growth, cuts were made daily both from the cellophane bags, and from the bouillon cultures. The culture was centrifugated for 30

minutes at 4000 r.p.m., it was washed three times with distilled water which contained 0.3% formalin.

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(p.125) One drop of the washed microbic bodies was put on the screen, covered with a thin layer of colloid.

For 16-18 hours of incubation, both with the electronic microscopy and with the regular microscopy, large burnt bacilli were found; the same cells could be observed for the length of a few subsequent days. In the intercellular space, first in a small number, sufficiently large corpuscles were observed. Already in 24-36 hours, the corpuscles with large accumulations almost occupied all the intercellular space, and they settled down on the microbic cells, by covering the surface of the cells partly or fully(Photo 1,2).

In the following time, the shell of the bacteria appeared as if corroded:- the flagella, covered with phage, remained undamaged(Photo 3).

At the ordinary microscopy of the smears from the same bacterial emulsion, the appearance of the lysed forms was noticed, the number of the flagella in the bacilli was increased.

In 72 to 96 hours, a large number of completely disrupted cells has been observed. Occasionally, cells were met with, filled up with corpuscles of the phages, and also with spherical formations(probably spores)(also filled up with corpuscles(Photos 4 and 5).

At this time, with the ordinary microscopy of the smears, the whole field of vision was covered with lysed cells.Only the individual bacilli were stained blue. Often, spores were found with long flagella. With further ageing of the culture, in the intercellular space the corpuscles were considerably less than at the start of the inoculation. In the majority of the cases, they settled down on the flagella(cilia?) which remained longer in the undamaged condition, or they settled down on the surface, or within the microbic cells and spores. The majority of the microbic cells was composed as if tehy were of separate tiny lumps, slightly connected with each other. Together with them, empty stainless shells were met with. However, even for a long period of tôme for the existence of the culture, whole undamaged cells and spores with flagella were found. It is evident that separate individuals were stable both in respect to the penetration of the bacteriophage corpuscles into the cells, and in respect of the lysogenic factors taking place in the culture(Photo 6 and 7)

By establishing the fact of the long existence of the bacteriphage in the culture of the Bac.edematiens, we decided to investigate the old cultures, which were kept for years on Tarozzi's medium and on brain medium at room temperature, in the Museum of Cultures of the Institute. The Stocks No. 4 and 277 have been subjected to investigation, which were of 4 and 6 years of age.

(p. 126 and p. 127) full-page Photos 1, 2, 3 and 4.

(p. 128) full-page Photos No. 5 and 6.

(p. 129) The old cultures were completely transparent, and only after an energetic shaking was it slightly cloudy. In order to liberate it from the small pieces of meat and from the other inclusions, the culture was filtered through a cotton-wool filter; thereafter, it was centrifuged, washed out, and worked up for the electronic microscopy.

The bacterial cells and spores of Stock No. 4 of seven years of age on the Tarozzi medium have become deformed. Occasionally, the bacteria showed themselves as a conglomerate of lumps, without shells, the flagella have not been changed. In the spores having shells (capsules), and in the residues of the microbic cells as well as in the intercellular space, phage corpuscles were observed.

The four-year old culture of Stock No. 277 was preserved on a brain medium.-

PHOTO No. 7 (no legend*)

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The microbic cells were as if filled up with bacteriophage corpuscles, they appeared alveolar, half-destroyed, the flagella were preserved, in the intercellular space small particles of bacteriophage were observed. The spores were filled up with phages and they had the cilia.

(p. 130) The obtained data prove that the phage which exists in the Bac.edematiens cultures, was preserved in them for the length of many years, and it possessed not lesser viability than the microbic cells of these cultures.

At the transfer (subculture) of the old stocks, the multiplication occurred not only in the bacterial cells, but also of the phage corpuscles which were collected on the surface of the microbes, they destroyed the shells of the cells, penetrated inside the cells, lysed and fully destroyed them. In the subsequent time, they were preserved in the spores, on the surface of the flagella and in the remnants of the microbic cells.

Beside the investigation of the bacterial cultures, the filtrate of the three-

-day cultures of B.edematiens has been investigated. The culture was filtered through a Chamberlain L_5 filter. The filtrate was tested for sterility with subculture on various media, under anaerobic and aerobic conditions. The sterile filtrate was centrifuged at 16,000 revolutions for 30 minutes, three times, it was [redacted] STAT washed in distilled water which contained 0.3% formalin. In the filtrates, small corouscles were discovered, in some preparations covering all the visual field. No other formations which would resemble microbic cells were observed by us. If our photos are compared with the photos of PENSO(Penso, 1955), tracing in the electronic microscope the attack of the phage at the moment of its addition to the culture and its penetration into the inside of the bacteria until the full destruction of the microbic cells, it is possible to assert(confirm) that we have observed the same phenomenon in the culture of the Bac.edematiens containing spontaneous bacteriphages.

The data which we introduced prove that in the cultures of the Bac.edematiens of Stock No.4 and No.277, cultivated directly in nutrient bouillons or in cellophane bags which are immersed into the nutrient medium, bacteriophages have been found. According to the degree of the reproduction of the microbic cells, the bacteriophage had covered their surface, it penetrated inside the microbic cells, and spores, it destroyed the microbic cells. Together with the lysis of the bacteria, toxins are getting free into the surrounding medium. Since at the cultivation, into the cellophane bags a diffusion of the fresh nutrient substances occurred from the surrounding medium, in the culture a reinforced reproduction took place on the part of the microbes which were subjected to a constant lysis by the bacteriophage, due to which the titre of the toxin in the bags reached many times above the titres which were obtained under the conditions of ordinary cultivation where the products of metabolism gather which hinder the further multiplication of the bacteria.

For the confirmation of this supposition it is necessary that, in the successive investigations of the toxin-producing cultures(tetanus, botulinus, and so on) which are capable of giving very strong toxins with the cultivation in cellophane bags, it should be studied the presence of the bacteriophage in these cultures, and the bacteriophages of these cultures should be separated in a pure form. The work in this direction is continued, the obtained results will be presented in a subsequent communication.

(over)

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PRODUCTION OF EDEMATIENS TOXIN AND ANATOXIN WITH GROWING OF THE CULTURE IN CELLOPHANE BAGS INMERSED IN THE NUTRIENT MEDIUM(p.133- 139)

* * *

Notwithstanding the large number of works in the area of study of the metabolism and of the requirement of the anaerobic microbes in nutritive substances, the problem about the possibility of the regular production of the toxins(of these microbes)with high effectiveness did not find its solution until the present time. It is well known that the amount of toxin entirely determines the amount of antitoxin obtained from it. For the creation of combined preparations for the immunization against gas gangrene , anatoxins of high quality are necessary which contain the least amount of ballast proteins.

For the achievement of this purpose, at the present time wide use is made of the purification and concentration of the native anatoxins which can be realized by very different methods.

In the 1946-1953 years, in the foreign literature, communications have been published by a number of authors(4-7) about the fact that, with the cultivation of the microbes in cellophane bags which are immersed in the nutrient medium, toxins can be successfully produced which excell by 20 to 100 times the strength of those toxins which are obtained by the ordinary methods of cultivation and which have considerably smaller amount of ballast proteins. Such toxins have been called by the authors dialysate toxins, and we henceforth will use this expression. From these toxins, anatoxins were produced which, in regard to their immunizing activity, have considerably excelled the ordinary anatoxins. In the years of 1951 to 1953, at the Gamalei Institute of Epidemiology and Microbiology, investigations were undertaken for the production of aerobic and anaerobic dialysate toxins. The results of these investigations have been reported at the Inter-Institutional Conference in the Ministry of Health of the U.S.S.R. in April 1954(1).

(p.134) In the same year, similar investigations have been undertaken by BORISONIK(1955).

In the present communication, the results will be exposed about the investigations on the production of the edematiens toxin at the cultivation of the microbe in cellophane saccules, as well as the data are given which are characteristic for the antigenic and immunogenic properties of the produced anatoxins.

For the cultivation of Cl.edematiens, a nutrient medium was taken whose basis was a hydrolysate of fish-bone meal. Under ordinary conditions, the cultivation of the microbe on this nutrient medium has produced toxins of the edematiens of the strength of from 400 to 4000 Dlm/ml(3).

The methodology of cultivation is summarized as follows(1). In the cellophane double-everted bag, a siphon was inserted to which the bag was completely attached. The bag, which was mounted in this manner, was inserted into a 3-liter flask with 2.5 l of nutrient medium. Into the bag, 200-250 ml of physiological solution was poured in. The flasks were sterilized at 100°C twice for 40 minutes each time. Directly before the seeding, to the medium surrounding the bag, 0.5% glucose was added. The seeding was done inside the bag with 0.5 - 1 ml of the culture.

In control, we poured nutritive medium in the same type of flasks with cotton. Before seeding, we added 0.5% glucose to the control, too. All together, nine series of experiments were carried out, and 73 flasks with bags and 20 flasks controls were seeded. The results of the first five experiments are given in Table 1.

From the Tables it is evident that the power of the dialyzed toxins was by 10 to 50 times above the power of the toxins with the ordinary method of cultivation. If at the time of the experiment, the surrounding medium has germinated, the earlier it has germinated the lower was the strength of the toxin in the cellophane bags.

From the Tables it is also evident that, with the other conditions being equal, the strength and the amount of the toxin in the separate bags has varied considerably. If the peak accumulation of the toxin in the nutritive medium has been observed with the usual methods of cultivation at 3-5 days, then with the cultivation in the cellophane bags, the maximum contents in toxin in the bags were by 7 to 10 days. As our experiments have shown, on the walls of the cellophane bags a large amount of toxin became adsorbed, therefore we have very carefully washed off the bags two or three times with physiological solution.

Later on, four series of toxin were produced which thereafter were transformed into anatoxin by means of the addition of 0.4% formalin and by keeping it in the thermostat at 37°C for 48 hours.

The results of the experiments are shown in Table 2.

TABLE 1

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TOXIN PRODUCTION OF CL. EDEMATIENS AT SEEDING UNDER ORDINARY CONDITIONS AND INTO CELLOPHANE BAGS IMMERSED IN THE NUTRIENT MEDIUM

No. of exper.	Mode of seeding	Time of culture	Strength of toxin Dlm/ml	amount of toxin	Annotation
1.	Flask	5	>2000<4000	2.5 l	
	Bag 1	7	>2000 < 40,000	45 ml	
	Bag 2	7	>2000<4000	200 ml	The surrounding medium germinated in one day.
2.	Flask	6	>2000 <4000	2.5 l	
	Bag 1	8	>100 000<200 000	25 ml	
	Bag 2	8	> 80 000<100 000	20 ml	
3.	Flask	6	>400 <1000	2.5 l	
	Bag 1	8	> 20 000<40 000	100 ml	
	Bag 2	8	> 2000 <4000	150 ml	The surrounding medium germinated in two days.
4.	Flask	5	>400 <1000	2.5 l	
	Bag 1	10	>40 000 < 60 000	100 ml	
	Bag 2	10	>10 000 <20 000	150 ml	The surrounding medium germinated in 3 days.
	Bag 3	10	>40 000 < 60 000	50 ml	
5.					
	Flask	4	>1000 <2000	2.5 l	
	Bag 1	7	>10 000 <20 000	150 ml	
	Bag 2	7	>10 000 <20 000	120 ml	
	Bag 3	7	>20 000 <40 000	200 ml	

TABLE 2.

CHARACTERISTICS OF THE EDEMATIENS DIALYZED TOXINS AND ANATOXINS

No. of series	Number of bags	Strenght of toxin in the bags Dlm/ml	Strength of toxin after addition of physiol. solution after washing of bags. Dlm/ml	Amount of toxin in l	% of formalin	No. of B.U./ml
188	10	>20000<40000	> 6000 <10000	3.8	0.4	20
			(Extent of harmlessness for all:48h)			
193	16	>40000 <60000	>40000 <60000	1.2	0.4	80
194	8	>20000 <40000	>5000 <40000	1.75	0.4	40
195	12	>40000 <60000	>20000 <40000	2.0	0.4	

These data also showed that the dialyzed toxins are considerably superior in their quality to the ordinary ones, and the anatoxins which are made of them contained 2 to 8 times larger number of combining(binding) units. In the three series of dialyzed anatoxins and in one series of ordinary anatoxin, the contents of

the total, of the protein, and of the amino nitrogen has been determined*(FOOTNOTE: These determinations were done by G.F.SHEMANOVA, collaborator at the Biochemical STAT Department, for which we thank her very much). The results are given in Table 3.

TABLE 3.

CONTENT OF TOTAL, AMINO, AND PROTEIN NITROGEN IN THE REGULAR AND THE DIALYZED ANATOXINS OF THE EDEMATIENS.

No. of series	Number of B.U. PER ml.	Type of anatoxin	Total N mg	Protein N mg	Amino N mg	Number of BU/mg of total N	Number of BU/mg of protein N
183	10	Regular on fish hydrolyzate	3.78	0.50	2.08	2.6	20
188	20	Dialyzate	2.02	0.27	1.85	10	74
193	80	Dialyzate	2.86	0.77	1.72	28	104
194 195	40	Dialyzate	1.51	0.30	1.72	26	133

From these data it follows that, with the evaluation in units of activity, the dialyzed anatoxins have contained 4-6 times less ballast proteins than the regular anatoxins.

The immunogenic properties of the three series of dialyzed anatoxins in comparison with the first series of ordinary anatoxins were determined in experiments on guinea pigs. The animals(12 guindea pigs in each group) were immunized with one shot of one ml of anatoxin precipitated by 0.5% potassium alum. Moreover, 10 guinea pigs(5 in each group) were immunized with one ml of native anatoxin, both dialyzed and ordinary.

The anatoxin content was determined 10, 20, and 30 days after the immunization. The results of the experiments are given in the illustration.

The experiments proved that the immunizing effect of the native dialyzed anatoxin is smaller than that of the ordinary one, notwithstanding the large content of antigenic units within it. The immunogenic properties of the precipitated dialyzed anatoxins proved to be somewhat better than those of the ordinary ones. Moreover, these differences(text continues later...)...

(p.137) FIG.1:(Full-page figure) DYNAMISM OF THE ACCUMULATION OF THE ANTITOXIN in guinea pigs immunized with dialyzed and with ordinary anatoxins of the edematiens

(over)

bacillus.

ORDINATA: - ; ABSCISSA: days.- On DIAGRAM: inoculated 1.0 anatoxin without alum; _____ series 183 , 10 B.U.(ordinary); - - - series 194 plus 195, 40 B.U.

- - - - - -

(p.134) FIG 2: (full-page illustration): COMPARISON OF THE IMMUNOLOGICAL EFFECTIVENESS of the different concentrations of aluminium hydroxide.

ORDINATA: - ; ABSCISSA: days.- On DIAGRAM: inoculated 1.0 anatoxin, plus 0.5% alum. _____ series 183...10 B.U.(ordinary); _ . _ . _ Series 188...20 B.U.; _ _ _ _ Series 193...80 B.U.; --- - - - - Series 194 and 195....40 B.U.

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(text continues...)...are not very great.Such results, evidently, are explained so that a considerable part of the activity was left in the supernatant fluid,and only an insignificant part was precipitated by the alums.

The produced dialyzed anatoxins were used by O.A.KOMKOVA for the immunization of horses. Three horses have been given dialyzed anatoxin precipitated with 0.5% of alums, during 1-3 cycles.

The advantage of these anatoxins,when compared with the ordinary anatoxins, could not be seen.

CONCLUSIONS.

1) At the cultivation of Clostridium edematiens cultures in cellophane bags which are immersed in nutrient media, toxins were obtained which were 20 to 50 times superior to those of the cultivation with the ordinary method and which contained 10 000 to 100 000 Dlm/ml.

2) However, notwithstanding the great power, the dialyzed toxins are transformed into anatoxins with the addition of 0.4% of formalin,and with keeping them in the thermostat at 37°C for the same length of time as the ordinary toxins(48 h.).

3) The dialyzed anatoxins contained ~~XXX~~ 4-6 times less ballast protein,and 4-10 times less total nitrogen per unit of effectiveness of the preparation.

4) In the work, data were introduced about the study of the antigenic and immunogenic properties of the dialyzed anatoxins.

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CONCENTRATION AND PURIFICATION OF THE GANGRENE ANATOXIN OF THE CLOSTRIDIUM OEDEMATIENS(p.141 - 148)

* * *

As it is known, the native gangrene anatoxins have small effectiveness; in the Cl.oedematiens, it makes 5 - 50/ml B.units. Moreover, they contain a large amount of ballast protein, pigment substances, and other remnants of the nutrient media.

These properties of the native anatoxins hinder their utilization for the purpose of immunization against infection, and they force us to submit them to purification and concentration.

The methods of purification and concentration of the Cl.oedematiens anatoxins are not elucidated in the literature, although generally there are descriptions of the diverse methods applied for the production of purified toxins and anatoxins of the pathogenic microbes of the anaerobe group:- the precipitation with alvohol at the isoelectric point(LOGAN), the method of adsorption and elution(VAN HEININGEN), the purification of the anatoxin with the use of the fractionation of proteins by salting out with ammonium sulfate$(NH_4)SO_3$ (VOROB'EV, VAN HEININGEN), the purification with methanol and ethanol(PILLEMER) and others.

For the purification of the Cl.oedematiens anatoxin we accepted the method of precipitation with 1-normal hydrochloric acid in the presence of table salt.

The material of investigation was the native anatoxin of Cl.oedematiens, cultivated on casein hydrolyzate with corn extract. All twenty series of the experimental material were different from each other in regard to effectiveness and amount of the protein nitrogen. For this reason, the work over each series had started with the determination of the isoelectric point of the anatoxin, the pH value of which varied from 3.3 to 4.9 for the different series.

For the determination of the isoelectric point, table salt was put into the anatoxin at the rate of 20%, by the solution of which 1-normal hydrochloric acid was flooded--at the rate of the test-tube analysis approximately from 60 to 80 ml of acid per one liter of the native anatoxin.

(p.142) TABLE 1. (No heading of table)

No. p/p	No. of series	1. INITIAL ANATOXIN a	b	c	d	e	f /	2. CONCENTRATED ANATOXSTAT a	b	c	d	e
1	4	5	265.1	37.8	13.0	25	100	113.1	84.8	117.0	88.8	80.0
2	9	25	274.1	11.2	223.0	30	600	175.0	111.0	541.0	66.6	80.0
3	11	25	306.6	12.6	198.0	30	600	161.0	126.0	477.7	96.7	77.7
4	12	25	345.8	40.6	61.3	30	600	294.0	126.8	473.2	89.5	80.0
5	41	10	267.4	18.2	55.0	21	200	120.1	66.4	301.0	82.6	95.0
6	201	10	278.6	19.6	51.0	25	200	118.7	97.6	204.0	80.0	80.0
7	Mixture of 208, 210, 214	30	315.0	15.4	129.8	28	800	128.8	91.0	879.0	78.8	93.6
8	212	50	347.2	32.2	155.0	30	800	90.4	67.0	1212.0	94.5	53.3
9	214	50	383.6	18.2	274.0	30	900	203.0	107.8	841.0	82.2	60.0

HEADINGS:

1a...activity, B.U. per 1 ml; 1b...total nitrogen, in mg%; 1c...protein N, in mg%; 1d...charge, B.U. per 1 mg of protein N; 1e...concentrate by total nitrogen; 1f...activity, B.U. in 1 ml.

2a...total N in mg%; 2b...protein N in mg%; 2c...charge, B.U. per 1mg of protein N; 2d...per cent of purification by protein N; 2e...percent of yield of antigen.

Then the mixture was left for the night at plus 4° to plus 6°C, and on the next day it was centrifuged or separated depending upon the amount of the material to be treated. The separated sediment was dissolved in buffer phosphate of pH 7.4, or in physiological solution with alkalization by NaOH(caustic sodium) up to a pH 6.8-7.0. The solvent was taken in an amount equal to 1/30 part of the native anatoxin which was taken for concentration.

The degree of purification of the anatoxin was checked as to its content in total and protein nitrogen by means of Kjeldahl's micromethod, at which the content of the protein nitrogen was determined according to the difference between the total nitrogen and the residual nitrogen.

The comparative results of the analyses of the native Cl. oedematiens anatoxin and of the from it obtained first concentrate are given in Table 1.

From the Table it can be seen that, already at the first concentration of the anatoxin, its considerable purification has been also taken place:- in ten of the twenty experimental series, the content in protein nitrogen did not exceed 100 mg%

after the concentration; the content in protein nitrogen has increased 2 to 10 times at the concentration of the anatoxin at a rate of 30 times of its original volume.

As a result of the anatoxin concentration, as the Table indicates, the activity has also been considerably strengthened, expressed in units of binding:- for instance, in the experiments Nos. 1, 5, 6, 7, the activity in one ml of the native anatoxins was 5, 10, 10, 30 B.U., and in the concentrates, correspondingly, 100, 200, 200, and 800 B.U., i.e., the output of antigen was 80 to 95% in these series as to B.U.

The concentrated anatoxin, after the first purification, had preserved its activity completely for 6-8 months when it was kept in the refrigerator. The concentrates which were obtained due to the first precipitation of the anatoxin with the 1-normal hydrochloric acid, were still strongly contaminated in the majority of cases, and their further purification was necessary.

The repeated purification of the anatoxin was carried out in different ways.

1. OVERPRECIPITATION WITH 1-NORMAL HYDROCHLORIC ACID.

The second and the third repeated precipitations of the concentrates were done with a minimum amount of hydrochloric acid at the rate of 1-3 ml per 100 ml of the concentrate, with addition of table salt up to 5-10% NaCl, each time the precipitate was dissolved in physiological solution, without change of the concentration.

The results of the purification of the anatoxin with triple overprecipitation by means of the 1-normal hydrochloric acid are given in Table 2.

TABLE 2.

(No specific heading)

No. p/p	Number of series	Activity B.U. in 1 ml	Protein N mg%	Charge in B.U. per 1 mg of prot. nitrogen	% of purification I. conc. of anatoxin	% of purification Native anatoxin
1	Concentrate of anatoxin of mixture of ser. 208, 210, 214 before purific.	800	91.0	879	-	-
-	After 2. precipit.	800	81.2	972	-	-
-	After 3. precipit.	800	35.5	2285	62.0	92.8
2.	Anatox. concentrate ser. 214, before purif.	900	107.8	841	-	-
-	After 2. precipit.	900	99.4	1006	-	-
-	After 3. precipit.	900	63.0	1426	41.7	88.4
3.	Conc. No. 212 anatox. before purif.	800	66.9	1212	-	-
4.	After 2. precipit.	800	64.05	1281	-	-
-	after 3. precipit.	700	40.25	2000	40.0	95.9

(p.144 cont.) 2. PURIFICATION OF THE CONCENTRATES OF ANATOXIN WITH N/10 BUFFER ACETATE.

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After the second precipitation of the concentrate with 1-normal hydrochloric acid, the precipitate is washed with buffer acetate at pH 4,0. In the case, if the first concentrate was strongly pigmented, the precipitate is washed with buffer acetate even after the third repetition of precipitation. The precipitate is dissolved in physiological solution, without a change of the concentration.

Under such conditions, the anatoxin of Cl. oedematiens is not dissolved in the buffer acetate.

The results of the work are given in Table 3.

3. PURIFICATION WITH ALCOHOL IN THE COLD.

After the second repetition of precipitation with 1/N HCl, the precipitate is twice wahsed with buffer acetate, and dissolved in the initial amount of the physiological solution, and thereafter the concentrate was precipitated with an equal amount of alcohol at pH 5,5, and at a temperature of -5° to -7° C, and with a rate of ionic strength (both the alcohol and the concentrate of the anatoxin was cooled down in advance to [illegible] -5°C). After an hour's settling in a cold room, the precipitate is centrifuged, and dissolved in the initial amount of the physiological solution.

