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The Formation of Specific 7 S and Macroglobulin Type Antibodies in Chickens

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The heterogeneity of antibodies has now been demonstrated in different characteristics of the antibody molecule, i.e. in serological properties, physico-chemical character and in the antigenic structure /6/. Considerable data have been collected on the formation of different types of antibodies. For example, the immunization of rabbits with foreign erythrocytes led to the formation of antibodies which were mainly of the macroglobulin type at the beginning of immunization, while after repeated immunization a large proportion of antibodies was formed with an electrophoretic mobility of $gamma_2$ and a sedimentation constant of 7 S /15, 14, 8/. This time course in the formation of antibodies of the $gamma_{1M}$ and $gamma_2$ type was demonstrated in further types of corpuscular and soluble protein antigens /2/.

These facts, pointing to the interrelationship between the formation of macroglobulin and 7 S antibodies, were further confirmed by the finding that both types of antibody are also formed together in the lower vertebrates /16, 17/ and bytthe fact that a similar time course in the formation of the two types of antibodies was found in the period of immunological immaturity /13, 11/.

Little is yet known of the serological properties of different types of antibodies. It has been shown that macroglobulin anti-

than 7 S antibodies /14, 8/. Macroglobulin antibodies are also essentially more effective in the haemagglutination reaction in which 700 times less macroglobulin than 7 S antibodies are necessary to produce the same effect /9/. These results, however, touch only a small part of antibody activity and it would be most important to carry out a comparison and different serological manifestations on reaction with antigen.

We have attempted to make a comparison of the activity and specificity of antibodies of the macroglobulin and 7 S type. Chickens were used as the source of antibodies since they are very good producers of antibodies to soluble protein antigens and produce macroglobulin antibodies in relatively high amounts /1, 4/. In addition antibody formation in chicken is very rapid, a high level appearing even after primary immunization.

A group of chickens /Leghorn, weight 1.5 kg/ were immunized with one dose of 50 mg. of human serum albumin p-azobenzoic acid /p-ABA-HSA /9/.

Antibodies to protein carrier determined by the haemagglutination of HSA sensitized erythrocytes appeared in all chickens as soon as on the third day after immunization, whereas antibodies to hapten, also determined by haemagglutination, did not appear until the seventh day after immunization and then only in low titres similarly as described by Gold and Benedict /7/. The birds were exsanguinated on the seventh day and the types of antibodies present in the serum determined by ultracentrifugation in a sucrose gradient and by haemagglutination of the separate fractions. In accord with the data in the literature /1, 4/ antibodies to HSA were of both the macroglobulin and 7 S type. On the other hand, antibodies to hapten were exclusively of the macroglobulin type /Fig.1/.

immunization, antihapten antibodies were of the 19 S type, in a further experiment we attempted to determine whether antibodies of two physico-chemical types can be formed to one determinant group, i.e. haptenic group bound to the protein molecule. We therefore investigated whether antibodies of the 7 S type were not also formed against hapten on repeated immunization. A further group of chickens was immunized repeatedly with 40 mg. of p-ABA-HSA given intravenously at weekly intervals. Blood was collected on the seventh day after immunization before giving the next immunization dose. As evident from Fig.2, antibodies to hapten in the first two collections were of the macroglobulin type and after the third immunization dose antihapten antibodies of the 7 S type appeared, so that after further immunization antibodies of both types were present in the serum. These antibodies to hapten were demonstrated by haemagglutination of p-ABA, bound by azo-linkage to erythrocytes. In this system, in addition to the actual hapten, the amino acids, with which diazotized p-ABA reacted, could form part of the determinant group, whereas the other carriers, i.e. HSA on immunization and erythrocytes on detection, are quite different. This denotes that both types of antibodies, macroglobulin and 7 S, are formed against the same determinant group and that hapten alone or possibly a hapten-azo-amino acid residue /12/ are sufficient in both cases to produce a positive reaction. Bauer /3/ demonstrated 19 S and 7 S antibodies to the same hapten in rabbits, similarly.

That the two types of antibodies have the same specificity does not, of course, denote that both must have the same immunochemical properties, i.e. an equally large combining site, the same space configuration of the combining site and therefore an equally

firm binding with antigen. Unfortunately, we could not used antihapten antibodies to obtain more details about macroglobulin combining site because these antibodies occured in too small amounts in the serum and attempts to concentrate them were unsuccessful. In further experiments, therefore, we used antibodies to BSA which were formed after immunization in sufficient amounts for serological study.

The anti-BSA antibodies were separated into macroglobulin and 7 S types by filtration on Sephadex G-200. Antibodies from the corresponding fractions were concentrated by precipitating the globulins with 30% saturated sodium sulphate, and dissolved in a small volume of saline. The sodium sulphate was removed by filtration on Sephadex G 25.