(see Tables 3 and 4 on other side)

4. PURIFICATION WITH ACETONE.

The cooled concentrate of anatoxin is poured out into centrifugal tubules, cooled off in advance, and then, with a constant stirring with the aid of a glass rod, the acetone is poured into it which was cooled off to -10° C, in half of the amount of the anatoxin. In avoidance of the denaturation of the protein, this operation was carried out at the rate of ionic strength from 0,07 to 0,2, with a pH of 5,7 - - 6,3, and at a temperature of -5°C to -7°C. At this temperature, the mixture was left for 30 minutes, after which the tubes were centrifuged with their content for the length of 10-20 minutes. The precipitation was dissolved in physiological solution without a change of the concentration.

The results of the purification are summarized in Table 5. (see further pages)

5. PURIFICATION ON ALUMINUM HYDROXIDE.

The concentrate of the anatoxin is acidified with an m/60 mono-substituted potassium phosphate of pH 5,5, and then one half amount of an $Al(OH)_3$ suspension

(from p.145)

TABLE 3

Headings: a...Number p/p; b...Number of series; includes: 1.1... concentrate of anatoxin in a mixture of series 208,210,214,before purification; 1.2...precipitate after the second precipitation,washed with buffer; 2.1...concentrate of anatoxin of series 214,before purification; 2.2...precipitate after the second precipitation, washed with buffer; 3.1...concentrate of anatoxin in the mixture of series 183 and 184,before purification; 3.2...precipitate,after second and third precipitation washed with buffer./ c...activity, B.U. in 1 ml; d...protein N,in mg%; e...charge,B.U. per 1 mg of protein N; f... PERCENT OF PURIFICATION,in relation to the first concentrate, g... same,in relation to the native anatoxin.

a	b	c	d	e	f	g
1	1.1	800	91.0	879	-	-
1	1.2	700	46.2	1515	49.3	89.5
2	2.1	900	107.8	841	-	-
2	2.2	900	74.2	1340	31.2	86.3
3	3.1	400	152.6	263	-	-
3	3.2	300	33.6	892	77.9	93.8

TABLE 4.

(NOTE: Headings c,d,e,f,g the same as for Table 3)

1	Anatoxin concentrate of series 15,before purif.	>400 <600	122.4	327	-	-
-	Precipitate after second precipitation,washed with buffer	>400 <600	56.8	714	55.0	86.7
-	After purification with alcohol	300	19.4	1546	85.0	95.5
2	Anatoxin concentrate of series 16 before purific.	600	134.3	447	-	-
-	Precipitate,after 2nd precipitation,washed with buffer	>400	48.5	825	65.0	90.0
-	After purification with alcohol	300	31.9	967	76.3	93.4

(over)

(from p.146)

TABLE 5.

Headings: c,d,e,f,g as for Table 3(see previous page)

STAT

a	b	c	d	e	f	g
1	Concentrate of anatoxin in a mixture of series 210,208, 214,before purification	800	91.0	879	-	-
	- After purification	600	25.2	2400	72.4	92.4
2	Concentrate of anatoxin ser.16,before purification	600	134.3	447	-	---
-	After purification	300	20.2	1435	85.0	96.0
3	Concentrate of anatoxin of series 216 before purif.	800	56.0	1428	-	-
-	After purification	700	24.5	2857	61.5	87.5
4	Concentrate of anatoxin of ser.38 before purification	300	79.8	380	-	-
-	After purification with acetone,with a 3-times increase of concentration	800	32.2	2484	87.2	96.8

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TABLE 6.

(Same headings as for Table 3 or above)

a	b	c	d	e	f	g
1	Anatoxin concentr. Ser.214	900	107.8	841	-	-
-	Concentr.purified,thrice precipitated with HCl	900	63.0	1421	40.2	88.4
-	supernatant fluid	M.10				
-	I.Elution	800	21.0	3809	67.2	96.2
-	II.Elution	300	13.5	2222	78.6	97.4
2	Anatox.concentr.Ser.212	800	66.9	1212	-	-
-	Supernatant fluid	M.10				
-	I.Elution	400	19.9	714	70.2 /XXXXXXX/	98.0
-	II.Elution	100	14.0	714	80.0.	-
-	Third Elution	M.100	5.6	-	-	-
3.	Concentrate of mixture of Ser.208,210,214	800	91.0	879	-	-
-	Supernatant fluid	M.20				
-	I.Elution	400	16.8	2369	81.6	94.9
-	II.Elution	300	15.4	2000	84.2	-

- - -

(cont.from p.146,text)...is added which contains 12 mg Al_2O_3 in one ml. The mixture is now and then stirred for 30 minutes, and then it is centrifuged. After the centrifugation, the supernatant fluid is analyzed for nitrogen and activity, and the precipitate is washed with m/60 potassium phophate,of a pH of 5.5.

The elution of the anatoxin from the adsorbate was done with buffer phosphate at pH 8.0, taken in the amount of half of the volume of the initial concentrate. We left the sample for 1-2 hours in the thermostat, (p.147) and for the night in the refrigerator. Thereafter, the precipitate was separated by centrifugation, and the process of elution was repeated. The initial concentrates of the anatoxin were very well sorbed in the acid zone,in a wide range from pH 5.5 to pH 6.8. The supernatant fluid of the sorption had less than 10 B.U. From 30% to 60% of the adsorbed antigen was successfully eluted.

The results of the experiments are summarized in Table 6(See previous page).

As it can be seen from the given data, by means of the adsorption of the antigen with successive elution, a highly purified preparation of the anatoxin can be produced. However, even with multiple successive elutions, not more than 60% of the anatoxin could be liberated from the adsorbed condition. It is evident that the adsorbed bonds(links) of the anatoxin purified with aluminum hydroxide are extraordinarily stable, and it is needed to find additional force for the full elution of the antigen.

(p.148) CONCLUSIONS.

1) The concentration of the Cl.oedematiens anatoxin, produced on a casein medium, at the first stage of purification is successfully realized by the precipitation at the isoelectric point,with a 1/N hydrochloric acid, with the addition of table salt up to 20%.

2) For the purpose of further purification of the concentrates, these were investigated:- repeated superprecipitation with hydrochloric acid, removal of the ballasts with buffer acetate, precipitation with alcohol and acetone in the cold, adsorption on Aluminum hudroxide, with subsequent elution.

3) The best results of purification are obtained at the adsorption of the anatoxin on Aluminum hydroxide,with subsequent elution of the antigen. However, the low output does not permit that this method should be recommended for the practice.

(over)

4) High degree of purification of the anatoxin with good output is reached by precipitation of the anatoxin with acetone in the cold, and with triple superprecipitation with hydrochloric acid, and at the isoelectric point.

5) The obtained concentrated anatoxins of Cl.oedematiens were investigated in immunological experiments in the Department of Wound Infections of the N.F.Gamalei Institute for Epidemiology and Microbiology, of the Academy of Medical Sciences, of the U.S.S.R.

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(p.149)

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SOME DATA ON THE PURIFICATION AND SORPTION OF THE EDEMATIENS ANATOXIN
(p.149- 157)

* * *

The invasion of the combined preparations into the practice of modern immunology dictates the necessity of the creation of antigens which possess a high immunological effectiveness in a small volume. The production of such antigens for the active immunization against tetanus and gas gangrene is possible by means of the purification and concentration of the native anatoxins, with their subsequent sorption on aluminum compounds.

The problems of the preparation of different purified sorbed anatoxins wage studied not at all at an identical degree. Thus, they are comparatively well worked out in respect to the tetanus antigen, and they are insufficiently worked out in regard to the anatoxins of gas gangrene, including also the edematiens anatoxin.

In the available literature, we have detected only two communications devoted to the purification and concentration of the edematiens anatoxin-- they are the works of BLAGOVESHCHENSKII and collaborators(1956), and of CHERKAS(1958). The problems of sorption of the purified edematiens anatoxin were not completely elucidated in the literature.

In connection with this, we made it out goal: 1) to study some of the methods of purification of the edematiens anatoxin for the reason that the most effective ones among them should be found; 2) to compare the sorption effectiveness of aluminum hydroxide and aluminum phosphate; 3) to study the immunological activity of the antigens purified by the various methods; 4) to explain, how the various content of aluminum hydroxide will influence the immunological capability of the purified sorbed edematiens anatoxin.

The work on the purification and concentration was carried out with four series of edematiens anatoxin which were prepared on casein media. The antigens of Series No.208 and 209 were obtained on the No.1a medium(pure acid-casein hydrolyzate). They were characterized(p.150) by the following data:

(p.150 cont.)

:- antitoxin combining capacity...15 and 18 B.U. in one ml; content in total nitrogen---251.17 and 261.61 mg%; content in protein nitrogen---1.STATand 4.79 mg%. The antigens of Series No.4 and No.217 were prepared on the No.1 medium(acid-casein hydrolyzate plys 3% corn extract). Their basic data are:- antitoxin combining capacity---50 and 23 B.U. in one ml; content in total nitrogen---361.20 and 410.51 mg%; content in protein nitrogen---27.20 and 18.04 mg%.

The isoelectric points of the various series corresponded to the pH values from 3.8 to 4.1 .

For the purification of the edematiens antotoxin the following methods have been used:

1) precipitation at the isoelectric point,with 1/normal hydrochloric acid at room temperature,with preliminary addition of 20% of NaCl;- the method was elaborated by BLAGOVESHCHENSKII and co-workers(1956) for the purification of the tetanus and the gangrene toxoids;

2) pretipitation at the isoelectric point with 10% metaphosphoric acid,prepared ex tempore,at a temperature of -5°C to -15°C,with the preliminary addition of 25% of NaCl;- the method of RAYNAUD and co-workers(1953) modified by us,and being used for the purification of diphtheria, tetanus and staphylococcus toxoids;

3) outsalting with ammonium sulfate at its 60-70% concentration for saturation, with subsequent dialysis;

4) precipitation at the isoelectric point with a 30% solution of trichloracetic acid at room temperature with preliminary addition of 20% of NaCl.

The precipitated preparations were separated with the centrifuge,or with the separator of the A-59-3-M mark, and they were dissolved in various amounts of distilled water,by alkalizing it with 1-normal NaOH(in case of purification with acids) at pH 6.5 to 6.8. The different samples of antigen were concentrated 10-14 times. The data about the single purification of edematiens antigen are given in Table 1.

We see that all used methods of purification have given results of approximately similar value. The output of antigen in all cases was shown to be sufficiently high---62.5 to 100%; however,the coefficient of purification,in regard to total nitrogen, was not satisfactory;---15.5 to 73.0--,from our point of view.

It is interesting to remark, that, at the concentration of the series No.

No.208 and No.209, with comparatively poor total nitrogen, higher coefficients of purification were obtained (31.7 - 73.0) than with the concentration STAT the Series No.4 and No.217 which were rich in nitrogen (15.5 - 22.8).

(p.151) TABLE 1

RESULTS OF A SINGLE PURIFICATION OF THE EDEMATIENS ANATOXIN

HEADINGS: A...method of purification; b...numbers of series; c...output of antigen, %; d...degree of removal of total nitrogen, %; e...number of B.U. per one mg of total nitrogen; f...number of B.U. per 1 mg of protein nitrogen; g...coefficient of purification by total nitrogen; h...coefficient of purification by protein nitrogen.

A	b	c	d	e	f	g	h
Hydrochloric acid	208	100.0	97.91	285	567	47.5	-
	209	94.4	98.53	443	1043	63.3	2.59
	-	90.6	98.68	219	347	73.0	-
	4	62.5	96.85	273	392	19.5	2.13
	217	100.0	95.85	137	260	22.8	2.13
Metaphosphoric acid	208	100.0	97.49	238	-	39.7	-
	209	92.3	97.29	234	478	33.4	1.27
	-	100.0	96.90	222	438	31.7	1.17
	217	85.4	96.04	121	224	20.2	1.84
Ammonium sulfate	209	73.1	98.20	280	505	40.0	1.34
	217	100.0	93.93	93	159	15.5	1.30
Trichloracetic acid	209	83.3	98.14	309	719	44.1	1.91

It must be supposed that the content in total nitrogen in the native anatoxins, and consequently also in the initial nutrient media, is apparently one of the important factors of getting good results at the purification of these antigens. In this connection, the replacement of the meat media, which was done in the Gamalei Institute of Epidemiology and Microbiology, may be considered entirely reasonable and logical; the media which were used for the production of the tetanus and for the gangrene anatoxins, were replaced by the less complicated and more standard casein media (VYGODCHIKOV and co-workers, 1957).

The small size of the obtained coefficients of purification instigated us to

subject the first concentrated preparations to a second purification.

For this purpose the following methods were used: STAT

1) adsorption to $Al(OH)_3$ with successive four-times elution with alkaline buffer phosphate(ph: 7.9) at a temperature of 10°C and 57°C,- the method suggested by BLAGOVESHCHENSKII and coworkers(1956) for the second purification of the tetanus toxoid;

2) precipitation with equal amount of a mixture of 3.5 M solution of KH_2PO_4 and K_2HPO_4 at a pH of 6.5 to 6.6, and a temperature of 4°C:- the method of TURPIN and coworkers(1956) which was modified by us and which was used for the second purification of the staphyloccic toxoid;

3) precipitation with a 30% solution of trichloroacetic acid,at a pH of 3.8 and at room temperature;

(p.152) 4) outsalting with ammonium sulfate at 32.5-35% concentration to saturation with subsequent dialysis;

5) quick precipitation by a double volume of acetone at 4°C temperature:- the method of AMOUREUX and YEN(1950) ,used for the second purification of the tetanus and the diphtheria toxoids.

A part of the obtained preparations was subjected to sterilization by filtration through a No.5-F filter,or Zeiss filter.This procedure was accompanied by noticeable losses of the antigen(in individual cases up to 39%), in connection with which its output was considerably lower in the filtered preparations.

The total data in regard to the double purification of the edematiens anatoxin are represented in Table 2.(see next page)

The very best results(p.153) were obtained with the precipitation of the antigen by means of the hydrochloric and metaphosphoric acids, with sibsequent purification with 3.5 M phosphates:- the output reached 31.4-88.9%; the coefficient of purification by total nitrogen was 76.9 -554.3. Other methods have conditioned an output of 21.9 - 72.2%,and a coefficient of purification of 33.8 - 399.6. Noticeable differences could not be found in the effectiveness between the first two methods. Since the precipitation with hydrochloric acid is technically easier than the precipitation with metaphosphoric acid, the use of hydrochloric acid was more appropriate at the first stage of purification.

In this manner,among all investigated methods of purification of the edema-

(Table from p.152)

TABLE 2

RESULTS OF THE DOUBLE PURIFICATION OF THE EDEMATIENS ANATOXIN

STAT

	METHOD OF PURIFICATION	b	c	d	e	f	g	h
		(Headings as in Table 1)						
I.	FIRST: Hydrochloric acid SECOND: Adsorption-elution	209	21.9	99.94	1169	1169	389.7	-
	" Phosphates	209	88.9	99.75	2472	3615	353.1	9.35
		4	31.7*	99.66	1291	1604	92.2	8.72
		-	60.0*	99.23	1077	1295	76.9	7.04
		217	34.8*	99.65	564	4890	94.0	40.08
	" Trichloroacetic acid	209	72.2	99.82	2797	2797	399.6	7.44
II.	FIRST: Metaphosphoric acid SECOND: Phosphates	209	31.4*	99.94	3880	5912	554.3	15.72
		-	88.9	99.84	3752	4306	536.0	11.45
		4	36.7*	99.55	1128	1474	80.6	8.01
		217	34.8*	99.61	504	892	84.0	7.13
	" Trichloroacetic acid	209	55.6	99.05	404	515	57.7	1.37
III	FIRST: Ammonium sulfate SECOND: " "	209	23.4	-	-	-	-	-
		217	39.1*	98.92	203	253	33.8	2.07
IV	FIRST: Trichloroacetic acid SECOND: Acetone	209	33.7	99.81	1182	1680	168.9	4.47

* These figures are lowered on account of the loss of antigen at the sterilizing filtration.

(text from p.153 cont.)...tiens anatoxin the most accessible and the most effective proved to be the method of precipitating the antigen with hydrochloric acid and with the mixture of phosphates. It gave a possibility to produce preparations containing from 564 to 2472 B.U. per one mg of total nitrogen, and from 1604 to 4890 B.U. per one mg of protein nitrogen.

The comparative study of the sorption activity of $Al(OH)_3$ and of Al phosphate was carried out with the edematiens anatoxin of Series No.4, purified with

hydrochloric acid and with the mixture of the phosphates, and containing 475 B.U. in one ml. The $Al(OH)_3$ suspension was prepared in the Department of Biochemistry, STAT according to the modified prescription(formula) of WILLSTETTER, the aluminum phosphate suspension was prepared by us independently, according to the method of HOLT(1950).

To the separate portions of the antigen, equal volumes of the sorbents were added, containing different amounts of $Al(OH)_3$(from 1 to 10.5 mg) and of aluminum phosphate(from 2 to 13.6 mg) per one ml. In this way, in the samples of the aluminum hydroxide, we produced 238 B.U. of antigen in one ml, and 0.5, 1, 2, 3, and 5.25 mg of the sorbent, and in the samples of aluminum phosphate -- also 238 B.U. of antigen, and 1, 2, 3, 5, and 6.8 mg of the sorbent per one ml. The adsorption preparation with the aluminum hydroxide was made at pH 6.7, with the aluminum phosphate at pH 6.1. After 18 hours, in the supernatant fluid, the amount of the non-sorbed anatoxin was determined.

It was detected that for a complete sorption of the antigen, 3 mg of $Al(OH)_3$ and 6.8 mg of aluminum phosphate was necessary. Consequently, the aluminum hydroxide proved to be a more active sorbent than the phosphate, which is in agreement with the data of a number of authors, data which were obtained in experiments with other anatoxins(HALONEN & JÄNTES, 1955; PETROSYAN and coworkers, 1956; BERGOL'TSEVA, 1957).

For the complete characteristics of the different methods of purification of the edematiens anatoxin it was necessary to conduct a comparative study of the immunogenicity of the preparations purified and concentrated by the various methods. We conducted this work with the antigen of Series No. 217, purified by three methods: 1) by precipitation with hydrochloric acid and phosphates, 2) by precipitation with metaphosphoric acid and with(p.154) phosphates; 3) double out-salting with ammonium sulfate. After the purification, all preparations were completely sorbed on $Al(OH)_3$ with a concentration of the latter of 5 mg in one ml.

Rabbits of six groups, weighing each 2.0 - 2.3 Kg(four rabbits in each group) were immunized with a single-shot under the skin of their flank, with two doses of each of three preparations -- 10 and 75 B.U. -- introduced in the same amount (one ml), and with a conctant content of 5 mg of $Al(OH)_3$ in one ml. After two months, all rabbits were revaccinated with the same doses of the corresponding

preparations. In the animals, for the determination of the content of antitoxin in their blood, blood was taken on the 15th, 25th and 60th day after the STAT immunization, and on the 5th, 15th and 118th day after the revaccination. Each serum was titrated separately. The obtained data were submitted to a statistical treatment which consisted in the following:- during the entire period of investigation, for each group of rabbits, the geometric average of the edematiens antitoxin titres and its logarithm, with the standard deviation, was calculated.

For the elucidation of the authenticity(significance) of the difference between the antitoxin levels in the different groups, a comparison was made of the logarithms of the geimetrical averages of the titres, with the aid of the t-test with a level of significance of 0.05.

In the course of the experiment, it came out that not a single one of the examined preparations had a true superiority above the other in regard to immunogenicity. In the group of rabbits immunized with 10 B.U., the average(geometric) of the titres after the immunization had reached a maximum of 3.42 - 5.12 A.U., after revaccination - 18.54 - 49.33 A.U.; in the groups of animals which were inoculated with 75 B.U., correspondingly, the values were 6.90 - 15.30 A.U., and 43.09 - 84.26 A.U. Four months after the revaccination, all rabbits were completely resistant to the intramuscular inoculation of 100 D.S.L. of a 2-day culture of Cl. oedematiens. Before the inoculation, the antitoxin level in their blood varied from 1.25 to 17.0 A.U.

In this way, the method of purification of the edematiens toxoid, with the aid of hydrochloric acid and of phosphates, having proved itself the simplest and the most effective in regard to its chemical indices, had allowed the production of a preparation which is not less immunogenic than the preparations purified by other and more complicated methods.

The following section of our work was devoted to the elucidation of how the amount of $Al(OH)_3$ ingluences the immunogenicity of the oedematiens anatoxin if the sorbent is in surplus in comparison with the needs for a full sorption. This question gained special interest in connection eith the researches of EISLER and EIBL(1953), which revealed that the immunological effectiveness of the sorbed anatoxins, apart from the completeness of the sorption of the antigen, is also determined by the strength of its combination with the adsorbent, and the latter, as

(p.155) FIG.(full-page) COMPARISON OF THE IMMUNOLOGICAL EFFECTIVENESS OF THE DIFFERENT CONCENTRATIONS OF ALUMINUM HYDROXIDE. STAT

ORDINATA: Titres in A.U. and their logarithms;

ABSCISSA: Days(0 to 129)

On DIAGRAM: Revaccination(arrow).

p.156)....it has been indicated by DEHMEL(1949) and HOLT(1950), depends upon the content of the depot substance in the preparation.

The rabbits of three groups, wighing each 2.0 to 2.3 Kg(three rabbits in each group) were submitted to a single-shot immunization under the skin of the flank, with preparations purified with the Series No.4 sorbed edematiens anatoxin, containing one and the same dose of antigen(25 B.U.), but different amounts of $Al(OH)_3$ - - -0.56, 5.0 and 10.96 mg, in one ml of the preparation. The degree of sorption of the antigens in all cases has been complete(full). The revaccination of the animals was done two months later. The content of edematiens antitoxin was examined in the blood of each rabbit dynamically. The obtained data were submitted to the usual statistical treatment(see above). Its results are given in the Figure.

After the first immunization, the preparations which contained 5.0 and 10.96 mg of $Al(OH)_3$ revealed definite immunological superiority over the antigen with 0.56 mg of aluminum hydroxide. Between the first two preparations, no noticeable differences could be found in the immunogenic capacity. After revaccination, the effectiveness of all three antigens was roughly identical. In this manner, the results of the first immunization of the rabbits indicated that the increase in the amount of $Al(OH)_3$ in the preparation over the amount which was required for the full sorption of the antigen(from 0.56 to 5.0 mg) has increased the immunogenicity of the purified sorbed edematiens anatoxin taken in the dose of 25 B.U. Further increase in the concentration of the aluminum hydroxide(up to 10.96 mg) proved to be ineffective.

In connection with this, we suggest that, at the choice of the dose of the adsorbent for the purified sorbed anatoxins, one should not stop at that minimum concentration of the adsorbent which gives a full sorption of the antigen. It is necessary to bring to light the optimum dose of the adsorbent which assures the easiest bondage of the latter(adsorbent) with the antigen, and, consequently, also the highest immunogenicity of the preparation, not raising, however, thereby the

"threshold of reactogenicity".

CONCLUSIONS.

STAT

1) Among the methods which we have examined for the purification of the edematiens toxoid the simplest and the most effective proved to be the method of precipitating the antigen at the first stage of purification with a 1½normal hydrochloric acid, with the addition of 20% NaCl to it, and at the second stage with a mixture of a 3.5 M solution of KH_2PO_4 and K_2HPO_4.