We worked with pooled sera from three groups of chickens, i.e. taken after primary immunization, after secondary immunization and finally with hyperimmune serum. The birds were immunized intravenously with 40 mg BSA at monthly intervals, hyperimmune serum was collected after the fifth immunization.

In accord with the data in the literature, in all three groups we found haemagglutinating antibodies in both the macroglobulin and 7 S fractions. Fig.3 shows the eluates of the primary and hyperimmune sera from the Sephadex column and haemagglutinating titres of the separate fractions. It is evident that in the primary sera most haemagglutinating activity was present in the fraction containing macroglubulin and in the fraction containing antibodies of the 7 S type anti-BSA antibodies were demonstrated only after concentration with sodium sulphate. In hyperimmune sera haemagglutinating antibodies were present in both peaks. A most interesting fact was discovered on determining the presence of cross-reacting antibodies. If a comparison is made of the titres of haemagglu-

tination of erythrocytes sensitized to BSA and cross-reacting with HSA in the separate fractions of the eluate, it can be seen that antibodies cross-reacting with HSA are mainly present in the fractions containing the macroglobulin antibodies, whereas in the 7 S fraction their titre is incomparably lower as compared with that of anti-BSA antibodies.

Antibodies were further determined by quantitative precipitation. In concentrated fractions of antibodies of 7 S or macroglobulin type the precipitin reaction was made in 0.15 M NaCl and in 1.5 M NaCl to determine both types of antibodies /4/. In concentrated antibodies of the macroglobulin type we were not successful in demonstrating the presence of detectable amounts of precipitating antibodies in any of the pooled sera tested. However, in the case of macroglobulin antibodies the sensitivity of the reaction was greatly decreased since these antibodies precipitate spontaneously so that the control values were so great that they did not permit the determination of amounts of antibody of less than 20-30 /ug Ab/ml.

The precipitation of antibodies of the 7 S type was positive in 1.5 M NaCl in all three pools of sera investigated. In 0.15 M NaCl the reaction was positive after the secondary immunization and in hyperimmune serum, but not in the primary serum. The amount of antibodies precipitating in 0.15 M NaCl or in 1.5 M NaCl varied so that the relatively highest amount of antibodies precipitating BSA at 0,15 M NaCl in the mixture of hyperimmune sera similarly as was described by Benedict et al. /4/. This also explains the increase in haemagglutinating antibodies in fractions corresponding to antibodies of the 7 S type during immunization, since only antibodies precipitating in 0.15 M NaCl

/4/ are effective in haemagglutination.

A comparison of the amount of type 7 S antibody precipitating in 0.15 M NaCl with haemagglutination titre of that fraction showed that the sensitivity of the haemagglutination reaction is within the usual range of sensitivity for passive haemagglutination. If we compare the high haemagglutination titres of antibodies of the macroglobulin type with the negative results of quantitative precipitation /what means that the amount of these antibodies was less than 30 /ug Ab/ml/, it is evident that the macroglobulin antibodies in chickens are much more active in haemagglutination than the 7 S type antibodies. Evidently in passive haemagglutination chicken macroglobulin antibodies react similarly as rabbit antierythrocyte γ_{1M} antibodies, where Greenbury et al. /8/ found that for producing the same haematolytic effect an incomparably smaller amount of macroglobulin antibodies is neededthan in haemagglutination with 7 S antibodies.

We next attemted to determine the specificity and binding power of haemagglutinating antibodies of the two types. For this purpose we used cross reactions with HSA and inhibition of haemagglutination. Inhibition was studied by adding 5 /ug of BSA or HSA to each tube of serial dilution of the sera tested and by comparing the result of haemagglutination with that of the control.

Fig.4 gives a comparison of the reactions of all three test sera. It is evident that in cross reactions the antibodies of the two types differ considerably. Antibodies of the anti-BSA macroglobulin type give cross reactions with HSA sensitized erythrocytes to a high degree in all three cases. In the case of antibodies of the 7 S type cross reactions were essentially less, i.e. these antibodies showed a far higher specificity to BSA. Similar differences were displayed in the haemagglutination in-

hibition reaction. Macroglobulin antibodies were only inhibited by homologous antigen to a small extent, whereas the reaction of the 7 S type antibodies was almost completely inhibited by homologous antigen. On comparing the results of cross reactions of macroglobulin antibodies from the samples tested no essential differences could be found; 7 S type haemagglutinating antibodies on the other hand cross react in hyperimmune and secondary sera less than do antibodies in the primary sera.