2) At the comparison of the study of the sorption activity of $Al(OH)_3$ and of aluminum phosphate, introduced into the experiments for the purification of the edematiens toxoid, it was revealed that the aluminum hydroxide is a more active sorbent than the phosphate.

(p.157) 3) The edematiens anatoxin which was purified with the aid of hydrochloric acid and of phosphates, in regard to its immunogenic power, was not inferior to the preparations which were purified by other, more complicated methods.

4) The increase in the concentration of the $Al(OH)_3$ above the amount which is needed for the full sorption of the antigen, has caused, within known definite limits, an increase in the immunogenicity of the purified, sorbed edematiens anatoxin.

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(p.159)

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ACTIVE IMMUNIZATION OF THE EQUINE PRODUCTORS OF THE ANTITETANUS SERUM WITH EDEMATIENS ANATOXIN(p.159 - p.166)

* * *

Among the equine productors of therapeutic sera, sometimes cases of gas gangrene are observed. Thus, ZANNOLLI and CATINO(1925) describe a few cases of gas gangrene in equine productors of various sera. In the majority of the cases, these authors have distinguished Cl.histolyticum as well as other anaerobes(Cl.septicum, Cl.perfringens, Cl.oedematiens). These anaerobes were sometimes isolated in association with each other as well as with other aerobes not identified by the authors. Anaerobic microbes were also isolated from abscesses at the site of the inoculation of an antigen. In a number of cases, upon the inoculation of Cl.histolyticum, a specific serum was used which showed a therapeutic effectiveness.

In the native literature, we were unable to detect data about the gas gangrene in equine productors of therapeutic sera. One of the authors of the present work (O.K.KOMKOVA) had observed cases of gas gangrene in equine productors of therapeutic sera. At the bacteriological investigation of the material, in a number of cases, a culture of the Cl.oedematiens was isolated; in some cases the specific serum showed therapeutic action.

From 1951 to 1955, among the equine productors of the antitetanus serum two cases of gas gangrene were observed. In both instances, a highly toxigenic culture of the Cl.oedematiens was isolated. One of the isolated strains(79) is used in our Institute and in a number of other institutes as the production strain for the making of edematiens anatoxin.

The above indicate cases prompted us to immunize the live-stock of the equine producers of the antitetanus serum--(p.160) with edematiens anatoxin. In this manner, at our disposal, a considerable live-stock of horses was found on which we were able to investigate the immunogenic properties of the native and of the concentrated edematiens anatoxins produced in the Gamalei Institute for Epidemiology and Microbiology.

In the years 1954-1955, in the Gamalei Institute of Epidemiology and Microbiology, formulas of nutritive media were worked out whose basis was the acid hydrolyzate of casein(VINOGRADOVA, VLASOVA, PALKINA, 1956). On these nutrient media, edematiens toxins were obtained of the power of 2000 - 10 000 Dlm, and anatoxins in which 10-60 B.U.-s were contained(VLASOVA, VINOGRADOVA, PALKINA, 1957). The native anatoxins were purified and concentrated in the Department of Biochemistry of the Gamalei Institute of Epidemiology and Microbiology(BLAGOVESHCHENSKII and others, 1956). The produced preparations contained 600 - 1500 B.U. per ml, depending upon the quality of the initial preparation and upon the degree of concentration.

The antigenic and immunogenic properties of the native and of the concentrated preparations were examined on small laboratory animals(VLASOVA, ZELEVINSKAYA, VOLKOVA, 1957). The conducted experiments showed the good immunogenic properties of the produced preparations. All the above discussed data permitted us to take the indicated preparations for active immunization of horses against gas gangrene provoked by the Cl. oedematiens. All together two experiments were set up(one in 1955, and the other in 1957), and 226 horses were immunized.

EXPERIMENT No.1:

* * * * * * * * In the first experiment, 154 horses were immunized. The immunization was carried out with native edematiens anatoxin of Series No. 203 containing 40 B.U. per ml. The immunization was done with 5 ml of anatoxin which was precipitated with 0.5% of potassium alum, or sorbed on 5% $Al(OH)_3$. In this manner, for the immunization, 300 B.U. of anatoxin were taken, or 0.4 - 0.5 B.U. per Kg of body weight. A portion of the horses was immunized with a single shot, a portion was immunized with two shots. The samples for the determination of the titre of antibodies were taken at different times--from 15 days to 3 months after the immunization. The results of the experiments are given in Table 1 and 2.

As it can be seen from Table 1, at the taking of the samples 15-30 days after the immunization, in the majority of horses(43 and 56) the titre was >1 A.U., and in 8 of them it was >10 A.U., and only in 4 horses was the titre $0 < 0.1$ A.U.

In the groups in which the samples were taken at a more advanced period of time, the number of horses with low titres was increased; thus, at taking the samples after 2-3 months, in half of the horses(in 8 out of 15) the titre was lower than 0.1 A.U.

(p.161)

TABLE 1.

SINGLE IMMUNIZATION OF HORSES WITH EDEMATIENS TOXOID

STAT

NAME OF ANTIGEN & DOSAGE	TIME OF TAKING SAMPLE	Number of horses	OF THEM....HAVING A TITRE,A.U. <0.1	>0.1<1	>1<10	>10<50
Edematiens anatoxin Ser.203, precipitated with 0.5% alum(200 B.U. in 5 ml)	15-20	24	1	3	15	5
	20-30	32	3	6	20	3
	30-60	35	7	9	16	2
	60-90	15	8	2	5	-
The same, sorbed on 5%(200 B.U. in 5 ml)	15-30	17	-	9	7	1

TABLE 2.

TWO-SHOT IMMUNIZATION OF HORSES WITH EDEMATIENS TOXOID

INTERVAL BETWEEN injections	Time of sample taking	Number of horses	OF THEM . . . HAVING TITRE,A.U. <0.1	>0.1<1	>1<10	>10<50	>50<100
30-40 days	15-20	22	-	-	8	13	1
	20-30	14	-	-	7	7	-
2-3 months	15-20	22	-	3	8	10	1

From the presented data it follows that, at a single-shot immunization of the horses with native alum-precipitated edematiens anatoxin, the peak of titres was observed at 15-20 days(30 days) after the immunization, and at a later period of time, the titres dropped. From the same data it is evident that, at a single-shot immunization, there is nevertheless a group of refractory horses(about 7%) which did not have even a minimum of titre in their blood.

The fact calls attention that, at the immunization with the same antigen, but sorbed on 5% $Al(OH)_3$, the refractory group was missing. These data were confirmed in the subsequent experiment(See Table 4, Group 1). Therefore, we think them possible, notwithstanding the small number of this group, compared with the previous one, attention should be paid to these results.

In Table 2, the results of the two-shot immunization at different intervals are presented.

The horses were immunized with the same dose(5 ml) of native-,alum-precipitated edematiens anatoxin. In the group of horses which received the second injection 2-3 months after the first injection, there were horses having > 0.1 and < 1 A.U. after the first injection.

As it is seen from Table 2, not a single horse had a titre < 0.1 A.U. All together, in three out of 56, the titres were > 0.1 < 1 A.U., and more than a half of the horses(32 out of 56) had titres > 10 A.U.

In this way, after a double immunization the group of refractory horses, which had not even a minimum of titre in their blood, had disappeared.

In the year of 1957, 29 horses(14 after the single shot immunization, and 15 after the two-shot immunization in 1955) were revaccinated. Before the revaccination, in 22 horses blood was taken for the determination of the antitoxin titre. In 6 horses the titre was > 0.1 < 0.5 A.U., and in 16 horses < 0.1 A.U.

For the revaccination, native anatoxin precipitated with 1% alum was taken. It contained 30 B.U. per ml. For the revaccination, 10 ml anatoxin, i.e., 300 B.U., were taken.

The results of the experiment are given in Table 3.

TABLE 3

REVACCINATION WITH EDEMATIENS ANATOXIN OF THE HORSES IMMUNIZED IN THE YEAR 1956

Group No.	Group name	Sample taking after revaccination, days	Number of horses	OF THEM...HAVING TITRE, A.U.				
				>1 <10	>10 <50	>50 <100	>100 <200	>300 <500
1	After 1-shot immunization	10-20	14	1	7	3	1	2
		30-45	7	4	2	1	-	-
2	After 2-shot immunization	10-20	15	-	6	8	1	-
		30-45	9	2	7	-	-	-

After revaccination, the results in both group were roughly identical:- more than a half of the horses(15 out of 29) had a titre > 50 A.U.; not a single horse had a titre < A.U. In the course of time, the titre was reduced. In one horse, the dynamism of the anatoxin titre was particularly followed up after the revaccination.

In 11 horses, blood was taken at 2-5 months after the revaccination. All horses had a titre > 0.1 & < 1 A.U. The experiment showed that, at revaccination when conducted after such a delayed period of time as two years, a considerable amount of

(p.163) antitoxin titre can be obtained(up to 300 A.U.). These data speak of the good immunological reconstruction of the organism after single shot and two-shot immunization, i.e., of the fact that the immunization was done with good antigens.

EXPERIMENT No.2

* * * * * * * * * In the second experiment, we set the task to compare the immunogenic properties of the native and purified concentrated edematiens anatoxin.

For the immunization of the horses, native edematiens anatoxin of Series No. 218 was taken; it contained 30 B.U. per ml. The purified concentrated anatoxin was also diluted to a 30 B.U. per ml content. The degree of purification of the anatoxin taken for the experiment may be judged from the following figures:

	NUMBER OF B.U./mg of total nitrogen	NUMBER OF B.U./mg of protein nitrogen
Native anatoxin, Series 218	3.4	166
Concentrated anatoxin Ser. 218	380	445

The immunization of the horses was carried out with the native and with the concentrated edematiens anatoxin, sorbed on $Al(OH)_3$ as well as with the native anatoxin precipitated by 1% of potassium alum. Both the native and the concentrated anatoxins were sorbed by 5% $Al(OH)_3$ (in the computation into Al_2O_3, the sorbed preparation contained 0.6 mg of Al_2O_3 in one ml). Before the immunization, the completeness and fullness of sorption and precipitation was checked. In the sorbed preparations, the antigen in the supernatant fluid was not present, i.e., the sorption was complete; at the precipitation with alum, about 20% of the antigen remained in the supernatant fluid.

All together 72 horses were immunized. Before the immunization, in 25 horses blood was taken for the determination of the natural antitoxins. Not in a single case was antitoxin found. These data are in agreement with the data of GUILLAUMIE and others(1956) who also could find no natural antitoxins in the blood of horses. The horses were immunized with a single shot of 10 ml anatoxin edematiens under the skin; in this way, 300 B.U. of anatoxin were inoculated into the horses.

All horses were assigned into three groups. The first group--had 23 horses. They were immunized with concentrated anatoxin, sorbed on 0.6 mg/ml of $Al(OH)_3$. The second group--had 18 horses. They were immunized with native anatoxin, sorbed on 0.6 mg/ml of $Al(OH)_3$. The third group--had 31 horses. They were immunized with na-

tive edematiens anatoxin, precipitated with 1% potassium alum.

The results of the experiment are given in Table 4.

STAT

(p.164)

TABLE 4.

ONE-SHOT IMMUNIZATION OF HORSES WITH NATIVE AND CONCENTRATED EDEMATIENS ANATOXIN

NAME OF ANTIGEN & DOSAGE	TIME OF SAMPLE TAKING, DAYS	NUMBER OF HORSES	OF THEM . . . HAVING TITRE, A.U.			
			<0.1	<0.1 >1	<1 >10	<10 >50 (fifty)
Edematiens anatoxin of Series 24, conc., sorbed on 0.6mg/ml $Al(OH)_3$ (300 B.U. in 10 ml)	10-15	8	2	3	3	-
	15-30	17	4	4	9	-
	30-60	19	-	16	3	-
	60-90	11	-	11	-	-
Edematiens anatoxin of Ser. 24, native, sorbed on 0.6 mg/ml $Al(OH)_3$ (300 B.U. in 10 ml)	10-15	8	4	2	2	-
	15-30	15	-	0	6	-
	30-60	12	-	10	2	-
	60-90	10	-	10	-	-
Edematiens anatoxin of Ser. 24, native, precipitated by 1% alum (300 B.U. in 10 ml)	10-15	8	3	4	1	-
	15-30	22	1	2	17	2
	30-60	12	-	8	4	-
	60-90	18	2	14	2	-

As it can be seen from the Table, the maximum accumulation of antitoxin took place between the 15th and 30th days after the immunization. The largest number of horses which at this time had >0.1 A.U. in their blood was in Group III, i.e., upon immunization with the native alum-precipitated anatoxin. The results in Group I and II were roughly identical. These data showed us that in the process of purification and concentration the antigenic and the immunogenic properties of the anatoxin do not change. These data had for us a great importance, since the purified and concentrated edematiens anatoxin enters as one of the components into the complex preparations which are used for the active immunization of people.

In the domestic literature, we were unable to find data about the active immunization of large animals. The available numerous works about the immunization of the small laboratory animals with edematiens anatoxin as such as well as in a mixture with other anatoxin agents of gas gangrene have shown the good immunogenic

properties of the edematiens anatoxins(PLETENEVA,1950; KOVTUNOVICH,1953; SHNEERSON,1954)(KHODOROVA,1954; BORISONIK,1955; VYGODCHIKOV,ZELEVINSKAYA,VOLKOVA and others,1957; ZIMINA,1957).(p.165) Similar results were also obtained by a number of foreign authors(ROBERTSON & KEPPIE,1943; ALTMEIER and others,1947,1956). In the works of the foreign authors, we were successful to find data about the immunization of large animals with the edematiens anatoxin. Thus, TUNNICLIFF(1939-1943) brings the results of immunization of sheep with alum-precipitated edematiens anatoxins. The author indicates that, after a single injection of immunization with 5 ml of anatoxin, the sheep happened to be protected from infection for the duration of 45 months, i.e., for almost about 4 years. GUILLAUMIE,KREUGER, and DEVISME(1956) have immunized hogs, mules, and horses with native edematiens anatoxin. In compliance with the data of these authors, in the blood of the immunized animals from 0.5 to 25 A.U. accumulated in relation with the number of injections and with the amount of the preparations which were used for the immunization.

In this way, the data obtained by us are in full agreement with the results of the investigation of the above cited authors.

CONCLUSIONS

1) After a one-shot immunization with the native alum-precipitated edematiens anatoxin in the dose of 200-300 B.U., per injection, or 0.5-0.8 B.U. per Kg of body weight, in the blood of the majority of horses(42 out of 56) $\geqslant$1 A.U. has accumulated. Only individual horses(4 out of 56) did not have 0.1 A.U.

2) Upon a single shot immunization with the native and concentrated edematiens anatoxin, sorbed on $Al(OH)_3$, identical results were obtained, but somewhat inferior to those received upon the immunization with native alum-precipitated anatoxin.

3) After a two-shot immunization with the same antigen(twice, each 200 B.U.), in the blood of the majority of horses(53 out of 56) $\geqslant$1 A.U. was found; in half of them .... $\geqslant$10 A.U., and only in 3 horses ... $>$0.1 $<$1 A.U.

4) After revaccination of the horses with native alum-precipitated edematiens anatoxin, carried out 2 years later after the initial immunization, in the blood of the majority of animals(28 out of 29) $>$ 10 A.U. has accumulated; in the blood of half of them... $\geqslant$50 A.U.

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INDIVIDUAL PECULIARITIES OF ANTITOXIN PRODUCTION AT THE ONE-SHOT IMMUNIZATION WITH PURIFIED SORBED ANATOXINS(p.167- 174)

* * * *

The wide perspectives of the practical use of purified sorbed anatoxins are creating the necessity for the study of the immunological laws which apply to the vaccination with these preparations. The investigations of this type of study are always timely, since up to now the basic ideas about the laws of antitoxic immunity had been chiefly founded upon data obtained in experiments with native anatoxins.

A big step forward in the matter of the study of the immunological peculiarities of the sorbed preparations is the work conducted in our Department(1958) in which, together with other problems, the general laws of the dynamism of antitoxic immunity were investigated in detail. However, the dynamism of the individual immunological reactions upon vaccination with the sorbed antigen is still very little studied for the present.

The study of the dynamism of the accumulation of the antitoxin in the blood of the animals is conveniently conducted by means of the construction(construction) of graphic curves of the relationship of "TIME-EFFECT", in which on the abscissal axis the time is put down that has past from the moment of immunization, and on the ordinatal axis- the height of the antitoxic titres. The juxtaposition of the individual graphs of antitoxin accumulation and the attempt at grouping them according to separate types, starting from the immunological similarity or difference, may represent certain theoretical and practical interest.

At the use of the purified sorbed anatoxins, the peculiarities of the immunological reaction of the organism are discerned in the best way after a single-shot initial immunizstion. The second inoculation of the antigen, carried out under conditions of raised immune reactivity, will(p.168) level out to a considerable degree the individual differences of antitoxic response.

We have studied the dynamism of antitoxin formation at single shot immunization of rabbits with purified sorbed antitoxins of the oedematiens, and the tetanus bacilli. 81 rabbits were used all together in the experiment.

26 were inoculated with various doses of the edematiens anatoxin(from 5 to 160 B.U.);
27 were inoculated with different doses of the tetanus anatoxin(from 20 to 200 B.U.)
28 were submitted to a combined immunization with these antigens(with doses of 25
and 160 B.U. of the edematiens anatoxin, and 40 and 200 B.U. of the tetanus anatox-
in, taken in different combinations). All doses of the antigens were introduced into
rabbits at a constant concentration of the $Al(OH)_3$ ... 5 mg in one ml. The content
of the blood in antitoxin was examined in the animals 5,15,25,45 and 80 days after
the immunization. For each rabbit, a graph of the accumulation of the antitoxin
was drawn(for the rabbits which were subjected to the combined immunization, two
graphs were made). On the basis of the average(arithmetic) of the titres, a common
curve was also plotted of the accumulation of both antitoxins-- individually for
the rabbits which were inoculated with one antigen, and for the animals which were
immunized with the sorbed preparation, a separate plotting was made. Thereby, it was
discovered that the forms of the common curves for both antitoxins were completely
identical at the separate and at the combined immunization. Consequently, the simul-
taneous introduction of a second antigen into the organism did not influence the
dynamism of the development of the immunological reaction in respect to the other
of the antigens.

/ There is reason to suppose that the amount of the produceable antitoxin after
a single-shot immunization with sorbed anatoxin at each given moment depends
to the utmost degree upon three factors:- 1) the levels of the immune reactivity
of the organism; 2) the amount of the antigen which approaches the cells that prod-
uce the antitoxin; and 3) the concentration of the antibodies in the tissues and
fluids of the organism, capable to some degree to neutralize the action of the anti-
gen. By the reciprocal interaction of these factors, the shape of the curve of the
antitoxin content in the blood of the inoculated animals will be also determined.

On Fig. 1A, the common curve of the accumulation of the edematiens antitoxin is
presented. We see that the antitoxin appears in the blood of the rabbits 5 days af-
ter (the immunization), and it has already reached its maximum content on the 15th
day. Thereafter, its level remains steady for 30 days (form the 15th to the 45th),
which is probably conditioned by a period of equilibrium between the acting factors.
After 45 days, a gradual reduction in the antitoxin level is observed. It is prob-
able that it is provoked by the decrease in the amount of the antigen which goes
to the cells which produce the antitoxin, and the slow tempo of decrease may be

partly explained by the drop in the concentration of the antibodies in the tissues (p.170) and fluids of the organism. We cannot say whether at this time the immune reactivity in regard to the edematiens anatoxin is lowered or not, since there are no data in the literature about this problem. In the experiment of KHALYAPINA and coworkers (1954, 1958) with the diphtheria and the tetanus anatoxins, such a reduction was not observed.

FIG. 1 (full page illustration on p.169): DYNAMISM of the accumulation of the edematiens antitoxin (4 graphs, marked A, B, V, G.) (V: low left; G: low right).

In this manner, the common curve of the accumulation of the edematiens antitoxin is characterized by the following phases:- quick rise, prolonged stabilization, and gradual reduction.

At the study of the individual antitoxinic response we have found that, in 36 rabbits out of 54 inoculated with edematiens anatoxin, the curve of the accumulation resembled the common curve to a larger or smaller degree. We called this the Type I Graph; it is presented in Fig. 1, B, with all its variants. For variant a, characteristic is the slight rise of the antitoxic level at the 25th day, and its subsequent decrease at the 45th day, without an expressed period of stabilization; for variant b, characteristic is the reaching of the peak on the 25th day and a somewhat shortened stabilization in connection with this phase; for variant v, characteristic is the beginning of the decrease of the level with the 25th day, and also a shortened phase of stabilization; for variant g, characteristic is the maintenance of the maximum level of the antitoxin up to the 80th day, i.e., a prolongation of the phase of stabilization. In our opinion, all these variants are not independent types of the curve, but only different shapes of one type characteristic for animals with a stable form of immunological reaction, steadily maintaining, for a shorter or longer period of time, the level of immune activity which they reached. The grouping of the rabbits according to the variants was the following:

Type I...9 rabbits; variant a...7; variant b...6; variant v...9; variant g...5. In 8 rabbits a graph was found which was sharply distinct from the graph of Type I, with all its variants. We called it Type II curve (See Fig. 1, V). For this curve, characteristic is the quick rise of the antitoxin level at the 15th day, which is followed by a sharp drop of the level at the 25th day and then a very slight decrease at the 45th and 80th days. Such a type of curve must be characteristic, accord-

ing to our opinion, for animals with a comparatively weak and unsteady character of the immunological reaction, animals in which the status of increased immune reactivity is quickly changed into a prolonged phase of relative immunological inertia.

Finally, in 10 rabbits we discovered Type III of the curve(See Fig.1) for which the characteristic features were two rises of the antitoxin level("to peaks")... on the 15th and the 45th days(on a variant, the second "peak" was noted in a later period of time— on the 80th day). The second rise of the graph can be explained by the presence of a second "stimulus"(GLENNY & BARR, 1931) which appears upon immunizations with the depot preparations. The clearness and the vigor of this phenomenon must depend to a large degree upon the level of the immune reactivity of the animals. In connection with this, the curve of Type III is characteristic, according to our opinion, for animals in which a brief period of stabilization or of some reduction of the level of immune reactivity is replaced by its sharp rise, which will also give the possibility of a distinct manifestation of a "second stimulus". These animals are characterized by the highest activity of the immunological reaction. Their distribution according to the variants was the following:

TYPE III...8 rabbits; variant a_...2.

In this manner, the study of the individual graphs of the accumulation of the edematiens anatoxin permitted to separate the inoculated animals into three groups, according to the three types of graphs:— first(10 rabbits)...with high intensity of the immunological reaction; second(36 rabbits)...with moderate intensity and marked stability of this reaction; third(8 rabbits)...with not sufficient intensity and with an unstable character of the immunological reaction.

The general common curve of the accumulation of the tetanus antitoxin is presented on Fig.2,A. It shows that the antitoxin in the blood of the rabbits appears 5 days after the immunization) and it reaches the maximum content on the 45th day, at which the tempo of growing of its level is visibly lessened after the 25th day. At the 80th day, some reduction of the antitoxin level is noted. Although here also a clear phase of the stabilization of the titres is not seen, nevertheless the difference between the amount of antitoxin an the 25th, 45th and 80th days is so small that this period may be considered the phase of the relative stabilization of the level of the tetanus antitoxin.

The study of 55 individual graphs allows to collect them into three basic types. CURVE TYPE 1(see Fig.2,B), with its variants, to a larger or smaller degree resembles

the common curve of accumulation of the tetanus antitoxin. For variant a. characteristic is the stabilization of the titres after 45 days, but in single STAT cases there may be also their slight increase; for variant b. characteristic is the reaching of the maximum peak level already on the 25th day, its stabilization before the 45th day and a gradual lowering at the 80th day; for variant v. characteristic is a maximum peak level at the 25th day, and its maintenance until the 80th day. The distribution of animals according to the variants was the following:- Type I...24 rabbits; variant a...4 rabbits; variant b...9 rabbits; variant v...8 rabbits. On the whole, the curve of Type I was observed in 45 rabbits. We assume that, here as well as at the immunization with the edematiens anatoxin, the Type I Curve defines (includes) animals of a stable character of their immunological reaction.

For the Type II Curve(See Fig. 2, V), characteristic is, after the rise of the antitoxin level at the 25th day, its sharp drop at the 45th day and/...

FIG. 2(Full-page illustration on page 172): DYNAMISM OF THE ACCUMULATION of the tetanus antitoxin. (4 graphs, marked A. B. V. G. (V: left low; G: right low) Ordinatas carry the A. U.-s; abscissas...days after the immunization)

(p.173) ...the further gradual reduction at the 80th day(for variant a, the curve of the drop of titres is somewhat leveled out). On the whole, the Type II Graph was seen in 7 rabbits; from them, variant a was present in 2. Such a curve may be characteristic, in our opinion, for animals of an unstable character of the immunological reaction.

The Type III Graph(See Fig. 2, G), with two "peaks" of the antitoxin accumulation--on the 25th day and on the 80th day(for variant a, on the 15th and 45th days)--is characteristic for animals having a high immunological reactivity. The curve of this type was present in 3 rabbits(one of them showed variant a).

In this manner, the rabbits immunized with tetanus anatoxin, according to the character of the immunological reaction, could be separated into the same three groups as the animals which were immunized with the edematiens anatoxin. It is true that here the quantitative correlations in the groups were somewhat different(3, 45, 7 rabbits, respectively).

We were unsuccessful in showing even a little expressed relationship between the values of the immunizing doses of the antigens and the types of the graphs of the accumulation of the antitoxins.

It must be said that in the rabbits which were subjected to the combined immunization the coincidence of the graph types was observed of the two STAT titoxins in 17 out of 28 cases. Eleven rabbits reacted in different ways to the simultaneous inoculation of the two antigens. This can be explained by the differences in the antigenic and immunogenic properties of the edematiens and tetanus anatoxins. In regard to the edematiens anatoxin, the immunological processes differed by a greater lability than in respect to the tetanus anatoxin. The level of the edematiens antitoxin had much quicker reached the maximum and it was reduced on the 80th day considerably more strongly than the level of the tetanus antitoxin(See Fig.1,A, and 2,A). The same is shown by the juxtaposition of the times of maximum accumulation of the antitoxins in the different rabbits; this is given in the table.

TABLE

TIMES OF PEAK ACCUMULATION OF THE ANTITOXINS AFTER A ONE-SHOT IMMUNIZATION WITH PURIFIED SORBED ANATOXINS.

TIME IN DAYS	NUMBER OF RABBITS WITH PEAK TITRES OF ANATOXIN	
	of edematiens	of tetanus
15	27	0
25	14	24
45	11	27
80	2	4

(p.174) It is possible that, during the examined period, the immune reactivity of the animals was not kept on a constant level in regard to the edematiens anatoxin, while this was the case in respect to the tetanus anatoxin; but, a certain tendency to drop was detected.

CONCLUSIONS.

1) At the single-shot immunization of rabbits with purified sorbed anatoxin of edematiens and tetanus, it was found that all individual curves of the accumulation of anatoxins could be basically referred to three types. The type of the graph is determined by the strength and the stability of the immunological reactions characteristic for the given animal.

2) There is a reason to suppose that in regard to the edematiens anatoxin the immunological processes are distinguished by greater lability than in regard

to the tetanus anatoxin.

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PRODUCTION OF TETANUS TOXINS WITH THE USE OF CELLOPHANE BAGS FOR THE GROWTH OF THE CULTURE AND STUDY OF THE PROPERTIES OF THE OBTAINED ANTIGENS(p.175-181)

* * * * * *

The obtaining of powerful toxins has a great practical importance. The works of a few authors showed that, with the use of cellophane bags for the growth of the culture, toxins of good quality can be produced.

For the purpose of producing powerful toxins of the tetanus bacillus, we have also employed the cellophane bags.

At the growing of a culture in cellophane bags, we used various utensils which we modified into corresponding suitable shapes. Into a 5-liter flask, with 3.5 liters of nutrient medium in it, we let down a doubly everted bag one end of which we tied tightly and fastened to the neck of the flask, and the other end we put on the neck of the flask. Then, we poured physiological solution into the cellophane bag, up to the level of the nutrient medium, and we inserted a siphon into it, with a cotton plu. For taking a sample from the nutrient medium, we also inserted a tubule into the flask; the tubule was placed between the cellophane bag and the neck of the flask.

We have arranged the fourth (flask) slightly differently. Into the fourth we poured two liters of nutrient medium, and we inserted a siphon into it to which we tightly fastened a doubly everted cellophane bag, and we filled it with physiological solution. We sterilized the thus adjusted vessels together with the medium at 110°C, after which we carried out the seeding in the cellophane bags. For this purpose, 100 ml of a liquid culture of the tetanus bacillus was diluted in 3 liters of physiological solution; with the aid of the siphon, we poured it into the bags to the level of the nutrient medium, and we incubated it for 9 days at 35°C.

In this way, nine bags were seeded. Each bag was controlled with a culture growing by the ordinary method. For this (p.176) we used the media of RAMON, GLUZMAN, and a medium prepared on hydrolyzate of fish meal. The determination of the

strength of the toxin as well as of the antitoxin combining capacity of the anatoxins was done by means of titration on white mice. The results of the experiments are given in Table 1.

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TABLE 1

PRODUCTION OF DIALYZED TETANUS TOXIN ON DIFFERENT NUTRIENT MEDIA

MEDIUM	TOXIN	NUMBER OF Dlm in 1 ml of toxin	RATE BY WHICH DIALYZED TOXIN is stronger than the normal
RAMON's	Dialyzed	100,000,000	200
	regular	500,000	-
"	Same	10,000,000	10
		1,000,000	-
"	Same	50,000,000	100
		500,000	-
FISH HYDRO-lyzate	Dialyzed fishy	10,000,000	10
		1,000,000	-
"	Dialyzed regular	50,000,000	100
		500,000	-
"	Same	50,000,000	100
		500,000	-
GLUZMAN's	Same	10,000,000	10
		1,000,000	-
"	Same	50,000,000	50
		1,000,000	-
"	Same	50,000,000	50
		1,000,000	-

From the conducted experiments it is evident that the strength of the dialyzed toxin is 10 to 200 times higher than the strength of the toxin prepared in the ordinary way.

During the experiment, it was noted that the growth of the tetanus bacillus, seeded in cellophane bags, was slowed down in comparison with the growth of the culture in the flasks. Therefore, experiments were arranged for the study of the dynamism of the toxin production under these circumstances. For this purpose, we seeded the culture of the tetanus bacillus into the cellophane bags immersed in Ramon's medium. As control, a culture was used cultivated by the ordinary method.

The tetanus culture, poured out from the bags and the flasks, was checked for 3-6-9 days for growth. In the toxin we determined (p.177) the amount of the lethal

doses and the units of combining(B.U.) per one ml. At the same time, we measured the pH of the toxin, we examined the glucose consumption and under STAT microscope we observed the tetanus bacilli for alterations.

Table 2 indicates how, according to the grade of the growth of culture on the Ramon medium in the cellophane bags, the strength of the toxin and its combining capacity had gradually increased, reaching the peak between the 6th and the 9th day. In the control flasks, the optimum toxin formation was observed on the 3rd(third) day of growth of culture. The pH of the dialyzed toxin and of the ordinary one increased from pH 7.2 to pH 7.5. The glucose, added to the nutrient medium in the amount of 0.75%(750 mg%) , was determined by the method of BERTRAND. The most energetic expenditure of glucose was noted in the culture seeded with the ordinary method in which it was consumed by the 6th day of the growth of the culture. In the medium, too, surrounding the cellophane bags, traces of glucose could be noted at the 9th day of the cultivation of the culture.

(p.178 text cont.) It should be remarked that on the first day of the growth of the culture, in the smears under the microscope, long threads of the tetanus bacillus were seen. It is evident that the culture had gwown so violently that it could not succeed in dividing, forming threads which were well stained with methylene blue. On the 6th day the culture had the form of bacilli with a small number of spores of the tetanus microbes, weakly susceptible to staining. On the 9th day a large portion of the microorganisms has undergone lysis.

With cultivation of the tetanus bacilli by an ordinary method, the appearance of the lysis will come later.

The nutrient medium surrounding the bag has not contained any amount of tetanus toxin. The inoculation of 1 ml of the nutrient medium under the skin of the paw of guinea pigs did not cause the symptoms of tetanus.

The dialyzed toxin , in comparison with the ordinary one, is rather quickly transformed into toxoid. For its complete neutralization it is enough to let it stay in the thermostat at 37°-38°C for 5-6 days and to add to the toxin 3% of formalin.

Having obtained anatoxins from the dialyzed toxin, we decided to verify their effectiveness on animals.

It has been noted that our further investigations, both with the ordinary to-

xins and with the dialyzed toxins, were done after their filtration through a Zeiss filter. Therefore, in view of the accumulation of a large amount of microbic bodies in the cellophane bags, at the preparation of dialyzed toxin we dissolved it in physiological solution.

TABLE 2

CHANGE OF THE PROPERTIES OF TETANUS BACILLI IN THE PROCESS OF GROWING IN CELLOPHANE BAGS AND AT CULTIVATION BY THE ORDINARY METHOD

Bag No.	DAYS OF INCUBATION	TYPE OF CULTURE	Dlm in 1 ml	B.U. in one ml	pH of toxin	Glucose in mg%
Bag No. 1	3	Thread form	1,000,000	100	7.2	400
Bag No. 2		Same	>500,000 <1,000,000	>50 <100	7.2	500
Control		Spores & many lyzed bacilli	1,000,000	>100 <200	7.4	200
Bag No. 1	6	Bacilli & spores	10,000,000	>200 <400	7.3	160
Bag No. 2		Same	10,000,000	>200 <400	7.4	100
Control		Spores & many lyzed bacilli	1,000,000	>100 <200	7.5	0
Bag No. 1	9	Spores & lyzed tetan. bacilli	50,000,000	>800 <1600	7.5	traces
Bag No. 2		Same	100,000,000	>800 <1600	7.5	traces
Control		Spores & lyzed tetan. culture	>500,000 <1,000,000	>50 <100	7.5	0

For the purpose of the elucidation of the immunogenic properties of the dialyzed anatoxins, an experiment of immunization was arranged on guinea pigs. The ordinary anatoxins served for control to the experiment. The dialyzed and the ordinary tetanus toxins, produced on RAMON's medium, had 1,500,000 Dlm in one ml, but the anatoxins prepared from this toxin had >150 <200 B.U.

The strength of the toxins, produced on GLUZMAN's medium, equaled 2,000,000 Dlm, in one ml, but the anatoxins prepared from them had 250 B.U.

We immunized the animals subcutaneously by means of a two-shot inoculation of one ml of tetanus anatoxin sorbed on Al hydroxide, with 21 days' interval between the first and the second injections. For the antitoxin content, blood was taken

on the 21st day after the first inoculation, and on the 30th day after the second.

TABLE 3 indicates that all the animals, both the experimental animals and the controls, had >0.1<1 A.U. on the 21st day after the first inoculation. On the 30th day after the second injection, the best results were obtained with immunization of the pigs, by using dialyzed anatoxins.

(p.179)

TABLE 3

IMMUNIZATION OF TEN GUINEA PIGS WITH DIALYZED AND ORDINARY ANATOXINS PREPARED ON RAMON'S AND GLUZMAN'S MEDIA

ANATOXIN	MEDIUM	Dlm of toxin in one ml	B.U. in one ml of anatoxin	pH	Al_2O_3 in 1 ml mg	B.U. in 1 ml of dialyzed fluid	A.U. on 21st d. after 1. inocul.	A.U. on 30th d. after 2. inocul
Dialyzed	RAMON	1,500,000	>150 <200	7.3	2.5	1	> 1/10	<19.8
regular	"	1,500,000	>150 <200	7.4	2.5	>33 <50	>1/10	<14.8
Dialyzed	GLUZMAN	2,000,000	250	7.6	2.5	>1 <5	>1/10	<11.2
regular	"	2,000,000	250	7.6	2.5	>33 <50	>1/10	<15.8

Thus, the pigs immunized with the antigen which was prepared on Ramon's medium after the second injection had an average of 9.8 A.U. per 1 ml of serum. The control group contained 4.8 A.U. in the same volume. The animals which were immunized with the antigen prepared on Gluzman's medium, had 12.2 A.U. in the experimental group, and 5.8 A.U. in the control group per 1 ml of serum. Comparing with each other, the dialyzed and ordinary antigens in this experiment which antigens were prepared either on Ramon's or on Gluzman's medium it may be said that the dialyzed anatoxins yield twice as large an accumulation of antitoxin, by comparison with the antigens prepared in the ordinary fashion. Having obtained good results at the immunization of the pigs with dialyzed anatoxins, we endeavoured to clarify for ourselves the cause of their great effectiveness in comparison with the ordinary antigens. From the literature it is known that the dialyzed anatoxins have a smaller amount of admixture than the ordinary anatoxins.

For the explanation of the purity grade of our antigens, the amount of Dlm per 1 mg of nitrogen was calculated in two series of dialyzed anatoxins (after an appropriate treatment, consisting in the solution of the culture in physiological

saline, separation and filtration through Zeitz's filter), and also in two series of anatoxins prepared in the ordinary fashion.

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Table 4 indicates that the dialyzed toxin of Series No.594 had 1,700,000 Dlm per 1 mg of total nitrogen, and the toxin of this series(p.180)prepared by the ordinary method had 200,000 Dlm. In the dialyzed toxin of Series No.606 the charge per 1 mg of nitrogen was equal to 1,450,000 Dlm, and the control toxin of this group had only 160,000 Dlm.In this manner,in the series No.594 anatoxin, the charge per 1 mg of nitrogen is 8.5 times higher in the dialyzed anatoxin; in series No.606, nine times larger than with the ordinary toxin. From this it follows that the dialyzed toxins have less ballast substances than the toxins which were prepared in the ordinary way. Therefore, they are the best sorbed on Al hydroxide, due to which they possess a great immunizatory stimulating power.

TABLE 4

CHARGE OF ANTIGEN PER ONE MG OF TOTAL NITROGEN

SERIES No.	ANTIGEN	Dlm per 1 mg of nitrogen in tetanus toxin
594	Dialyzed	1,700,000
	regular	200,000
606	Dialyzed	1,450,000
	regular	160,000

Having obtained good results in the preliminary experiment, the laboratory prepared 17 series of dialyzed tetanus toxin, designated for the immunization of horses. All together 235 bags were seeded, and 27.6 liters of tetanus culture were prepared, which contained from 500,000 to 1,000,000 Dlm in one ml of the filtrate. After the solution of the culture in physiological saline, after separation and filtration through the Zeitz filter, 51.8 liters of tetanus toxin were produced of a strength of 2,500,000 Dlm and 320 B.U. per one ml.

This toxin, precipitated with alum, was successfully used for the overimmunization of horses.

CONCLUSIONS

1) The strength of the dialyzed tetanus toxins was 10 to 200 times higher than the strength of the toxins obtained with the ordinary procedure.

(over)

2) The peak toxin-production of the culture of the tetanus bacillus, grown in cellophane bags, happens to be between the 6th and 9th days of the growth of the culture.
(p.181)

3) With the cultivation of the culture in the cellophane bags as well as with cultivation of the tetanus bacillus in the usual way, a lysis of the microbic bodies has been noted.

4) The nutrient medium surrounding the bags of culture does not contain tetanus toxin.

5) The dialyzed toxin is quicker changed into toxoid than the ordinary toxin.

6) The dialyzed anatoxin, comparing it with the ordinary anatoxin, is better sorbed on $Al(OH)_3$, creating a most powerful depot at the immunization of the animals, duw to which it has a great immunisatory stimulating power.

7) With the cultivation of cultures in the cellophane bags, high-quality antigens could be produced for industrial purposes.

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p.183.

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ACTION OF ULTRASOUND WAVES UPON THE TETANUS TOXIN(p.183-185)

* * *

Works devoted to the action of ultrasound upon the bacterial toxins excreted by pathogenic microorganisms are exceedingly few. KASAHARA and TAKAGI(1938) studied the action of ultrasound waves upon the diphtheria toxin and found that the toxicity of the latter weakens to a considerable degree under the action of the ultrasonic waves. BEUMER and BEUMER-JOGHANS(1950) as well as RAYNAUD, WISMAN and PRUD'HOMME(1950), by studying the action of ultrasound upon the diphtheria and the tetanus toxins, observed that the diphtheria toxin is very resistant to ultrasound, but the tetanus toxin is resistant only in the V_f medium(high-frequency medium?); in case of the action of the ultrasound upon tetanus toxin which was solved in physiological solution the action resulted in a partial breakdown of the toxin. STEPANCHENOK-RUDNIK, NEKHOTENOVA, BLAGOVESHCHENSKII and PAVLOV(1958) studied the action of the ultrasound waves upon the diphtheria toxin, and the results which they obtained prove the resistance of the diphtheria toxin to the action of the ultrasonic waves.

It is of interest to investigate the action of the ultrasonic waves upon the tetanus toxin--, one of the powerful toxins of the pathogenic microbes of the anaerobe group, which was also the topic of the present research.

MATERIALS AND METHODS.

Native tetanus toxin was prepared on a medium of an acid casein hydrolyzate basis. For cultivation, the toxogenic Stock KOLLE-8 has been used. The various series of the toxin(Nos.231, 321, and 350) possessed a very high toxicity(2-3 million Dlm) and a very high antigenicity(200-300 A.U.)

For the concentration of the toxin, the method of precipitation was employed at the isoelectric point with 1-normal HCl, with advanced addition of NaCl up to 15%.

(p.184) The concentration was made 20 times to the volume. The native toxin had

155.4 mg% of total nitrogen, the concentrate had 52.5 mg%. The purification as to total nitrogen was realized at a rate of 98.32%. The sound exposure of the toxins was made with the domestically prepared ultrasocin apparatus UL-1 of construction SKTB of the Ministry of the Instrument-Making Industry, U.S.S.R. As emitters of the ultrasound in the UL-1 apparatus, piezo-ceramic discs of barium titanate are used, with the resonance frequencies of 400, 800, 1200 and 2400 kilocycles. The power of ultrasound at frequencies of 400 and 2400 kilocycles is equal to 5 watt/cm^2, and at frequencies of 800 and 1200 kilocycles it was 10 watt/cm^2. The ultrasonic chambers are cooled by a current of water taken from the water conduit. The cuvette in which the sound-wiring is conducted represents a glass vessel with double walls(which makes it possible to bring supplemental cooling by means of alcohol at ℞ -25° to -20°C), and with a bottom from nylon film. The cuvette is fastened on a special stand, it is immersed into the chamber that is filled with distilled water, and ,in this way, the ultrasonic waves pass along in a water medium.

The sound exposure of the toxin was done with samples of 50 ml. The sample of toxin was placed in the cuvette;it was covered with a rubber cap in the chamber of the appropriate frequency of ultrasonic wave.The sound exposure was kept c 30 minutes long and for an hour and a half. The temperature of the water in the chamber was from #(plus)14°C (at the start of the experiment) to #(plus) 27°C (at the end of the experiment). The temperatur of the toxin in the cuvette at the end of the experiment was equal :--at 400 kilocycles of frequency, to plus 5°C; --at 1200 kilocycles of frequency, to + 27°C; and at 2800(2400) kilocycles, to +16°C. The samples of the sound-exposed and non-sound-exposed toxin were thereafter investigated on animals(determination of Dlm (minimum lethal dose) and BU.)

RESULTS

In Table 1, the data are presented in regard to the action of the ultrasonic waves upon the native tetanus toxin.

As it can be seen from the table, the native tetanus toxin did not change its properties under the action of the ultrasonic waves, inasmuch as identical values of Dlm(Minimum lethal dose) and B.U.(binding units) are evidenced both in the non-sound-exposed toxins and in the sound-exposed toxins. It is possible that in the present case, a protective effect of the ballast colloids is taking place which

colloids are present in the native toxin. For the verification of this, the effect of the ultrasonic waves upon the purified concentrated tetanus toxin was studied. The findings of this experiment are presented in Table 2.

The results obtained in the experiment with the examination of the concentrated tetanus toxin prove that the concentrated toxin as well as the native toxin are resistant to the action of the ultrasonic waves and, evidently, this resistance of the toxin is not connected with the presence of ballast colloids in the native toxin.

(p.185)

TABLE 1

ACTION OF ULTRASOUND UPON THE NATIVE TETANUS TOXIN

Series of toxin	NON-EXPOSED TOX. Dlm (Min. let. dose)	B.U.	SOUND EXPOSURE frequ. / kilocycles	for minutes	TOXIN EXPOSED TO SOUND Dlm	B.U.
231	2,000,000	200	-	-	-	-
"	-	-	1200	30	2,000,000	150
321	2,000,000	200	-	-	-	-
"	-	-	400	30	2,000,000	200
"	-	-	800	30	2,000,000	200
"	-	-	1200	30	2,000,000	200
"	-	-	2400	30	2,000,000	200
"	2,000,000	200	-	-	-	-
"	-	-	400	90	2,000,000	200
"	-	-	800	90	2,000,000	200
"	-	-	1200	90	2,000,000	200
"	-	-	2400	90	2,000,000	200
350	3,000,000	100	-	90	-	-
"	-	-	400	90	3,000,000	300
"	-	-	800	90	3,000,000	300
"	-	-	1200	90	3,000,000	300
"	-	-	2400	90	3,000,000	200

(p.185 cont.)

TABLE 2

ACTION OF ULTRASOUND UPON CONCENTRATED PURIFIED TETANUS TOXIN
STAT

SERIES OF TOXIN	NON-EXPOSED TOX. Dlm	B.U	SOUND EXPOSURE frequ. / for kilocycles/ minutes		TOXIN EXPOSED TO SOUND Dlm	B.U.
350	15,000,000	1500	-	-	-	-
"	-	-	400	90	15,000,000	1500
"	-	-	800	90	15,000,000	1500
"	-	-	1200	90	15,000,000	1500
"	-	-	2400	90	15,000,000	1500

(p.186)

CONCLUSIONS

1) We investigated the action of the ultrasound upon preparations of tetanus toxin(frequency of the ultrasonic waves 400,800,1200,and 2400 kilocycles; the power of the ultrasound was 5 watt/cm^2 and 10 watt/cm^2).

2) The toxic properties of the preparations of the native and of the concentrated toxin did not change by the action of the ultrasonic waves at an exposure of 30 and 90 minutes.

3) The ultrasonic method of breaking up the microbic bodies is suitable for the investigation of the problems of the genesis of the toxin in the microbic cell.

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(p.187)

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IMMUNIZING PROPERTIES OF PURIFIED SORBED TETANUS ANATOXIN (p.187-195)

* * *

The literature has many data corroborating the great effectiveness of the sorbed tetanus anatoxin in comparison with the native preparation.

In this work we set it our task to confirm this situation on the material of production, to study dynamically the effectiveness of the native and of the purified sorbed tetanus anatoxin as well as to follow up for the course of a longer time the immunizing properties of small and medium doses of the purified sorbed tetanus toxoid on guinea pigs in cases of one-shot and two-shot immunizations.