The high degree of cross reactivity of macroglobulin antibodies can be explained in two ways. First, by a different mechanism of formation of these antibodies and thereby a different degree of response to separate determinant groups of antigen used for immunization, which could lead to a higher formation of antibodies to determinant groups common for related antigens. The second possibility explanation is that the combining site of macroglobulin antibodies is less complementary to the determinant group of antigen which would permit a higher degree of cross reactivity. The lower degree of complementarity could be due to the combining site being smaller in macroglobulin antibodies than in 7 S antibodies or to its being less exactly delimited in space /less rigidity of the structure of the site of linkage/. If the second explanation of the high cross reactivity of macroglobulin sera, i.e. difference in the combining site of antibody were true, this property would necessarily manifest itself in greater dissociation of the antigen-antibody complex. This corresponds to the results of the inhibition reactions which show that the system macroglobulin antibody-BSA-erythrocyte is only very little inhibited in the presence of free antigen which is evidently due to the high dissociation of the antigen-antibody complex.

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Summarizing our results we can say that macroglobulin antibodies have different properties from 7 S antibodies. Both types of antibodies can arise to the same determinant group but the specificity and firmness of the binding of macroglobulin antibodies is lower than that of 7 S antibodies.

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S-sulphonated Anti-dinitrophenyl Antibodies. Some Specific Features of the Interaction between Isolated H and L Subunits.

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The existence of an active site on the protein molecule composed of several types of subunits gives rise to the question as to which type is the carrier of the active site and whether it is one type or whether more types participate. If we consider antibodies, which are known to made up of two types of subunits, H and L, it is clear that there are three basic possible answers to this question. The carrier of the active can be subunit H, subunit L or both of these subunits can participate in its formation. These basic alternatives comprise within themselves further cases expressing the possibility of the interaction of several subunits of the same type or the differentiation of the role of carrier of specificity and activator. It is not difficult to consider these possibilities and to discus. their consequences, but such considerations do not of themselves lead to any advance in the solving of the problem: no possibility can a priory be designated as impossible. In 1961, on the basis of the electrophoretic analysis of different guinea pig antibodies, Edelman et al. /1/ expressed the opinion that antibody specificity is given by the L chain. This opinion, still not supported experimentally by testing activity, was maintained by Edelman and Benacerraf in a further publication /2/ in 1962, in which other possibilities are discussed, e.g.

the interaction of two different or similar L chains. Before this in 1962 Porter /3/ has already published experimental results placing the antibody active site into the H chain. Our work published at the beginning of 1963 /4 - 7/, however, pointed to a different conclusion. No appreciable activity was found in any of the isolated chains but reappeared after mixing them. On mixing H and L chains, the activity of antibodies of different specificities was also covered but only partically and the hybrid possessed a specificity corresponding to the H chain used. From these results we reached the conclusion that the participation of both H and L chains is needed for the formation of the active site and that specificity is determined by the H chain, whereas the L chains may be partly replaced in their function by the L chain from another antibody. A very important finding was that isolated H and L chains are capable of mutual interaction even without the formation of covalent bonds. The model of equine antitoxin used, however, was not ideal from all aspects. Although the recovered activity was equal to the activity of the preparation which was subjected to the same treatment without separation of the chains, this activity was very low, maximally 5% of the protein in the sample was active. Apart from that the determination of activity by means of an immunosorbent did not permit the extension of the study to the measuring of the physico-chemical parameters of the interaction.

For the further study of this problem we chose antibodies to the dinitrophenyl group, whose preparation holds out the possibility of a high yield with a relatively narrow specificity. Big animals-bulls and pigs - were chosen as immunization objects,

for a large series of experiments and partly because it permits working with antibodies from one individual so that some aspects of heterogenicity can be excluded. The immunizing antigen was gamma-globulin, modified to a high degree by 2,4-dinitrobenzene sulphonic acid /8/, was given in adjuvant. Bulls were immunized with pig gammá-globulin, pigs with bovine gamma-globulin. In pigs a single immunization with 100 mg. antigen 2 - 3 weeks before slaughter proved satisfactory. The concentration of antibodies obtained was 0.1 - 1.0 mg./ml. In bulls an antibody concentration of 0.1 - 1.0 mg./ml. was attained two weeks after one dose of 200 mg. antigen per animal. Repeated doses of antigen in adjuvant always produced approximately the same increase in the concentration of antibodies so that blood could be collected after each reimmunization containing antibodies in concentrations which were suitable for isolation.