In the preceding works of the Department of Wound Infections of the N.F.Gamalei Institute, in the experiments on animals, the high immunizing activity of the sorbed tetanus anatoxin has been proved.

After a work of many years, with the native tetanus toxoid, and after a work of two years with sorbed tetanus toxoid, under conditions of mass production, it was interesting to compare the effectiveness of these preparations with a check of their immunizing properties on guinea pigs.

By comparing the productivity findings about 83 series of native toxoids and 43 series of sorbed toxoids which were released by us during the past few years, we have arrived at the following results.

In TABLE 1, the material is presented on the testing of the immunogenicity of the native anatoxin during the period of 1952-1957, and of the sorbed anatoxin for the period of 1957-1958.

Eightythree series of native anatoxin were tested on 393 guinea pigs each of which was inoculated with 5 ml of the native toxin, containing in this volume a number of from 150 to 1000 B.U.-s. Thereafter, 30 days later, each of the animals was given 100 M.L.D. of tetanus toxin.

From the TABLE it is evident that the native anatoxin of 54 series(p.188) at its inoculation into 240 guinea pigs in the amount of 5 ml(150 to 100 B.U.) imparts an immunity to the animals which protects them from 100 M.L.D of tetanus toxin.

(p.188 cont.)

TABLE 1

IMMUNOGENICITY OF THE PRODUCED SERIES OF NATIVE TOXOID FOR 1952-1957 AND OF THE SORBED TOXOID FOR 1957-1958

ANTIGEN →	NATIVE			SORBED
	xx	xx	TOTAL /	xx
Number of examined series	54	29	83	43
Number of pigs taken for experiment	240	153	393	174
Inoculated antigen in B.U.	150-1000	150-1000	150-1000	40-200
At what time was the decisive does given?	21-30	21-30	21-30	13-27
Amount of toxin, in M.L.D.	100	100	100	1000 10000
RESULTS:				
a) "smooth"(100% immunization	240	112	352	178 (174)
b) tetanus	-	41	41	-
Percentage of non-immunized guinea pigs			10.4%	
Percentage of immunized guinea pigs			89.6%	100%

However, the anatoxin of 29 series, inoculated into 153 guinea pigs, could not protect all animals from 100 M.L.D. of the toxin. The guinea pigs, having obtained the same amount of toxin(41 animals) died or gave symptoms of tetanus.

Consequently, 41(10.4%) out of 393 guinea pigs happened to be not immune.

The industrial materials of 43 series of purified sorbed tetanus toxoid were examined on 174 guinea pigs. The animals received from 40 to 200 B.U. antigen. Then, 13, 15, 16, 19, 20, 21, 22, and 27 days after the injection, from 1000 to 10,000 M.L.D. of tetanus toxin was inoculated with a single shot into the animals. After the period of 6 days after the inoculation of the toxin, not a single case of tetanus could be noted among the guinea pigs. These observations permit to announce that a considerable portion of the animals(10.4%), at a single shot inoculation of a comparatively large dose of native anatoxin(150 to 1000 B.U.), remained unprotected from 100 M.L.D. of tetanus toxin, and meanwhile a considerably smaller amount of the sorbed anatoxin(40 to 200 B.U.) protects the animals from a considerably larger dose of the toxin(1000 to 10,000 M.L.D.), moreover the protective properties in the animals are elaborated earlier(by 13-15 days) than in case of the immunization with the native anatoxin.

Table 2 represents the immunogenicity of the 43 industrial series (p.189) of the purified sorbed tetanus toxoid released by the Institute in the years 1957-1958. In the process of the industrial investigations it was observed that the guinea pigs which were immunized with sorbed tetanus anatoxin, already on the 13th to 15th day, had sufficient amount of antitoxin to protect them from large doses of tetanus toxin(See Table).

Table 2 indicates that the pigs which received 200 B.U. of antigen, on the 13th day have been protected from 2000 M.L.D. of tetanus toxin; it also shows that the animals which were immunized with anatoxin in the amount of 40-200 B.U., on the 15th day did not show any symtpoms of tetanus when they were inoculated with 10,000 M.L.D. of the toxin.

From the literature it is also known that the immunization of the animals with sorbed tetanus toxin has produced a greater resistence to the toxin than the immunization of the animals with native preparations.

Thus, VOROB'EV(1951) informed about the high resistance of mice to tetanus toxin, which mice were immunized with a single shot of sorbed tetanus anatoxin, while the immunization of animals with the native preparation did not protect the mice from ONE M.L.D. of the toxin.

PILLIMER, GROSSBERG, WILLER(1946) immunized mice with(one ml) sorbed tetanus toxoid, and observed a resistance of 90% of the animals to 10 M.L.D. of the toxin.

MARKOVICH and VOROB'EV(1956) remarked that in case of a single-shot immunization of the animals with small doses of anatoxin(0.005-0.01 ml) changes in the resistance of the animals were observed after 14 to 21 days, but with larger doses (0.05 to one ml) such changes occurred already on the 7th day.

In this way, both the experimental material, given by us earlier, and the industrial findings gathered during a few years as well as the literaure on this problem indicate the great effectiveness of the sorbed tetanus anatoxin in comparison with the native anatoxin. According to the data of PRICE, the effectiveness is greater at least by 70 times, according to the data of VOROB'EVA--by 100 times.

Above we showed the high resistance of the guinea pigs which had been immunized with sorbed toxoid , to the tetanus toxin. The resistance of the guinea pigs to tetanus toxin is considerably weaker, however, after a single-shot immunization with purified non-sorbed tetanus anatoxin.

Out of 11 guinea pigs which received from 6 to 7 ml of purified non-sorbed anatoxin, at the subsequent inoculation of them, (30 days later) with 10,000 M.L.D. of the toxin, ten pigs survived, and one died from tetanus; but from the ten pigs which were immunized with a single-shot of non-sorbed purified tetanus anatoxin, after their inoculation with 2000 M.L.D of the toxin, 6 pigs remained healthy. Four had tetanus of the second and third degree(Table 3).

(p.190)

TABLE 2

IMMUNOGENICITY OF THE INDUSTRIAL SERIES OF PURIFIED SORBED TETANUS ANATOXIN

for the years 1957-1958

Serial No.	Number Of pigs	B.U.	Days after which the toxin was given	Amount of Dlm(M.L.D) of toxin	Results
7	16	200	27	10,000	smooth
1	4	40	22	10,000	"
3	11	200	21	2,000	"
2	8	200	21	5,000	"
1	4	200	20	5,000	"
1	4	200	20	2,000	"
1	4	200	19	10,000	"
1	4	40	19	10,000	"
5	15	200	16	1,000	"
5	20	200	16	2,000	"
2	12	40	16	5,000	"
2	12	40	15	5,000	"
2	12	40	15	5,000	"
2	12	100	15	5,000	"
2	12	200	15	2,000	"
2	12	40	15	10,000	"
6	11	200	13	2,000	"
43	174				Smooth

TABLE 3.

RESISTANCE OF GUINEA PIGS IMMUNIZED WITH PURIFIED NON-SORBED TETANUS TOXOID

(RESISTANCE TO TETANUS TOXIN)

Number of pigs	Anatoxin per ml	B.U.	Day on which toxin was given	Dlm of toxin	Result
11	5-7	10,000	30	1,000	10 healthy 1 died
10	5-7	10,000	30	2,000	6 healthy 4 tetanus of II. and III degree.

(p.191) In the following experiment, we have observed for eight months the dynamism of the accumulation of the antitoxin in the guinea pigs which were immunized with two shots of the native and of the sorbed tetanus toxoids(Table 4).

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TABLE 4.

TWO-SHOT IMMUNIZATION OF THE GUINEA PIGS WITH NATIVE AND WITH SORBED TETANUS ANATOXIN

ANTIGEN	MONTHS AFTER 1st. Injection/A.U.	AFTER THE SECOND INJECTION, A.U.		
		1½mo. after	5 months after	8 months after
Native, 75 B.U.	8/ >0.1<1	8/ 4.1	5/ 1.2	4/ 0.1
Sorbed, 3 B.U.	15/ >0.1<1	14/12.3	8/ 6.8	6/ 4.5

ANNOTATION: Numerator...number of pigs; denominator...amount of antitoxin.

Eight guinea pigs were immunized with tetanus native anatoxin, with two shots, at 30-days' interval, each injection being 75 B.U. of antigen.

Thirty days later, (after the first injection), one ml of serum of the animals contained >0.1<1 A.U.

One and a half months after the second injection, the titre of the animals' sera rose to 4.1 A.U. Five months after the second injection, the amount of antitoxin in the blood of the animals dropped to 1.2 A.U., and at the end of the eighth month --to 0.1 A.U.

Fifteen guinea pigs were immunized with two shots of the sorbed tetanus toxoid, receiving at each injection 3 B.U. of the preparation. One months after the first injection, the guinea pigs had the same amount of antitoxin as it had been in the pigs immunized with the native anatoxin. In the subsequent period of investigation in the animals which were immunized with sorbed anatoxin, the amount of antitoxin in one ml of serum has been considerably greater than in the guinea pigs which were immunized with the native preparation.

Thus, 1½ month after the second injection, the guinea pigs had 12.3 A.U.. After 5 months, they had 6.8 A.U., and after 8 months, they had 4.5 A.U. in one ml of serum. From the experiment it can be seen that the accumulation of antitoxin was increased by 3 to 45 times above the level in the guinea pigs which had been immunized with the native preparation, notwithstanding the fact that the dose of

(p.192) the native antigen taken for immunization has been 25 times larger.

The experiment shows the advantage of the sorbed toxoid abSTAT the native anatoxin. It may be thought that the native antigen is quickly eliminated from the organism; that is why it is also relatively a weak stimulus for the immunisatory apparatus. But the sorbed anatoxin, due to the depot, enters the organism of the animal slowly(30-60 days), and during this entire period it provokes an immunizatory stimulus.

From Table 4, the considerable rise in the antitoxin titre can be seen after the second injection of both the native and the sorbed toxoids. From the literature it is known that the sensitiveness of the organism to the antigen and the effectiveness of the doses of the second stimulation depends upon the intensiveness of the prepared immunization, i.e., upon the power of the first immunizatory stimulation(GOLINEVICH, 1938).

As it is apparent from our experiments, the potential power/possibility of the small doses of the sorbed antigen is higher than the potential power of considerably larger doses of the native anatoxin .

Since in the previous works we have studied the immunizing action of the sorbed tetanus antigen, by giving the animals mainly large doses of this antigen and observing them(the animals)for a comparatively short period of time, it seemed to us necessary to track down, in an experiment on guinea pigs, the immunizing effect of small and moderate doses of the antigen during a prolonged period of time in cases of a one-shot and a two-shot immunization. The one-shot and the two-shot immunizations were emploued so that the effect of one injection and of two injections of antigen could be compared with each other in a single experiment. The two-shot immunizations permitted also the explanation of the potential power of the antigen and of its doses.

In the experiment, 61 guinea pigs were used. All animals were divided into four groups.

I. 15 pigs were immunized with a single shot, by giving each of them 60 B.U. of sorbed anatoxin;

II. 16 pigs were also given a single shot, 2 B.U. antigen for each;

III. 15 animals were given two shots, each 30 B.U.

IV. 15 pigs were inoculated with two shots, each 3 B.U. of anatoxin.

As a result of the experiment, it was proved (see TABLE 5) that the guinea pigs which received a single injection each, one month later had the following accumulation of antitoxin:- in group I, an average of 3.1 A.U.; in group II, >0.1<1 A.U.; in group III, 4.8 A.U.; and in group IV, >0.1 < 1.A.U. One month later, each of the guinea pigs of Group III was still given an additional 30 B.U. of sorbed anatoxin, and each guinea pig of Group IV was given an additional 3 B.U. Then, 2½ months after the second injection, in the pigs of Group III and Group IV (p.193) the amount of antitoxin has considerably increased:- in 14 pigs, each of which had received 30-30 B.U. of antigen in two shots, the average antitoxin amounted to 14.4 A.U., and in 14 pigs which were immunized with 3 B.U. of anatoxin in two shots (a total of 3 plus 3 B.U.), the titre of the antitoxin equalled an average of 11.2 A.U.

TABLE 5.

DYNAMISM OF ANTITOXIN FORMATION AFTER ONE-SHOT AND TWO-SHOT IMMUNIZATIONS WITH TETANUS SORBED ANATOXIN, WITH SUBSEQUENT REVACCINATION

Group No.	B.U. per injection	ONE MONTH after inj.	SECOND inj. B.U.	ANTITOXIN after 2½mo.	6months	9 mo.	REVACCINATION (see below)
I	30	15/ 3.1	----	14/5.3	11/2.8	7/2.5	
II	3	16 >1 /10 <1	----	13/15.	9/1.4	5/0.5	
III	30	15/4.8	30	14/14.4	8/4.5	7/3.5	
IV	3	15/ >1 10/ <1	3	13/11.2	8/52.	6/4	

(from above)

REVACCIN.	AMOUNT OF ANTITOXIN AFTER REVACCIN. 10 days	5 months	13 months
(I) 3 B.U.	6/15.5	5/13.1	2/20
(II) 3 B.U.	2/45	1/10	1/15
(III) 3 B.U.	4/27.5	1/20	-
(IV) 3 B.U.	5/56	1/30	-

ANNOTATION: Numerator. . . number of guinea pigs;

denominator. . . amount of antitoxin (in original: "anatoxin")

- -

In case of further investigations of the titres after 6 and 9 months, in the animals of this group the amount of antitoxin was maintained roughly at the same level. Thus, 6 months after the first injection, in 8 guinea pigs of Group III it equalled 4.5 A.U., and in 8 pigs of Group IV...it was 5.2 A.U. After nine months, in seven guinea pigs of Group III the titre of the serum dropped to 3.5 A.U., and in the pigs of Group IV it dropped to 4.0 A.U. per one ml of serum.

In the animals of Group I which had been immunized with a single shot of 30 B.U. of sorbed anatoxin, 2.5 months after the first injection, in 14 guinea pigs the amount of antitoxin was equal to 5.3 A.U.; after 6 months, in two pigs the antitoxin level dropped down to 2.8 A.U.; after 9 months, in seven pigs, it reached 2.5 A.U.

In the pigs of Group II which received a single shot of 3 B.U. of the antigen, at the examination it appeared that after 2.5 months the antitoxin level was 1.5 A.U. in 13 animals; after 6 months, it was 1.4 A.U. in 9 animals, and after 9 months it was 0.5 A.U. in 5 animals.

(p.194) By comparing the obtained results it can be shown that, in case of a single shot immunization with sorbed anatoxin the large amounts of antigen impart a higher immunity to the animals than the one we have noticed in the preceding works.

As it can be seen, the amount of antitoxin in the two following groups was almost identical.

It is evident that in case of a two-shot injection it is not necessary to strive for large doses of antigen. It is likely that there esists some kind of limit in this respect; it can be also thought that the amount of antitoxin does not accumulate in proportion with the increase in the antigen, which is already pointed out by TOPLEY, WILSON(1936) and EDSALL(1953).

The revaccination of the animals of the fourth group 9 months after the first injection, made with 3 B.U. of the sorbed antigen, has given the best results in the animals which were immunized with single shots of small doses of the antigen.

Thus, 10 days after inoculations of 3 B.U. of the sorbed anatoxin, 6 guinea pigs of Group I had an average of 15.5 A.U.; two pigs of Group II---45 A.U.; 4 pigs of Group III---27.4 A.U., and 5 pigs of Group IV...56 A.U.

Later on, we had a possibility to track down the results of the revaccination

on a very small number of animals from 13 months.

Five months after revaccination, 5 pigs of Group I had 13.1 A.U., and after 13 months, in 2 pigs of this group the antitoxin level was an average of 20 A.U. In one pig of Group II at the same times, it was 10 A.U. and 15 A.U. In each of the Groups III and IV, only one pig remained alive, and 5 months after the revaccination the pigs of Group III had 20 A.U., and those of Group IV had 30 A.U.

The completed work shows the high immunizing activity of the purified sorbed tetanus anatoxin, its advantages in comparison with the native preparation.

CONCLUSIONS.

1) The findings obtained with the mass preparation of the native and of the purified sorbed tetanus anatoxins attest the great effectiveness of the latter.

2) In case of the two-shot immunization of the guinea pigs with native(75 B.U.) and purified sorbed(3 B.U.) tetanus anatoxins, in a long period of time(8 months) the animals retain higher immunity in case of their immunization with the purified sorbed preparation(by 3 to 45 times higher).

3) The potential power of small doses of the sorbed tetanus anatoxin is higher than the potential power of large doses of the native antigen.

4) In case of a single-shot immunization with sorbed anatoxin, larger amounts of the antigen impart higher immunity to the animals.

5) In case of the two-shot immunization with 30-and 3 B.U. of the sorbed anatoxin, the pigs respond with an almost equal immunizatory stimulation. The increase in the antigen up to 30 B.U. does not yield a larger accumulation of antitoxin.

6) After revaccination, the amount of antitoxin is maintained at a sufficiently high level for 13 months.

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RESISTANCE OF GUINEA PIGS TO TETANUS BACILLUS SPORES AFTER IMMUNIZATION OF THE ANIMALS WITH TETANUS TOXOID(p.197 - 204)

* * * *

In view of the fact that data are available in the literature about the change of the specific properties of the native anatoxins in case of their chemical treatment(IZARD,RAMON,RICHOUT,PILLINER and others) we set the goal to track down whether the by us employed method of concentration and purification of the preparation would change the specific action of the native tetanus anatoxin.

For this, we investigated,in respect to the spores of the tetanus bacillus,the immunizing effect of the concentrated, purified and of the purified sorbed tetanus anatoxins on the guinea pigs which were under the experiment for testing the harmless ness of the tetanus toxoid.

The effectiveness of the concentrated purified sorbed tetanus anatoxin was examined on 60 guinea pigs(Table 1).

The animals were divided into 3 groups. In the first group were put 13 pigs each of which received 7 ml of concentrated tetanus anatoxin; into the second group ---16 pigs each of which was inoculated with 7 ml of purified non-sorbed tetanus anatoxin; into the third group--- 31 pigs each of which received 5 ml of purified sorbed anatoxin. All together 60 series of tetanus anatoxin were checked.

Twentytwo days after the inoculation of the antigen,in 50% of the animals which were under the experiment of testing the harmlessness, we determined the accumulation of the antitoxin.

In 8 pigs,each of which was given 7 ml(10,000 - 15,000 B.U.) of concentrated tetanus anatoxin, slight amount of antitoxin was noted in the sera:- in 2 pigs $<$ 1/100 A.U., in 4 pigs 1/100 A.U.M, and in 2 pigs $>$ 1/100 $<$ 1/10 A.U.

In 8 pigs each of which was inoculated with 7 ml(10,000 - 15,000 B.U.) of purified anatoxin, the antitoxin titre also happened to be very low:- one pig did not have 1/100 A.U.; in 2 pigs it was 1/100 A.U. In 2 pigs it was $>$ 1/100 $<$ 1/10,

In eitgh guinea pigs each of which was inoculated with 7 ml(10,000 - 15,000 A.U. of purified anatoxin, the antitoxin titre also happened to be very STAT: one pig did not have 1/100 A.U.; in two pigs it was 1/100 A.U. In two other pigs it was >1/100 <1/10, and in three pigs it was 1/10 A.U.

(p.198) TABLE 1.

RESISTANCE OF GUINEA PIGS, WHICH WERE IMMUNIZED WITH TETANUS ANATOXIN, TO SPORES OF THE TETANUS BACILLUS.

ANATOXIN CONCENTRATED	ANATOXIN Purified	ANATOXIN SORBED	Number of pigs	Antigen in 1 ml in A.U.	A.U. after 22 days	Given on which day	Amount	Result
13 series			13	7 (10,000-15,000)	2 - 1/100 4 - 1/100 2 >1/100 <1/10	24	2.5-5	death on 4.-8 day
	16 series		16	7 (10,000-15,000)	1 - 1/100 2 - 1/100 2 >1/100 <1/10 3 - 1/10	24	2.5-5	death on 4.-8 day
		31 ser.	31	5 (1000)	3- 5	24	12.5 - 25	no sign of tetan.
CONTROLS			3				1	died in 2-3 hours

(p.199) But in 16 guinea pigs which were under the experiment of examining the harmlessness of the sorbed anatoxin and each of which had received 5 ml of the preparation (1000 B.U.), at the determination of the antitoxin, it happened to be >3 <5 A.U., in one ml of the blood serum.

Twentyfour days after the inoculation of the antigen, spores of the tetanus bacillus were intramuscularly injected into all animals. The spores were in a 10% solution of calcium chloride.

The preliminary titration of the spores on guinea pigs showed that the determination of 1 M.L.D. of the spores is very difficult. At the sowing of the spores upon an agar column the following results were obtained: seeding

½ M.L.D. contained 3-5 spores;

5 M.L.D. " 5-15 spores;

25 M.L.D. " 25-75 spores.

In view of the fact that the guinea pigs are very sensitive to the microbe of tetanus, it is likely that one spore--inoculated with an appropriate irritant in the form of calcium chloride-- proves to be sufficient to provoke disease. But together with this tube to be sure that, in case of its introduction into the animal, only one spore will get in, does not seem to be possible. Hence, it is necessary to prepare such a suspension that 1 M.L.D. would contain not less than 5 spores(at the seeding of a medium) which also will assure a 100% mortality of the control animals.

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The guinea pigs which were given concentrated and purified tetanus anatoxin, at their intramuscular injection with 2.5 - 5 M.L.D. of the spores have died of tetanus on the 4.-8 days, somewhat surviving the control animals.

But the guinea pigs which completed the experiment of testing the harmlessness of the purified sorbed tetanus anatoxin and had received subsequently an intramuscular injection of the tetanus spores in individual doses from 12.5 to 25 M.L.D., all remained alive for 14 days without the clinical symptoms of tetanus.

The intramuscular introduction of the spores in a 10% solution of calcium chloride allowed us in all cases to notice, after 2-3 days, painful infiltration which caused contractures (of the muscles) in the animals.

In such cases when the guinea pigs did not die the infiltrated tissue became necrotic on the 10, - 14.day.

As it can be seen from the experiment, the inoculation of a large amount of concentrated and purified tetanus anatoxin had not protect the animals from 2.5 - 5 M.L.D. of the tetanus spores. This evidently is explained so that the purified preparation is quickly eliminated from the organism and that, for the time of staying in the organism(p.200) of the animal, the immunizatory apparatus does not elaborate sufficient amount of antibodies, which would be powerful to cope with the developing infection. Apparently, the single-shot inoculation of the concentrated and purified anatoxin in large amounts is not enough. Since the antitetanus serum does not possess either a bacteriostatic or a bactericide effect(DANTON, FIL'DENS), therefore in case of infection, in the organism of the animal the phagocytes are of the chief protective role, by phagocytizing and lysing the spores. In cases of deficiency in the anatoxin, however, in case of a heavy trauma, with large necrosis of the tissues and with the presence of blood clots which facilitate the reproduction of the bacteria and hinder the phagocytosis, the increased development of the microbes and the

the excretion of a large amount of toxin by the bacteria is possible, and the antitoxin may not show a satisfactory effect.

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In our experiments with the concentrated and purified anatoxin, as it has been already indicated, in the experimental animals we had a small amount of antitoxin which finally, in such an acute experiment and in the presence of such a massive dose of the infection, could not protect them from tetanus. Therefore, in the present case, it is impossible to talk of a change in the structure of the anatoxin in consequence to its purification. At the same time, the purified adsorbed anatoxin has imparted a high immunity to the animals, which immunity permitted to protect them from 12.5 - 25 M.L.D. of the tetanus spore.

Since the effectiveness of the native anatoxin, for the preparation of which the Stock Kolle No.8 of the B.tetani has been used, was epidemiologically approved, the following experiment was staged for the purpose of a comparative study of the immunizing activity and effectiveness of the native sorbed and of the purified sorbed tetanus anatoxins in regard to the tetanus spores.

The native sorbed tetanus anatoxin was used for the reason because the effectiveness of the non-sorbed anatoxins is considerably lower than that of the sorbed native antigens, which was found by us in the preceding work.

For the comparative study of the antigens an experiment of single-shot and two-shot immunizations, with identical immunizing doses, was arranged, with subsequent infection of the animals with the spores.

In one ml. the sorbed tetanus anatoxin of Series No.30 contained 200 B.U., completely sorbed on 2 mg of Al_2O_3. The supernatant fluid had less than 1 B.U. in one ml.

The native tetanus anatoxin of Series No.309 had 150 B.U. in one ml, and after sorptions on 2 mg of Al_2O_3 the supernatant fluid contained 5 B.U. of the antigen.

In the experiment of the single-shot immunization, each of six guinea pigs received purified sorbed anatoxin in the amount of 20 B.U. One month after(p.201) the injection, in one ml of the blood serum of the animals, three pigs had >0.5 A.U. and three had 0.1 A.U.

Two months after the inoculation, in five pigs the amount of anatoxin increased to $>1<3$ A.U.(TABLE 2).

~~TWO~~ Six pigs received the same amount of sorbed tetanus native anatoxin (20 B.U.). One month after the injection in one of the pigs the titre was <0.01 in

one ml of the serum, in another guinea pig, it was > 0.01 < 0.1 A.U.; and in another — > 0.1 < 0.5 A.U.; another contained exactly 1 A.U., and one pig yielded > 1 < 3 A.U. (TABLE 3).

Later on, two months after the introduction of the antigen, the amount of antitoxin increased: — in three guinea pigs it was > 0.1 < 1 A.U., in one pig it was > 1 < 3 , and in another pig it was 2 A.U.

Two months after the injection, each animal was intramuscularly inoculated with 12.5 to 25 M.L.D. of the spores of the tetanus bacillus/

Out of five guinea pigs immunized with purified sorbed tetanus anatoxin, one pig died from tetanus(on the 4th day); the rest remained healthy for 14 days(see TABLE 2)

Out of 5 pigs which were given sorbed native tetanus anatoxin, one pig also died of tetanus. The remainder had no symptoms of this infection up to the 14th day (TABLE 3).

In this way, the experiment with the single-shot immunization of the pigs with the purified sorbed and with the native sorbed tetanus anatoxin did not show any advantage of any of the antigens.

Then, we immunized five guinea pigs with two shots of the purified sorbed anatoxin, and seven pigs with the native sorbed anatoxin, by giving 5 B.U. of antigen at each injection, with an interval of 30 days, between the injections(TABLE 2 and 3).

One months after the injection, the animals each of which received 5 B.U. of the purified sorbed tetanus anatoxin, in one ml of the serum, had a slight accumulation of the antitoxin: — in four pigs it was < 0.01 A.U., and in one pig it was > 0.1 < 0.5 A.U.(TABLE 2).

After the same period of time, in the serum of the guinea pigs which were immunized with native sorbed antigen, a slight amount of antitoxin was also established — in 4 pigs — < 0.01 A.U., and in 3 pigs — 0.01 A.U.(TABLE 3).

Then, again each of the animals was given 5 B.U. of antigen more. Thirty days after the second injection the amount of antitoxin rose in both groups of pigs.

Thus, in the animals immunized with the purified sorbed preparation, one ml of serum contained: — in one pig, > 0.1 < 1 A.U.; in 2 pigs, > 1 < 3 A.U.; — in one pig, > 3 < 5 A.U.; — in one pig, > 5 < 10 A.U.; but in the animals which were given sorbed native antigen, one pig had > 0.1 < 1 A.U., 4 pigs had > 1 < 3 A.U.; one pig had > 3 < 5 A.U.(TABLE 2 and 3) and one had 5 A.U. Two months after the first injection and

(p.202)

TABLE 2.

SINGLE SHOT AND TWO-SHOT IMMUNIZATION OF GUINEA PIGS WITH PURIFIED SORBED TETANUS ANTITOXIN, WITH THEIR SUBSEQUENT INFECTION WITH SPORES OF TETANUS BACILLI

Serial No.	B.U. OF first inject.	ONE MONTHS AFTER first injection A.U.	TWO MONTHS AFTER first injection A.U.	SPORES M.L.D.	RESULTS
1	20	>0.5<1	>1 3	1.25-25	smooth
2	20	>0.5<1	1<3	1.25-25	"
3	20	>0.5<1	>1<3	1.25-25	"
4	20	<0.1			
5	20	<0.1	>1<3	1.25-25	dead on 4. day
7	20	<0.1	>1<3	1.25-25	smooth
		SECOND Inj.	THE ONE MO. aft.		
9	5	<0.01	5 B.U.	>5<10	12.5-25	"
10	5	>0.01<5	5	>1<3	12.5-25	"
11	5	<0.01	5	>1<3	12.5-25	dead on 14. day
12	5	<0.01	5	>0.1<1	12.5-25	smooth
13	5	<0.01	5	>3<5	12.5-25	"
Three control guinea pigs				1	dead on 3. day	

TABLE 3.

SINGLE SHOT AND TWO-SHOT IMMUNIZATION OF GUINEA PIGS WITH NATIVE SORBED TETANUS ANATOXIN, WITH THEIR SUBSEQUENT INFECTION WITH SPORES OF THE TETANUS BACILLUS

Serial No.	B.U 1. inj.	One months after first injection A.U.	Two months after 1st injection A.U.	SPORES M.L.D.	RESULTS
15	20	>1<3	>1<3	12.5-25	smooth
16	20	=1	=3	same	"
17	20	>0.1<0.5			
18	20	>0.5<1	>0.1<1	same	"
19	20	>0.01<0.1	>0.1<1	same	"
20	20	>0.01	>0.1<1	same	dead on 6. day
		SECOND INJ. B.U.	ONE MONTH after 2. inj		
22	5	<0.01	5	>0.1<1	12.5-25	dead on 6. day
23	5	<0.01	5	>1<3	same	smooth
24	5	<0.01	5	>3<5	"	"
25	5	<0.01	5	>1<3	"	"
26	5	<0.01	5	>1<3	"	dead on 14. day
27	5	<0.01	5	=5	"	smooth
28	5	<0.01	5	>1<3	"	dead on 14. day
THREE CONTROL GUINEA PIGS				1	dead on 3. day	

(p.204 cont....) one month after the second, all animals were intramuscularly given 12.5 - 25 M.L.D of the spores of the tetanus culture.

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Out of five guinea pigs which were immunized with purified sorbed anatoxin, one pig died on the 14th day; four stayed alive for 14 days without the symptoms of disease.

Out of seven guinea pigs which received the native sorbed preparation, one pig died of tetanus on the 6th day, and 2 pigs died on the 14th day, 4 pigs did not show tetanus during the period of two weeks.

The completed experiment with the two-shot immunization of the pigs, both with the purified sorbed and with the native sorbed anatoxins, had showed no advantage of the sorbed anatoxin.

The provided findings permit to suggest that the purified sorbed tetanus anatoxin keeps all its specific properties due to which it protects the animals from tetanus as well as as it is done by the sorbed native anatoxin.

CONCLUSIONS.

1) The single inoculation of large doses of the concentrated and purified tetanus anatoxin up to 10 000-15 000 B.U. does not protect the guinea pigs from 5 to 10 M.L.D. of the tetanus spores.

2) The single inoculation of 1000 B.U. of the purified sorbed tetanus anatoxin protects 100% of the guinea pigs from 25 to 50 M.L.D. of the tetanus spores.

3) The comparative study of the effectiveness of the native sorbed and purified sorbed anatoxins showed no advantage of any preparation above the other, either at the single-shot or at the two-shot immunization.

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PREVENTION OF EXPERIMENTAL GAS GANGRENE IN MICE (p. 205-213)

* * *

The study of antibiotics and other various chemotherapeutic preparations in the treatment of experimental gas gangrene undoubtedly showed that the combination of chemother. means with specific sera gives more hopeful results (ZEL'MANOVICH, 1946; SOLOV'EV, 1954; P'ANSYMEOIZE, 1955, and others).

The purpose of the presentwork has been the study of the effectiveness of various antibiotics for the treatment of gas gangrene, but also of their combination with specific sera in cases of infection with separate cultures of the perfringens & edematiens bacilli and of the septicus and histolyticus vibrios.

The experiments which were done on guinea pigs concerning the treatment of gas infection with biomycin(LARINA, VOLOKOVA, ZELEVINSKAYA, 1959) indicated that the treatment with biomycin did not give positive results if their employment started later than 3-6 hours after the infection.

Further researches were done in the laboratory for the study of the new antibiotics in the treatment of gas gangrene. Such antibiotics were the tetracycline, SK, P_6(or R_6?) which were obtained from the Dept. of Infectious Pathology and Experimental Therapy of Infections. The researches were conducted in trials in vitro and in vivo. The titration of the activity of the antibiotics in the in vitro experiments were done according to the methodology described in our preceding work.

These experiments showed that in the in vitro experiments the antibiotic tetracycline possesses a somewhat larger bacteriostatic effect than the biomycin (Table 1). The Antibiotic SK (or CK?) has given the best results, by causing a slowing down in the growth in doses of 3.5 microgram, while the P_6, even in a dose of 1000 micrograms proved to be entirely ineffective.

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FIG.: GRAPH of the Change of concentration of tetracycline in the blood in mice at different ways of its introduction.

ORDINATA: Amount of the antibiotic in micrograms in one ml of blood.

ABSCISSA: Time of the blood taking (3-6-12-24)

On diagram: - - - By the mouth

_____intramuscularly.

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- - - - - - - -

(p. 207) The effectiveness of the above mentioned antibiotics in the treatment of gas infection has been studied in experiments on mice.

In advance, for 3-4 days in succession a whole series of experiments had been conducted for the determination of the doses of tetracycline which can be tolerated by mice in case of the various ways of their introduction. With this set of experiments it was established that the tetracycline is harmless in doses of 100-200 mg per Kg of body weight given by the mouth, or 500 mg per Kg of body weight by the mouth, 100 mg per Kg of body weight given intramuscularly.

Moreover, we studied the time entry (admission) of the antibiotic into the blood and its amount in case of the different ways of its introduction. The determination of the tetracycline in the blood has been carried out according to the method of SHEERSON, in the modification of KIVIAN. The conducted researches showed that, in case of introducing 100 mg per Kg of body weight into mice with a single shot, with an intramuscular injection its maximum amount appears after 3 hours and it equals 4.8 micrograms per ml of blood, while with peroral administration it appears after 6 hours only, and moreover, in an amount which is just one half of that measured after i.m. injection (2.4 micrograms per ml), and this is in agreement with the data which were obtained by TISCH, CULL and others (1955).

The mice were infected both with spores and with dry culture, in a mixture of Calcium chloride, by means of an intramuscular inoculation into their small paws.

The treatment started simultaneously with the infection, and three and six hours later. The antibiotic was administered at the rate of 100 mg per Kg of body weight per day (twice by mouth and intramuscularly).

As the experiments have shown (Table 4), in case of the treatment of a single infection caused by the B.perfringens, we have about 50% of survival of the mice with giving the antibiotic, either by mouth or intramuscularly, simultaneously with the infection, while if treatment starts 3 or 6 hours after the infection the mice die.

The absence of any kind of effect with the treatment that started after 3 and 6 hours provided the reason for using specific sera simultaneously with the employment of the antibiotics.

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These experiments showed that in the invitro experiments the antibiotic tetracycline possesses a somewhat larger bacteriostatic effect than the biomycin (Table 1). The Antibiotic SK (or CK?) has given the best results, by causing a slowing down in the growth in doses of 3.5 microgram, while the P_6, even in a dose of 1000 micrograms proved to be entirely ineffective.

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FIG.: GRAPH of the Change of concentration of tetracycline in the blood in mice at different ways of its introduction.

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(p. 207 cont.)

In the experiment, such doses were selected of the specific sera which themselves, with a single injection, have resulted in not larger than 50% survival. As it can be seen from Table 5, one antibiotic given intramuscularly results in a 25% survival; 2 A. U. of the serum result in 65% survival, but the combination of one with the other assures a 100% survival of the animals. At beginning of the treatment 3 hours after the infection, the mice died in 100% of the cases regardless whether the treatment is with antibiotics (per os and intramuscularly) or with the injection of large doses of serum. The combination of one with the other will reduce the death rate, and there is a 20-30% survival. The tetracycline treatment of the mice which were infected with dry cultures of the edematiens produces only a very low percentage of survival regardless whether the treatment started simultaneously with the infection or from 3 to 6 hours later (Table 6). In case of infection with spore-bearing culture which washed clean from the toxin, when the treatment starts simultaneously with the infection or 3 (p. 213) hours later, the mice show a survival rate equal to 100% (Table 7).....

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TABLE 1

EFFECT OF ANTIBIOTICS UPON THE AGENTS OF GAS GANGRENE

(IN VITRO EXPERIMENT)

CULTURE	ANTIBIOTIC	DOSE OF ANTIBIOTIC, MICROGRAMS PER ML / CONTROL								
		100	50	25	12	6	3	1.5	0.7	
Perfringens	Tetracycline	0	0	0	♁	+	+	+	+	+
Edematiens	Tetracycline	0	0	0	0	0	0	♀	+	+
Septic vibric	Tetracycline	0	0	0	0	♀	+	+	+	+
Histolyticus	Tetracycline	0	0	0	0	♀	+	+	+	+

TABLE 2

EFFECT OF ANTIBIOTICS UPON THE AGENTS OF GAS GANGRENE

(IN VITRO EXPERIMENT)

CULTURE	ANTIBIOTIC	DOSE OF ANTIBIOTIC IN MICROGRAMS PER ML									CONTROL
		1000	500	200	120	60	30	15	7	3.5	
Perfringens	SK	0	0	0	0	0	0	0	0	♁	+
Edematiens	SK	0	0	0	0	0	0	0	0	♁	+
Vibrio	SK	0	0	0	0	0	0	0	0	♁	+
Histolyticus	SK	0	0	0	0	0	0	0	0	♁	+

Table 2 (Cont'd)

CULTURE	ANTIBIOTIC	DOSE OF ANTIBIOTIC IN MICROGRAMS PER ML STAT 1000	500	200	120	60	30	15	7	3.5	CONTROL
Tetanus	SK	+	0	0	0	0	0	0	0	0	+
Perfringens	P6	+	+	+	+	+	+	+	+	+	

Marking: Absence of growth ... 0

presence of growth ... +

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TABLE 4

EFFECTIVENESS OF TETRACYCLINE IN TREATMENT OF MICE

INFECTED WITH A CULTURE OF THE B.PERFRINGENS

SINGLE DOSE OF ANTIBIOTIC	FREQUENCY OF INTRODUCTION	ROUTE OF ADMINISTR.	BEGINNING OF TREATMENT	NUMBER OF EXP. ANIMALS	SURV.	DIED
50 mg/Kg	twice in 3 days	intram.	at once	30	17	13
		per os		30	18	12
		i. m.		30	0	30
		per os	3 h. later	30	0	30
		i. m.		20	0	20
		per os	6 h. later	20	0	20
	CONTROLS			20	0	20

TABLE 5

EFFECTIVENESS OF COMBINED TREATMENT WITH TETRACYCLINE AND SPECIFIC SERUM

IN MICE INFECTED WITH B.PERFRINGENS CULTURE.

SINGLE DOSE	Frequ. of administr. of antibiotic	Route of introduct.	Dose of serum	Beginning of Treatment	No. of Exper. Animals	Surv.	Died
50 mg/Kg	twice in 3 days	i.m.			25	9	16
		-	2	at once	25	15	10
		i.m.	2		25	25	0
		i.m.			25	0	25
		-	80	3 h. later	25	0	25
		i.m.	80		25	5	20
		by mouth	-		25	10	15
		-	2	at once	25	10	15
		by mouth	2		25	25	0
		by mouth	-		25	0	25
		-	8	3 h. later	25	0	25
		by mouth	80		25	9	17
	CONTROLS				20	0	20

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TABLE 6

EFFECTIVENESS OF TETRACYCLINE IN THE TREATMENT OF MICE INFECTED WITH STAT

DRY CULTURE OF B. EDEMATIENS

Single dose	Frequency of Injection	ROUTE of adminstr. of antibiotic	Beginning of Treatment	No. of exper. animals	Surv.	Died
50 mg/Kg	twice in 3 days	i. m. by mouth	at once	20	2 3	18 17
		i. m. by mouth	3 h. later	20	1 2	19 18
		i. m. per os	at once	20	2 3	18 17
		i. m. per os	3 h. later	20	1 2	19 18
		i. m. per os	6 h. later	20	3 3	17 17
	CONTROLS			20	0	20

TABLE 7

EFFECTIVENESS OF TETRACYCLINE IN THE TREATMENT OF MICE INFECTED WITH

SPOR BEARING CULTURES OF B. EDEMATIENS

Single dose	Frequency of admin.	Route of admin.	Dose of serum in A. U.	Beginning of treat.	No. of Exper. Animals	Surv.	Died
50 mg/Kg	twice in 3 days	i.m. per os	- -	at once	17	17 17	0 0
		i. m. per os	- -	3 h. later	17	17 17	0 0
		i.m. per os	- -	at once	17	17 12	0 5
		- per os	100 100	same	17	4 17	13 0
	CONTROLS				17	0	17

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TABLE 8

EFFECTIVENESS OF TETRACYCLINE IN THE TREATMENT OF MICE INFECTED BY HISTOLYTICUS CULTURE

Single dose	Frequency of admin.	Route of admin.	Beginning of treat.	No. of Exper. Animals	Survived	Died
50 m./Kg	twice in 3 days	i.m. per os	at once	20	20 16	0 1
		i.m. per os	3 h. later	20	20 3	0 17

Table 8 (Cont'd)

Single Dose	Frequency of admin.	Route of admin.	Beginning of treat.	No. of Exper. Animals	STAT Survived	Died
		i.m.		20	17	3
		per os	6 h. later		4	16
	CONTROL			20	0	20

TABLE 9

EFFECTIVENESS OF THE COMBINED TREATMENT WITH TETRACYCLINE AND SPECIFIC SERUM OF THE MICE INFECTED WITH B.HISTOLYTICUS CULTURE

Single Dose of Antibiotic	Freq. of admin.	Route of admin.	Dose of serum A. U.	Beginning of treatment	No. of exper. animals	Survived	Died
50 mg/Kg	twice in		-	at once		16	4
	3 days	per os	-	3 h. later	20	3	17
			-	6 h. later		4	16
			0.5	at once		11	9
		-	20	3 h. later	20	12	8
			20	6 h. later		12	8
			0.5	at once		20	0
		per os	20	3 h. later	20	14	6
			20	6 h. later		14	6
	CONTROL				20	0	20

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TABLE 10

EFFECTIVENESS OF THE COMBINED TREATMENT WITH TETRACYCLINE AND SPECIFIC SERUM IN MICE INFECTED WITH SEPTIC VIBRIO

Single dose of antib.	Freq. of admin.	Route of admin.	Dose of serum A. U.	Beginning of treatment	No. of exper. animals	Survived	Died
50 mg/Kg	twice in	i.m.	-		18	10	8
	3 days	-	50	15 h. later	16	4	12
		i.m.	50		17	13	4
		per os	-		18	10	8
		-	50	same	16	1	12
		per os	50		13	4	9
		CONTROL			15	0	15

TABLE 11

CULTURE	ANTIBIOTIC	BEGINNING OF TREAT.	NO. OF EXPER. ANIMALS	SURVIVED	DIED
Perfringens	SK 100 mg/Kg	at once	10	1	9
		3 h. later	10	0	10
		6 h. later	10	0	10
	P_6 25 mg/Kg	at once	10	4	6
		3 h. later	10	0	10
		6 h. later	10	0	10
EDEMATIENS	SK 100 mg/Kg	at once	10	0	10
		3 h. later	10	0	10
		6 h. later	10	0	10
	P_6 25 mg/Kg	at once	10	0	10
		3 h. later	10	0	10
		6 h. later	10	0	10
	CONTROL	perfringens	10	0	10
		edematiens	10	0	10

(p. 21[illegible] cont'd)

.....At a treatment which started 6 hours after the infection, 30% of the animals died. The treatment with serum in the dose of 100 A. U., when given simultaneously with the infection, gives a 25% survival rate, and if the antibiotic and the serum are injected together, the survival rate is 100%. In case of a treatment which started simultaneously with the infection, when histolyticus culture was used, are it started 3 hours later, the survival is 100% in case of an intramuscular administration. If the antibiotic is given by the mouth, the survival rate is 75%, in case of a treatment which started simultaneously with the infection, but it is only 25% if the treatment was begun 3 hours after the infection. In case of intramuscular administration which was delayed 6 hours after the infection, the survival rate is 75%; if the antibiotic was given by the mouth, the rate is 25% (Table 8). The mixed treatment with antibiotics and serum in doses of 0.5 A. U. (in case of treatment simultaneously started at time of the infection) and 20 A. U. (in case of treatment delayed by 3 or 6 hours after the infection), assures a 100% survival rate if the treatment was begun at the same time with the infection; in the remaining cases the effectiveness of the combined treatment does not have any success (Table 9). In case of antibiotic treatment of the mice which were infected with cultures of Vibrio septicus, fifteen hours after the infection, at any routes of administration, we have a 100% survival rate. The treatment which starts later than 15 hours gives only

an about 50% survival rate. With the simultaneous administration of a 50 A. U. dose of serum, only in the group of mice which was given antibiotic intramuscularly at the same time when the serum was injected, seems the mortality to be reduced.

The treatment of mice with the antibiotic SK at a dose of 10 mg/Kg (24 hour dose) and with P_6 at a dose of 25 mg/Kg showed that in both cases their effectiveness is insignificant. The antibiotic SK which proved itself highly effective in the in vitro experiments was completely ineffective in the experiments on the animals (Table 10).

CONCLUSIONS

The conducted work permits to draw the conclusion that the treatment of gas infection by means of antibiotics should be combined simultaneously with the treatment by the specific serum which neutralizes the toxin and this itself facilitates the course of the infection.

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MORPHOLOGY OF THE S- and R- VARIANTS OF TYPE-A Bac. BOTULINUS (p. 215-220)

The study of the variants of the anaerobic microbes, in particular the study of the variants of the different types of B. botulinus, is but slightly represented in the literature. The data which were obtained in this direction show that the anaerobe microbes, when they are seeded on the surface of the agar medium or in the depth, produce different forms which are distinguished by morphological and biological characteristics (VAN ERMENGEM, 1896; BEUGSTON, 1922; MEYER & GUNNYSON, 1929; CHERVYAKOVA, 1946; MATYASH & ASKALANOV, 1941; FRENKEL8, 1943). The study of the cultures of the anaerobe microbes showed that, similarly to the aerobe bacteria, in the process of growth they are forming smooth and rough colonies characterized by a whole series of properties. In addition to these forms, there are transitional ones which bear in themselves the properties of both variants.

For the cultivation of the B.botulinus on compact culture media, a 1.5% agar is used, prepared with bouillon of the tryptic digestion of meat, with addition of glucose and from 10% to 15% of defibrinated blood of horses or lambs. By observing the B.botulinus of Type A (Memphis strain) for growth on the surface of the agar, we were able to notice the development of two types of colonies:- smooth ones and rough ones (Fig. 1 and 2). The smooth variants were convex; they had a round shape, an even edge and a smooth surface, they weakly permitted the light, and they had a sticky consistency. The colonies did not exceed 1-2 cm. On the blood agar, clear zones of hemolysis resulted. The colonies of the rough variant were flat; they had very differently shaped forms, with uneven surface and edges. The colonies markedly varied in form and size.

In addition to obtaining variants of the Type A B.botulinus on agar in Petri dishes, we have also used other methods applied in the anaerobe bacteriological practice for the cultivation on high column of agar.

(p. 216) (full page illustrations)

MICROPHOTO 1: Peripheral portion of the site of the anatoxin inoculation which anatoxin was sorbed to 10 mg of $AlPO_4$; after

24 hours (x 106).

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MICROPHOTO 2: Peripheral portion of the site of inoculation of anatoxin which was sorbed to 4 mg of $AlPO_4$; after 10 days (x106).

(p. 217) If the Type A B.botulinus is sowed in a high agar column, more often only colonies grew out in the shape of flakes (fluffs) with a thick compact center. The colonies possessed a diffuse growth, and very rapidly they enlarged their sizes when growing in the thermostate or at room temperature (Fig. 3).

The second type of colony which we got at the cultivation in high agar column had the shape of a disk with completely smooth flatness, of a rather compact consistency (Fig. 4), sometimes in the form of two intersecting disks, which gave them the shape of an aeroplane.

MICROPHOTO 3: The same as on Microphoto 2 (x 345).

The colonies of the smooth variant in the high agar column and on the dishes proved to be slightly stable only, and they had the ability to quickly undergo a lysis. The researches made for the study of the causes of this lysis indicated that it is linked with the presence of a bacteriophage in the S-variants.

By using the method of silver impregnation proposed by Prof. MOROZOV for the study of the morphology of the cultures of the variants, we were in the position to notice that, side by side with the bacilli of the usual form, there were also unusual shapes in the cultures. Especially many of them could be noticed in the older, 7 to 10 days' old cultures.

(p. 218) The observed forms of the microbes presented themselves with small heads in the form of points whose diameter several times was less than the diameter of the bacilli. As a rule, the head was provided with a flagellum. This form was called a punctiform flagellated form.

Side by side with the above described forms, other shapes were also met with. These shapes presented themselves as rings surrounded by a larger amount of flagella of a diameter equal or larger than the diameter of the bacilli. This form received the designation of anular flagellated forms (Fig. 6).

MICROPHOTO 4: Site of inoculation of the anatoxin, sorbed to 4 mg of $Al(OH)_3$, after 10 days (x 345).

The number of these forms was increased according to the age of the culture.

In case of a transfer (replanting) of old bouillon cultures which contain a considerable number of them, we have noticed that again the growing young culture consisted of bacilli of the ordinary shape and it did not contain the mentioned formations. The punctiform and flagellated forms did not pass through the bacterial filters. For the purpose of detecting the existance of similar formations in other anaerobes, we have made observations with cultures of Type B B.botulinus, and of B.perfringens and B.tetanus, and others.

(p. 219) (full page illustrations)

MICROPHOTO 5: Peripheral portion of the site of inoculation of the antitoxin, sorbed to 10 mg of $Al(OH)_3$; after 30 days (x.345).

MICROPHOTO 6: Site of inoculation of anatoxin sorbed to 10 mg of $AlPO_4$; after 96 days (x.345).

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The investigations showed that in these cultures, also, the indicated shapes exist. In the work of SKAVINSKII (1940), according to the investigation of the developmental stages of the spirocheta of the recurrent fever, there is an indication about the fact that at definite stages of the spirochete's development forms will appear which somewhat remind us of the above described forms. The appearance of these forms is related by the author to unfavorable conditions for the microbes' development.

It is possible that the indicated forms represent one of the stages of development of the microbes at their transition from the visible into the invisible, since these forms were observed in dissolved lyzed cultures when the vegetative forms happen to disappear and the filtrates of these cultures acquire the properties of the phage.

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USE OF CASEINE-PLANT MEDIA FOR THE PRODUCTION OF TOXINS OF THE AGENTS OF THE ANAEROBE INFECTIONS. (p. 221 - 227) *

* (FOOTNOTE: Report at the Conference in Kharkov in 1956).

* * *

To everyone of us--, both to him who prepares the culture media and to him who uses them, it is well known what a great importance the quality of the nutritive media has at the manufacture of bacterial preparations. That is why it is also necessary to continuously improve the nutrient media, to make the better, to elaborate new and more rational methods of their manufacture so that at the same time it should answer the demands of the developing production.

Until the present time the production of anaerobic and aerobic toxins of the agents of wound infections in the Institutes of the Ministry of Health Defense was practiced on meat nutrient media.

The question about getting rid of the necessity of using meat media remains therefore one of the basic problems at the manufacture of the mentioned preparations.

In the GAMALEI Institute, especially acute has been the question to search for a non-meat nutrient medium in connection with the new method of purification and concentration of the anatoxins.

For this purpose, at the Institute the nutrient bouillons proved to be essential which are less saturated with inert (ballast) protein compounds than the meat media.

From the raw materials which are accessible for the bacteriological institutes, in the GAMALEI Institute three casein-plant media were obtained and used under conditions of production for the making of staph. toxin, tetanus toxin and of the toxins of perfringens and septicus and edematiens.

In distinction from the overwhelming majority of casein media known in the domestic and foreign literature, and used under (p. 222) industrial conditions, the caseine-plant media do not require for their preparation either meat or other meat products, including animal ferments or extracts from the liver or from the heart.

The casein-plant media are free of ballast protein substances which would make difficult the process of purification and concentration of the anatoxins according to the new method.

In this way, we succeeded to solve two tasks at the same time: to get rid of the need of using meat media and to improve the nutrient media which are suitable for obtaining toxins, with subsequent purification and concentration.

Before characterizing the casein-plant media, one general problem should be dealt with, which refers to all casein media regardless of their formula, of the method of their preparation or ultimate assignment.

This problem is about casein itself, about its quality, about those requirements which must be made to the quality of casein at the manufacture of bacterial preparations. This question must be raised since during the recent times the casein media attract greater and greater attention of the bacteriological institutes which endeavour to produce standard nutrient media, and this is impossible if due attention is not paid to the quality of the casein.

The employment of any which casein will not only deteriorate the quality of the media but it will also lead to waste in the production.

Thus, the complaints of the institutes on account of the complexity of the filtration of the casein media are explained by the use of badly soluble abomasum casein. The latter is meant for the production of glue, and it is not fit for the preparation of media.

A large precipitate of unsolved or undigested casein in the process of hydrolysis even attests the incorrect choice of casein. A mandatory condition for production of highly active toxins on meat media is the employment of fresh meat & the preparation of the [illegible] bouillons.

The presence of putrid products of a microbic decomposition is not permissible in the casein either.

We have done some special work to which we invited The Vologod Dairy Testing Laboratory which controls the quality of casein in all factories of the Vologod District, the Upper Vologod factory which prepares the casein for us, and the controlling laboratory of the Orlov Trust which is responsible for the quality of the casein in the factories of the Orlov District.

The results of the conducted works indicate what requirements the casein must

satisfy at the manufacture of the bacterial preparations.

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These conditions are the following:

1) Duration of the process of producing the caseine, including the drying, is not more than 2 days. (48 hours)

2) The casein must be white or of light yellow color, homogenous, with grains of sharp edges, odorless.

3) The fat content should not exceed 2%.

4) The acidity should not be higher than 150° (under the conditions of maintaining all other requirements).

5) The solubility, moisture, and ash content must be according to standard.

6) Absence of putrefying bacteria.

The brown or dark-yellow coloration of the grains proves the over-heating of the casein or the presence of iron salts; the gray color of the casein indicates putrefactive admixture; the melted edges of the grains speak of flushing with water of increased hardness; such a water will dissolve casein. The casein with such defects is unsuitable for the preparation of media.

To the above enumerated requirements the following caseins will answer: the acid technical casein of a higher and first rate (GOST-1211-41), and, as we have established, the alimentary acid casein (TU-153-54), under the condition that it is made only from skimmed milk.

In this case the percentage of fat in the alimentary casein does not exceed the fat content in the technical acid higher and first class casein.

Identical is also the cost of the food acid caseine and of the technical acid casein of the higher grade. However, the food acid caseine has a great advantage above the acid technical casein of the higher grade.

The technological process of the preparation of the food acid casein permits to put a control for the quality of the casein at all phases of its preparation, including also the process of drying which is done in closed furnaces with steam or fire heating.

This is why it is also easier to prepare a practically standard food acid casein under factory conditions than to produce technical acid casein of higher grades. The technical casein dries under the sun, and the higher grades quicker separate from

the total manufactured casein than they are knowingly prepared. The advantage in the technology of the manufacture of the food acid casein permits to secure such a casein as the requirement of all bacteriological institutes by the forces of a single and not even too large a factory.

The manufacture of a standard casein should be sought for by all the bacteriological institutes at one or two factories determined according to the indices of the casein's quality, in agreement with (p. 224) the requirements for "casein used in the preparation of bacterial preparations". At the preparation of the casein-vegetable medium we have spent some time on the food acid caseine and the technical acid casein of the higher grade and even of the first class.

The caseine-vegetable medium for the production of the staphylococcic toxin and of the tetanus toxin is composed of acid hydrolysate of caseine, yeast extract and autolysate of bran.

At the preparation of the hydrolyzate, such conditions of hydrolysis are established which permit to avoid the removal of hydrochloric acid by means of vacuum. The dark humin compounds which form at the hydrolysis and which inhibit the growth and reproduction of the microorganisms are removed by means of adsorption to activated charcoal. At this step, one should be sure in advance that the given brand of coal is suitable for this purpose. Although the coal is graded according to a single standard, nevertheless, not all portions of the activated charcoal are identical in their quality. Adsorption should not be arranged from concentrated solutions. At this, even with a charcoal of good quality, the clarifying of the hydrolyzate cannot be successfully reached. Distilled water must be added in the beginning to the hydrolyzate at such a rate that the pepton content should not exceed 2.5 grams, and afterwards the coal must be also put in.

The activated charcoal partly absorbs the vitamins. Therefore, in the process of the preparation of media, yeast extracts and the autolyzat of bran is put into the clarified hydrolyzate.

The caseine-vegetable medium for the production of the edematiens toxin consists of the acid hydrolyzate of caseine and of corn extract. If the corn extract is delivered from the factory immediately after its preparation, then only for 2-3 months will such an extract usually stay fit for the preparation of the nutrient media. Should the extract be used in the industry only after this period of time,

then a preliminary test can reveal the suitability of the extract.

The caseine-vegetable medium for the production of the perfringens STAT toxin and septicus toxin consists of the caseine-fungus hydrolyzate (it is prepared by means of hydrolysis of the protein by fungal protease which is employed in the form of a dry culture of Aspergillus terricola on bran), of yeast extract, salt solution, of the salt of potassium, sodium and magnesium, and of whole wheat. (We refued the introduction of the corn extract).

In the caseine-vegetable media, the contents in pepton-midmolecular products of the split of the protein molecule shows a decisive influence upon the process of toxin formation (these products give a positive biuret reaction). The optimum is a pepton content of 1.5 - 2.2 g%.

(p. 225) The caseine-vegetable media do not contain ballast (inert) protein nitrogen determinable by the method of POPE and LINGUT, which is confirmed by the absence of precipitation with trichloracetic acid (Table 1).

TABLE 1

CONTENT OF INERT (BALLAST) PROTEINS IN THE CASEIN-VEGETABLE AND THE MEAT MEDIA.

CULTURE	MEDIUM	PROTEIN NITROGEN mg% (by the method of Pope and Lingut)	AT BOILING WITH Trichloracetic Acid
Bac. edematiens	Caseine-vegetable	0	Transparent solution
	Meat peptic	27	Precipitate
Bac. perfringens and Bac. septicus	Caseine-vegetable	0	Transparent solution
	Meat tryptic	61	Turbidity
Bac. tetanus	Caseine-vegetable	5	Transparent solution
	Meat after RAMON	109	Precipitate
	Meat after GLUZMAN	100	Precipitate

The fungal protease causes a profound split of the molecule of the protein, and in the acid hydrolyzate of caseine the unsplit protein is removed by precipitation of the protein at the isoelectric point with hydrochloric acid (at p^H = 4.7).

The data of Table 1 give an explanation why the caseine-vegetable media proved to be suitable for the preparation of the purified concentrated toxins-anatoxins.

The GAMALEI Institute has initiated the use of the fungal proteases, of the Aspergilli in the production of bacterial preparations in the year [illegible]STAT. Since 1953, the fungal protease is utilized...(p. 226) in the preparation of nutrient media which are to be used in the production of the toxins of the anaerobes.

In this way, the field of the use of the fungal protease is gradually widened, and at the present time the question arises about the production of a purified concentrated preparation of the enzyme.

However, for this purpose we think it adequate to use, not the surface culture on bran, but the deep culture of the Aspergillus terricola cultivated in a reagent tube on liquid, polysynthetic medium of simple composition.

TABLE 2.

CASEIN-FUNGAL MEDIUM PRODUCED WITH THE USE OF DIFFERENT PREPARATIONS OF THE PROTEASE OF ASPERGILLUS TERRICOLA

SOURCE OF ENZYME	ENZYME	COEFFICIENT OF ENZYME in g by 1 ml of hydrolyzate	STRENGTH OF TOXIN M.L.D. in 1 ml.		
			Bac. perfr.	Bac. sept.	Bac. histolyt.
Deep aerated culture from reagent tube	Alcohol-purified concentrated from mycelia	2	300	500	1000
	Alcohol purified concentrated from culture fluid	2	300	500	1000
Dry surface culture on bran	Alcohol purified concentrated	2	300	500	750
	Non-purified - used in industry	20	300	500	500

This culture is purer in respect to content of ballasts in relation to the enzymes, and its manner of production of technically more perfect than the surface method of cultivation on bran, finally, the reagent is a basic apparatus in the bacteriological institutes.

At the present time, we produce deep culture of Aspergillus terricola in a reagent vessel with a volume of 500 liters.

Not only the mycelia have a proteolytic activity but also the cultural fluid. In this manner, two ways are noted for the production of a purified concentrated preparation of fungal protease:- from the mycelia and from the cultural liquid.

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Under the conditions of the experiment, we made alcohol-purified concentrated preparations of the fungal protease from the mycelia and from the cultural liquid which had an activity of 40,000 units (according to Ful'd-Gross). These results can be undoubtedly improved, and we are working on this problem.

However, even with such a degree of purity, 1.5-2 g of the purified concentrated enzyme replaces 20 g of fungal proteases in the form of dry culture of the fungus on bran. The produced hydrolyzates do not differ as to the degree of splitting of the protein molecule.

As it is evident from Table 2, the titres of the toxins of the histolyticus, of the perfringens and of the septicus are identical on both media.

The employment of the concentrated enzyme (fungal protease), just as much as the use of the standard caseine, pursues one goal to increase the standardization of the caseine-vegetable media and, in this sense, to bring them nearer to the polysynthetic media.

The caseine-vegetable media have been manufactured under industrial conditions in the amount of 20,000 liters.

According to the data of the Department of Anaerobic Infections, the preparations produced with the use of the caseine-vegetable media, are considerably better as to strength of toxin, activity of anatoxin and as to the other indices than the toxins and anatoxins produced on meat media.

At the preparation of the caseine-vegetable media we have been using several types of raw material. Perhaps, such a combination of the initial substances also creates a relationship (proportion) of all nutrient substances which are advantageous for the toxin formation, by not oversaturating them with any single compounds with the deficiency of other substances.

The caseine-vegetable media have also other advantages above the meat media:- they are very inexpensive, they permit to abstain from the use of the meat media and of the animal enzymes, they are always sure to get raw material since all basic raw materials are preserved in the dry form and they do not spoil. Therefore, it can be controlled in good time.

All this permits the recommendation of the caseine-vegetable medium for the use in the production of aerobic and anaerobic toxins of the agents of wound infections.

(No literature given)

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ON THE QUESTION OF THE ORGANIZATION OF THE PRODUCTION OF THE ALIMENTARY ACID CASEIN (p. 229 - 236)

* * *

During the last few years, a new trend appeared in the Soviet Union in the utilization of the domestic alimentary acid caseine--for the production of nutrient media to be used in the large-scale manufacture of bacterial preparations which are to be employed in the prophylaxis and treatment of infectious diseases of man (pertussis, tetanus, brucellosis, etc.)

In the contemporary practical bacteriology, one of the basic problems is how to get the high-quality purified concentrated combined preparations.

In connection with this, special importance is gained by the casein media whose use showed a great advantage when compared with the meat media, which is corroborated by the practice of the domestic institutes (1, 2) and of foreign laboratories (3).

In the casein, as raw material for protein, the nutritive value is high which is manifested in the full value of protein as to its amino acid composition, and the optmum quantitative ratio of the component amino acids is joined with the homogeneicity of the protein, with its capacity of hydrolyzing easily, and with the absence of any substantial amount of extraneous admixture and contamination in it.

The use of casein media enables the raising of the quality of the preparations (heightening the titre of the toxins, of the activity of anatoxins, increase of the output of bacterial mass) and, which is very important, it facilitates the purification and concentration of the preparations.

However, at the present time (and until now), there is no casein in the Soviet Union which is specially predestined for bacteriological purposes.

(p. 230) In the U. S. A., the "Difco" Firm issues a few types of casein which can be recommended for bacteriological works, but the methods of production and the standards are not indicated. Moreover, there are no data about from kind of casein the media are

manufactured that are released by the same Firm (4).

Hence, an urgent need arose(a) in the organization of the production of a high-quality casein which is specially designated for bacteriological purposes, and (b) in the elaboration of the corresponding standards.

The present report has the following purpose:

1) to acquaint with the results of a wide use of the food acid casein at the manufacture of nutrient media in the production of bacterial preparations (biological products);

2) to report the preliminary findings about the research into the indices of the fitness of casein;

3) to outline the steps for the organization of the production of high-quality casein for bacterial preparations.

The question about the assured supply of the bacteriological institues with special casein had been first raised in the Soviet Union on the Conference on the Anaerobes by the Gamalei Institute of the Academy of Medical Sciences, U.S.S.R. (5). In 1956, in view of the extreme importance of this problem, joint investigations were started in this direction by the Gamalei Institute, the Vologod Dairy Institute, and the Vologod Regional Dairy Testing Laboratory(6).

We have investigated 30 batches of edible acid casein which are produced by different factories of the Vologod District according to the available instruction, and ten batches of casein (from the Votchin Factory of the Vologod Trust) with a minimum content of fat. Such a casein was also examined which was specially prepared by the Experimental Department of the "Molochnoe" (Dairy) Factory, with various deviations from the technological instructions. At this, different methods of the preparation of food acid casein were studied:- granular, ejectoral and grainy (the usual method with decocting).

As a control, the technical acid casein of high and first grade was taken.

In the casein these were determined:- fat, acidity, moisture, solubility and the titres of intestinal bacteria.

In several experimental series, in addition to these examinations, determination was made also of the following:- ash content, pH, oxydation-reduction potential, protein content according to Kjeldahl, and microbiological examinations (titre of the intestinal group of bacteria, with identification of B. coli commune, the number of

putrefying (decomposing) bacteria).

(p. 231) From each batch of casein, acid and enzymatic hydrolyzates STAT were prepared (with fungal protease of Aspergillus terricola). From the hydrolyzate, nutrient media were prepared according to the methodology accepted by the Gamalei Institute.

The nutrient media were examined (tested) in the different departments of the Institute for their suitability to the production of bacterial preparations (of toxin-antitoxins of the anaerobic and aerobic agents of wound infection, of pertussis and dysentery vaccines, and of other preparations).

In the Gor'kov Institute of Epidemiology, Microbiology and Hygiene, the casein media were examined for their suitability to the preparation of tuberculin.

The technological process of the production of the alimentary acid casein permits to introduce a much tougher control of the casein's quality at all phases of its preparation, including the process of its drying which must be done in dryers.

Since the chance of a natural drying at the sun is excluded from the production of the alimentary casein, the production of a casein of constant indices becomes more real. Hence, we made it our goal to examine the fitness of this casein, and, in case of positive results, to introduced it in the practice of preparing the media.

In result of the conducted investigations, it was found that the food acid casein is completely fit for the manufacture of bacterial preparations. As an illustration, we quote the data represented in Table 1.

At the present time, the alimentary acid casein, thanks to the conducted researches, has obtained a wide use in the manufacture of tetanus anatoxin, of toxins-antitoxins of the agents of gas gangrene, of the pertussis vaccines and some other preparations.

Positive (favorable) results were also obtained in the experiment with the tularemia vaccine, with diphtheria anatoxin.

During the past one year and a half, we have used food acid casein at the preparation of the bacterial products, not paying any attention to the relatively high content of fat (up to 3%) in it. Meanwhile, in the high-grade types of the technical acid casein the fat content does not exceed 1.5%.

The presence of large amount of fat complicates the preparation of nutrient media. In the process of the hydrolysis of casein in alkaline medium, saponification of the fat occurs, which makes difficult the filtration and proteolysis, and, at the manufacture

of an acid hydrolyzate, -- the process of purification by coal (charcoal. Hence, the alimentary acid casein has been prepared with a minmum content of fat. STAT

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TABLE 1

QUALITY OF THE BIOLOGICAL PRODUCTS OBTAINED ON NUTRIENT MEDIA MADE FROM ALIMENTARY AND TECHNICAL CASEIN (Average data)

BIOLOGICAL PRODUCT	OBTAINED ON CASEIN MEDIUM	ACID CASEIN	
		Alimentary	Technical
Pertussis vaccine	a) Number of casein batch	50	15
	b) Morphology of culture	normal	normal
	c) Yield of microbic bodies, billions per on ml	38-45	38-45
Staphylococcus toxin	No. of casein batch	15	5
	Hemolytic titre (Lh)	0.3	0.3
Tetanus anatoxin	No. of casein batch	50	25
	Activity of anatoxin (B.U. per one ml)	100-300	100-300
Perfringens anatoxin	No. of casein batch	15	20
	Activity of anatoxin (B.U. in oneml)	200-300	200-300
Septicus anatoxin	No. of casein batch	15	15
	Activity of anatoxin (B.U. in one ml)	300-500	300-500

From this it was successfully gained, i.e., it was established in advance, that at the preparation of the media, in addition to the earlier employed grain casein (with decoction) the casein can be also utilized that is obtained by the method of ejection (Table 2).

After it has been established that the employment of the alimentary acid casein of a minimum fat content does not have an effect on the quality of the produced bacterial preparations (Table 3), the Votchin Butter Factory introduced in its practice in the manufacture of such a casein for the Gamalei Institute.

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TABLE 2

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TITRE OF THE TETANUS TOXIN AND ACTIVITY OF THE ANATOXIN ON NUTRIENT MEDIA WITH THE EMPLOYMENT OF EJECTED AND ORDINARY CASEIN

ALIMENTARY ACID CASEIN	Fat %	Acidity in o of Terner	Moisture in %	Toxin titre Lf/ml	Anatoxin activity B.U./ml
Ejected	3.0	48	10.1	40	250
	2.75	28	9.8	40	250
	3.0	24	9.8	24	20
With decoction (grainy regular)	3.0	48	10.1	35	250
	1.92	56	9.8	21	250

TABLE 3

TITRE OF TETANUS TOXIN AND ACTIVITY OF THE ANATOXIN ON NUTRIENT MEDIA WITH THE USE OF CASEIN OF DIFFERENT CONTENT IN FAT

CASEIN (food, acid)		Titre of toxin Lf/ml	Anatoxin activity in B.U./ml
% of fat	Acidity in Terner degr.		
3.0	20°	24	200
3.0	28°	24	200
3.0	48°	35	250
1.5	36°	24	200
1.4	20°	30	250
1.1	16°	28	200

At the same time it was shown that even this casein does not fully satisfy us.

In the practice of the preparation of the nutrient media, even with the use of indentical indices as provided by TU 153-54, it occurs sometimes (not so rarely) that the quality of the media is subject to considerable variations (Table 4).

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TABLE 4

QUALITY OF CASEIN AND TOXIN FORMATION OF THE BAC. TETANI AND BAC. PERFRINGENS

FOOD ACID	CASEIN			Titre of tetanus toxin Lf/ml	ACTIVITY OF ANATOXIN in B.U./ml	
% of fat	Acidity in ° of Terner	Moisture in %	Solubility		Tetanus	Perfringens
3.0	48	10.1	0.1	35	250	-
3.0	48	10.1	0.1	21	150	-
27	60	9.5	0.1	16	100	-
30	60	10.4	0.1	24	150	-
1.1	20	9.7	0.1	14	50	-
1.1	o6	9.8	0.1	28	200	-
3.0	16	9.7	0.1	-	-	400
3.0	12	9.5	0.1	-	-	100

TABLE 5

INFLUENCE OF THE REGIME OF PRODUCTION OF THE CASEIN UPON THE QUALITY OF MEDIUM AT THE PREPARATION OF THE PERTUSSIS VACCINE

(Output of bacterial mass in billions per ml)

CASEIN	Normal techn.	DISTURBANCE OF TECHNOLOGY AT VARIOUS PHASES				
		a	b	c	d	e
With decoction	38	25	24	30	46	13
Ejected	44	16	23	28	39	-
Granular, by food technology	39	16	23	48	29	-

HEADINGS: a...increased fat content; b...single washing; c...preservation in cold water; d...disturbance of temperature of drying; e...subsequent sterilization of casein

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(p. 235) For the purpose that the cause of this variation could be explained, the food acid casein has been worked out in the Experimental Department of the "Molochnoe" (Dairy) Factory according to the normal technology and with factory devications from the technological process at various phases of the manufacture.

Thereby, three types of casein were made:- grainy (the ordinary acid casein with decoction), ejected and granular (7). In each of these types six variants were included:- normal technology, with increase of the fat content in the mixture (0.9%), single washing out, increased temperature of drying, preservation with cold water for a day before drying, and sterilization after drying at one atmosphere for 10 minutes.

The quality of the nutrient media we judge according to the yield of the bacterial mass at the preparation of the pertussis vaccine (Table 5).

The obtained results show the dependence of the quality of the nutrient media upon the defined parameters of the technological process, namely:-from the casein of all three types which was elaborated according to the normal technology, nutrient media were obtained of the greatest yield of microbic mass; at the same time, as all the other variants have given rather bad results.

The obtained results also prove that deviations from the normal technological regime will show a substantial influence upon the quality of the casein which is designated for the manufacture of nutrient media, even though these deviations would not disturb the standard. In this way, the experimental investigation and the practice of the wide employment of casein in the production of bacterial products corroborates the necessity of having an organization for the production of a special type of casein for these purpose. Such a casein may be obtained on the basis of the food casein, with the elaboration of the separate details of the technical process and of the chemical bacteriological control.

CONCLUSIONS.

1. At the present time, a new trend appeared in the use of the domestic food acid casein at the manufacture of nutritive media to be used in the production of bacterial preparations.

2. Thanks to the conducted examinations, the domestic food acid casein has been

used the first time for the manufacture of nutritive media (TU 153-54).

3. It was established that deviations from the normal technologSTAT. regimen of the casein's production will have a substantial influence upon the quality of the bacterial preparations. Together with (p.236) this, in case of identical indices of the quality of the casein of the stipulated standard, the titres of the toxins and the yields of microbic bodies do not seem to be identical.

4. The casein which completely satisfies the requirements for the manufacture of bacterial products may be obtained under the condition of elaboration of the separate details of the technical regimen of the production of the food acid casein, furthermore under the condition of making the standard of casein for these purposes more accurate and of organizing a special department which is outfitted with modern equipment.

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RESULTS AND PERSPECTIVES OF THE ACTIVE IMMUNIZATION WITH COMBINED PREPARATIONS (p. 237-247)

* * *

One of the more important problems of modern immunology, of both its theoretical and practical branches, is the problem of active immunization with combined preparations. From the view point of theory, the basic ideas of the laws of antitoxic immunity are chiefly based upon the results of research obtained with native anatoxins (RAMON, GLENIN, ZDOROVSKII, VOSKRESENCKII, PONOMAREV, KHALYAPINA, ROZHKOVSKAYA, KLIMENTOVA). At the present time, a considerable number of facts is accumulating which point out the significant differences in the immunological processes that can be provoked by native and by purified sorbed preparations.

From a scientific practical point of view, the creation of the facility to immunize with a single combined preparation which contains a number of antigens acting simultaneously against a few diseases makes much easier the task of the valuable performance of massive prophylactic inoculations. In this manner, the staging of the problem itself has required the carrying out of important experimental researches, on the one hand for the purpose that a theoretical foundation should be created for the employment of the combined preparations, on the other hand with the goal that the advantages of the use of combined preparations should be practically realized.

Numerous data in the literature of the recent times about the pathogenesis and immunity in case of wound infections prove that the most effective and prospective trend in the fight with these infections is the solution of the problem of active immunization. If we consider that in the pathogenesis of the tetanus, of the gas gangrene and of the staphylococcic wound infections the leading factor is the intoxication by the corresponding toxins, then, of course, not only the road towards immunization and the immunogenic factor are determined, but also the end result of the immunization--the creation of a stable immunity.

(p. 238) During a number of years the collaborators of our Department have studied the various aspects of the problem of combined immunization against wound infections

(such as by tetanus, gas gangrene, staphylococcus, and others.)

At the present time, there is an ample opportunity to sum up some of the results of the scientific in the field of spedific prevention of infections which can be realized with the aid of immunization by using the preparations composed of a few components, which assures the creation of non-susceptibility at the same time to several infections.

Together with this it is appropriate to critically consider a number of problems which arise before us and before other investigators as a result of experimental works and which demand a solution in further researches.

The considerations which we presented are a short generalization of the results of scientific investigations which had been conducted by the collective of the Department of Wound Infections of the N. F. GAMALEI Institute of Epidemiology and Microbiology, and also by other collectives of our Institute.

These results have been the consequence of a harmonious work for the solution of the fundamental questions of the inoculational prevention of infections which questions were set in accordance with the contemporary theoretical ideas in the study of immunity and in accordance with the scientific practical interpretation of these concepts in the creation of new combined preparations which assure a non-susceptibility, first of all, to wound infections. The fundamental questions which were subjected to study have been:

FORM AND QUALITY OF THE ANTIGENS USED FOR ACTIVE IMMUNIZATION by means of combined preparations.

It is generally known that the native preparations--both anatoxins and also corpuscular vaccines--possess an ability to immunize against a number of corresponding infections. However, the native preparations have a series of deficiencies:

1) they contain different substances which do not have any immunoganic activity, i. e., which are ballasts (inert substances), since the nutrient media on which these inoculational preparations are produced are themselves in a great part a mixture of different substances that can be hardly defined chemically and that is unstable in respect to its chemical characteristics and composition; here it should be remembered that, in case of manufacturing the anatoxins on meat nutrient media, the separation of the fractions which are the most active in an immunological relationship is accompanied usually by great losses of antigen;

2) at immunization, many native preparations do not stimulate sufficiently strong immunity, inasmuch as a rather large (up to 15% of the inoculated) group remained refractory (p. 239...according to the pattern of the native diphtheria anatoxin). We agreed also with the results of the experimental researches which KASHINTSEVA carried out in reference to the native tetanus anatoxin in which experiments the group of the refractory people was also rather high (10.4% of the inoculated persons);

3) in reference to gas gangrene, the immunization with native preparations (anatoxins)--not speaking now of the anacultures--as the previous investigations of ZELEVINSKAYA (1935), PENFORD and TOLKHORS (1937), of KONSTANTINOVA (1944), of LOGAN and TAITL (1945), of WOTERS and MOLONI (1949 and of others had shown--does not create a satisfactory immunity mostly with respect to the Cl. perfringens. Even the production of the "dialyzed" toxins at the cultivation of the toxigenic stocks of the anaerobes in cellophane bags, as ventured by ZELEVINSKAYA, GIL'GUT, KASHINTSEVA, VLASOVA, has not solved the problems of immunization against wound infection by means of native preparations.

It is at present well known to us that the quality of the antigen which is used for the initial immunization has a rather important meaning for the ultimate immunological effect that can be reached under the compulsory condition of the employment of a remote revaccination, which had been shown by ZDRODOVSKII and his coworkers;

4) there is also no ground to prove that the employment of the native anatoxins is irreal for the creation (making) of combined preparations on account of the large volume of the injected (introduced) preparation.

In connection with what was pointed out the need arose to employ a number of methods which concern: 1) the purification of the native anatoxins from the ballast substances, and 2) the concentration of the produced antigens.

It should be remarked, however, that the highly purified anatoxins which can be used for immunization do not create sufficiently long immunizatory stimulations, and they possess a weak immunogenicity. This is in agreement with the results of the researches which were conducted by ALYMOVA, KRYZHANOVSKII and PEVNITSKI in relation to the immunizing effect of the perfringens anatoxins. As our investigations showed, the immunogenicity of the gangrene anatoxin is in direct ratio to

the complex of conditions, and not only to the high level of its purity, although the latter also has a very important meaning. STAT

The contemporary status of the studies of the antigens and the broad development of the immuno-chemical researches gives an opportunity to considerably strengthen the range of activity of the preventive preparations, by the employment of the purified and concentrated preparations simultaneously against several infections. The combined active immunization has a profound importance and it represents the only reliable method of the prevention of such multi-microbic infections as the gas gangrene.

The inclusion (p. 240) of the tetanus anatoxin and of others in the complex of the gangrene anatoxins makes it possible to obtain a preparation for the active prevention of wound infections. The serious course and the high mortality, which are characteristic for the wound infections, and also the defective effectiveness of the therapeutic sera justify the demand for the creation of a combined preventive preparation.

The problem of combined preparations opens an effectively new stage in the field of practical immunology. It guides to the need of producing new, more modern and more effective antigens.

The production of immunogenic combined preparations for the immunization against wound infections must proceed simultaneously along the following four directions and it must be connected with: 1) search for new nutrient media which are the simplest and the best for toxin production, and the obtaining of the toxins; 2) study of the procedure of making the toxins harmless which were produced on the above indicated nutrient media, for the purpose of producing the least denaturated antigens (anatoxins); 3) elaboration of the methods of purification and concentration of the toxins and the anatoxins which methods would allow that, with a small volume of the preparation the maximum immunological effect is achieved as a result of the active immunization; 4) study of the process of sorption of the anatoxins for the purpose of reinforcing their antigenicity and immunogenicity.

The investigations which had been conducted in close harmony with the Departments of Biochemistry (V. A. BLAGOVESHCHENSKII) and of Nutrient Media (I. N. VINOGRADOVA) for the search of the simplest (non-meat) culture media, allowed us to remain with the nutrient media which in their basis have the hydrolyzate of casein.

As a result of the utilization of less complicated (non-meat) culture media, the produced toxins of the agents of gas gangrene, of tetanus and of the STAPhylococcus are sufficiently strong, capable for a rapid detoxication and for a transition into anatoxins, which is connected with less denaturation of the produced antigens. For the production of the purified and concentrated anatoxins, all existing methods of precipitation and of the purification of the antigens have been investigated (VOLGIN, AKATOV, DAVYDOV, and coworkers of the Biochemical Department). At the final score, for the production of the purified and concentrated anatoxins, V. A. BLAGOVESCHEN-SKII's suggested methods have been used which are based upon the initial precipitation of the antigens at the isoelectric point with hydrochloric acid, with a compulsory subsequent purification by acetone or alcohol at low temperatures.

Methods of purification, concentration and sorption of the anatoxins have been worked out which permitted to make preparations of high degree of purity, containing a sufficient immunogenicity in a small volume. (p. 241) In this way, as a result of the investigations which had been conducted during a number of years by the co-workers of the Gamalei Institute of the Academy of Medical Sciences, U. S. S. R., methods have been worked out for the preparation, and the immunological effectiveness of a number of preparations was studied for active immunization--of complex anatoxins and of vaccines of different composition, such as, for instance, the trianatoxin, tetraanatoxin, and others which are composed of the anatoxins of tetanus and gangrene in different combinations.

ZELEVINSKAYA, VOLKOVA, KONSTANTINOVA, BULATOVA, 1944-1950; VYGODCHIKOV, VOLKOVA, ZELEVINSKAYA, VINOGRADOVA, GIL'GUT, KASHINTSEVA, LARINA, VLASOVA, 1954-1956, had been working on methods for the manufacture of the poly-synthetic culture media, and they showed the possibility of producing on these media active tetanus and gangrene anatoxins.

By the researches of BLAGOVESHCHENSKII and collaborators, in 1954-1956, methods were worked out for the purification, concentration and sorption of the different anatoxins, and, together with ZELEVINSKAYA, VOLKOVA, LARINA and others, preparations were manufactured which are highly active in immunological respect. In subsequent investigations, the high immunological effectiveness of the manufactured preparations was demonstrated in experiments on animals and at the immunization of people.

As a result of these researches, the suggested combined preventive preparations

have been also studied against tetanus, gas gangrene and against other wound infections:- the trianatoxin, tetra-anatoxin, the Preparation SPES, the penta-anatoxin, and others.

The results of the studies of the antigenicity and immunogenicity of the indicated preparations were repeatedly supplied which permits me to omit the details. It is necessary, however, to underscore that the immunological effect in reference to each component which goes into the composition of the combined preparation does not noticeably differ from the effect which can be obtained at the immunization with the same components when taken in the form of single antigens.

At the present time the following results have been reached:

1) Completed are the investigations on the tolerance and the immunological effectiveness of the purified sorbed tetanus anatoxin. They revealed the high effectiveness of the preparation which was introduced into the practice of the Public Health Service.

2) Suggestion was made about the purified sorbed preparation for the prevention of wound infections--, the trianatoxin, which possesses andimmunological effectiveness on experimental animals and for the human contingents (Dissertation of KONDRAT'EV, works of PONOMAREV, BRYZGALOVA, and others).

A continued instruction is maintained about the manufacture and the control of this preparation.

(p. 242) Finished are the investigations about the tetra-anatoxin, and its immunological effectiveness has been investigated. A temporary instruction is kept on about the manufacture and control of this preparation.

3) A method was elaborated for the production, and a purified sorbed penta-anatoxin was created which is composed of the trianatoxin and the Type A and B botulinus components.

Investigation was done on its immunological effectiveness in experiments, and proofs were produced about the absence of an immunological concurrence (competition!) of the antigens in the taken doses. The tolerance and the immunological effectiveness for human beings will be studied in the joint work of the Gamalei Institute of Epedemiology and Microbiology and of the No. 1 Laboratory of the Kirov Military Medical Academy (Director: I. I. DAL'BERG) in 1959.

4) A study was conducted of the preparation which is composed of the penta-

anatoxin in combination with chemical vaccines made from the antigens of the microbes of the intestinal group (polyantigen, Institute of Epidemiology and Microbiology), which were produced with the aid of the water-phenol method. The immunological effectiveness of the preparation was experimentally established, and no immunological concurrence was found between its components.

5) In the year 1959 the experimental study of the tetrapolyvaccine of the Gamalei Institute is planned which is composed of these components: typhoid O and Vi, paratyphoid B, cholera and tetanus. In 1958 a study was made of the immunological effectiveness of the polyantigen of the Gamalei Institute on human beings, which polyantigen consists of the antigens of typhoid O and Vi, of paratyphoid B and dysentery (Flexner and Sonne). By a comparison of this polyantigen with the N I I S I polyvaccine, the study gave the following results: in respect to tolerance, the Gamalei Institute's polyvaccine is 2-3 times less reactogenic than the N I I S I polyvaccine.

The Gamalei Institute's polyvaccine repeatedly caused a lowering of the morbidity from typhoid fever in 6-8 times. In the blood of the inoculated, sufficient amount of the tetanus anatoxin was detected (0.1-3.0).

6) Experimental preparations were produced for the prevention of childhood infections: -a) against diphtheria and tetanus; instruction is continued for the preparation and control of this product; b) against diphtheria, tetanus and pertussis.

The immunological and epidemiological study of this product is conducted on human contingents.

7) A start was made in the research for making a product against wound infections and botulism. The preparation consists of the trianatoxin and the botulinus components of Types A. B. C. E.

At the present time, a method is being worked out for the purification and concentration of the botulinus components of Types C and E, and work is progressing for the perfection of the methods of purification and concentration of the botulinus anatoxins of Types A and B.

(p. 243) A rather important question for the construction of combined preparations that are valuable in immunological respect is the question of the quality and the ratio of the antigens in connection with the appearances of an immunological concurrence among them. At the present time many observations heaped up which show that,

in case of a simultaneous immunization with a mixture of antigens, the immunological effectiveness of one or of several of them may be lowered. This observation has already long ago attracted the attention of the investigators, and at the present time it can be considered proved that, under definite conditions, as a result of the "immunological concurrence of the antigens", considerable decrease happens to occur in the immunogenic properties of the separate components of the combined preparations (MICHAELIS, 1904; BARR & LLEWELLYN-JONES, 1953 - 1956; ZDROVSKII, 1950; VOSKRESENSKII & LEBEDEVA, 1956).

The amount of the component also has a significance, which in its turn depends upon the quality of the initial anatoxin.

Thus, for instance, three components go into the composition of the trianatoxin:- that of tetanus, of edematiens, and of perfringens--, which may be characterized as "strong ones" the first two, and as a "weak" antigen the third.

In conformity with this, at the start of the work with the trianatoxin, even the volume ratios in one ml of the preparation have been correspondingly 1:1:8. It is natural that, with such ratios, the creation of multivalent preparations, without the increase of the total volume, had been an exceedingly difficult job. We wish to mention the great importance of the quality, of the volume and of the correct ratio of the components in connection with the circumstance that lately several investigators have formed the opinion that the quantity of the components in the inoculational preparations may be enlarged, without any thought about their immunological characteristics.

It is entirely natural that, in the mentioned aspect, decisive role is played by the optimum ratios between antigens that can be determined by large number of experiments, which was also done by us previously before recommending the above indicated tri-, tetra- and penta-anatoxins. In the ratio of antigens which we suggested in the trianatoxin, in which the perfringens component includes 25 B. U., the edematiens component 40 B. U., the tetanus component 200 B. U. in one ml, we have not observed the appearance of an immunological concurrence of the antigens. No such appearance of immunological concurrence was observed at the immunization with tetra-anatoxin either, with a ratio of the components as follows:- perfringens 25 B. U., edematiens 40 B. U., septic vibrio 15 B. U., and tetanus 200 B. U. in one ml. However, in the experiments of B. B. VOSKRESENSKII at immunization with the SPES

preparation, the staphylococcus component which went into the combination was inhibited, and a considerable increase in the dose of this component was STAT essary. (p. 244) About the question of the effectiveness of the doses of the perfringens antigen in the combined products we have contradictory findings. The findings of SHNEERSON and KRESTOVNIKOVA demonstrate the inhibition of the effectiveness of the perfringens component in the complex preparations.

CHERKAS and his coworkers have established that the perfringens antigen, when taken in large doses, provokes an inhibition of the production of antibodies by all the other antigens. Our works proved the effectiveness of the perfringens antigen, with 3.5 and 7 antigens, if it is taken in doses not lower than 25 B. U. in one ml.

I. A. LARINA showed that the increase of the dose of the perfringens antigen in the trianatoxin up to 80 B. U. per one ml did not result in a substantial increase in the titres of the antitoxin at the trials of immunization and revaccination, in comparison with lower doses of this antigen (25 and 50 B. U. in one ml), and it did not have any effect upon the immunological effectiveness of the other components of the trianatoxin (tetanus and edematiens).

Interesting are the experiments for the establishment of optimum and average effective doses of the antigen, which is important for the construction of combined preparations. The results of these observations may be formulated in the following shape.

As the optimum dose of a given type of antigen, in case of the immunization of a given type of experimental animal, must be considered that dose after which, if surpassed, the intensification of the immunological effect would cease (the level of antitoxin in the blood, or the level of resistance in respect to the corresponding toxin), i. e., it is the smallest amount of the doses which will give the maximum immunological effect.

By the works of JERNE and MAALE, FRIGGE, LEVINE and STONE, HOLT and BURNES, and AKATOV it was proved that the proportion between the dose of the anatoxin and the obtained immunological effect is expressed by a curve which demonstrates the dependence "dose-effect" and which first is proceeding under this or that angle to the axis of the Abscissa, and then it assumes the character of an asymptote, i. e., a line which infinitely is approaching the horizontal, thus symbolizing the cessation of the growth of the effect with the further increase of the antigen's dosage.

As AKATOV demonstrated, in the combined preparations these optimum doses of two antigens (tetanus and edematiens) will give a worse immunologic[STAT] effect than in case of separate immunizations with the optimum doses of these antigens. In this case, evidently, a mutual inhibitory influence of these dosages takes place upon the production of the corresponding antibodies.

In case of the combination of the doses of antigens in the combined preparation, which antigens gave an average immunological effect in case of separate immunization with them (average effective doses), (p. 245) the accumulation of the level of the corresponding antitoxins will reach the same level ~~xxxxx~~ as with the employment of the optimum doses of both antigens.

Consequently, in the given case, no mutually supplemental actions can be observed with these doses upon the production of the corresponding antibodies. In this way, it is appropriate and logical not to put the optimum doses, but only the average effective doses, of the antigens into the composition of the combined preparation.

The method of active immunization with the combined preparations has a great significance for the final immunological effect. The experimental study of the significance of the original (initial) immunization orients us to the reinforcement of the initial immunization which, under the conditions of revaccination, will determine the final immunological effect. This reinforcement may be realized first of all with the use of the purified, sorbed preparations which, by their immunogenicity, considerably surpass the native preparations. However, even in this case, a two-shot initial immunization, with the stipulation of a delayed revaccination, has assured a considerably greater immunological effect than a single-shot initial immunization.

In our combinations (associations), antigens which are different in immunological respect are combined. By studying finally the importance of the use of the method of the single-shot initial immunization with a delayed revaccination, we are conducting investigations both as to the improvement of the quality of the native antigens and as to the perfecting of the methods of purification, concentration, and sorption. On the problem of combined immunization, a great and beneficial work was unfolded in our Institute. However, it cannot be accomplished by the efforts of one department or of one laboratory, and it will be solved by joint efforts.

Actually, in a relatively short time, the problem has advanced both in the

theoretical and in the practical field. The question is now not only about a combined preparation which consists of three components; works were unSTALled about combined vaccines which consists of antigens of microbes of the intestinal group and of the wound infections. The pediatric combined vaccines begin to be shaped.

And above all, as a result of the theoretical and scientific practical researches, such a scientific build-up was created which permits us to get results, such results of which we could not even day-dream a few years ago. Before, there were still many unsolved and difficult questions, but we are profoundly convinced that the elaboration of the problems of active immunization with the combined preparations is on the right way and it will bring great advantage to the theory and practice of the Soviet Public Health Service.

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