We considerably modified the method used by Farah et al. /9/ for the isolation of antibodies. After absorbing the antibody to the carrier protein, antibodies to the hapten were precipitated by dinitrophenylated gamma globulin and the washed precipitate was dissolved with 0.05 M dinitrophenol in 0.1 M phosphate buffer, pH 7, containing 0.5 NaCl. The solution containing antibody, antigen and hapten was applied on a DEAE-Sephadex column equilibrated with the same buffer without dinitrophenol. Antigen was adsorbed firmly on the top of the column whereas antibody ran through without adsorption and was thus separated from the dinitrophenol whose zone moved down the column very slowly. The antibody yield on the DEAE-Sephadex reached up to 75% of antibodies determined in the serum by the quantitative immunosorbent method. In other cases the precipitate was dissolved with the buffer which contained

1×10^{-4} M ε-DNP-lysine. Antibody was separated from hapten and antigen in the same way as from dinitrophenol. However, it was only possible to remove excess hapten, a portion of the ε-DNP-lysine remained bound to the antibody.

The chemical character of bovine antibodies is 7 S gamma-globulin as determined by starch gel electrophoresis and by immunoelectrophoresis. At the same time the extent of electrophoretic heterogeneity is less than that of 7 S gamma-globulin. It displays no indication that the antibody could be present in two types, as was reported, for example, in guinea pigs /10/. The average association constant of the interaction of bovine antibodies with ε-DNP-lysine was within the range of 10^5 to 10^7 litre/mols in various preparations. It was determined by equilibrium dialysis and by polarography. The polarographic method of determining the association constants of antibody to the dinitrophenyl group was found very useful; ε-DNP-lysine gives a well measureable polarographic double-wave. By adding gelatine to the analysed mixture the height of the ε-DNP-lysine wave is made directly proportional to the concentration of free ε-DNP-lysine bound ε-DNP-lysine being not registered polarographically. The great advantage of the polarographic method is that it gives results in the course of a few minutes.

The breaking down of the disulphide bond was done by S-sulphonation. On the basis of previous work on the reactivity of the disulphide bonds /11/ and on the nature of the intermediates of limited cleavage of disulphide bonds /12/ we worked at a pH 8.6, i.e. under conditions in which 8 disulphide bonds in the molecule are cleaved in pig gamma globulin. As the second alternative we selected S-sulphonation at pH 5.7, at which seven

subunits - intermediates of the reaction -, which contain both H and L chains /12/, remain in the reaction product. The electrophoretic pattern on starch gel shows that in pig antibodies there is no difference in the position of the zones or in the relative amounts between nonspecific gamma-globulin and antibodies. In bovine antibodies the mobility of the two types of chains is somewhat less than in nonspecific gamma-globulin, the width of the diffusion zone of the L chains is smaller and in addition to this there is more incompletely split material in the resulting preparations /Fig.1/. S-sulphonation was also carried out in the presence of excess of dinitrophenol or ε-DNP-Lysin. As seen from Fig.2 the hapten bound on the antibody does not affect the splitting of interchain disulphide bonds. The electrophoretic pattern of subunits is the same as after S-sulphonation in the absence of hapten. Fig.3 confirms that neither on S-sulphonation at pH 5.7 is the character of antibody subunits significantly different from the character of subunits of nonspecific gamma-globulin.

None of the electrophoretic patterns obtained in urea starch gel at pH 3 - 4 showed banding of the L chain S-sulpho antibodies which were described by Edelman et al. /1/ in pure guinea pig antibodies. Banding did not appear in bovine or pig antibodies even when they had been subjected to reduction and alkylation, exactly according to the procedure described by Edelman and Poulik /13/. The large amount of antibody from single bulls made it possible to examine the heterogenity of L chains coming from different individuals by electrophoresis in starch gel at pH 8 /14/, where sharp bands actually appear. Fig.4 shows that L chains of antibodies yield a smaller number of bands and that there is hardly any difference among individuals.

As in the previous work Sephadex G-100 in 0.05 M formic acid with 6M urea was used for the separation of subunits, Fig 6 shows the distribution of protein after the separation of bovine S-sulpho gamma-globulin and antibody on a Sephadex column. The peak of higher subunits appears first overlapping partly with the peak of the H chains, which is followed by a well resolved peak of L chains. Under the conditions of separation any bound hapten was completely released from the protein and moved as a peak very distant from the L chains. The fractions containing subunits which were taken into the experiment were pooled and concentrated with dry Sephadex G-25. The subunits were then transferred into 0.1 M borate buffer pH 8.0 using a Sephadex G-25 column. All subunits were soluble in this buffer.

Binding activity was expressed by the quantity r denoting the number of moles of hapten bound by a mole of protein, and for comparison, in cases not stated otherwise, the molecular weight of protein was taken to be 160,000; i.e. even in the case of isolated H or L chains which actually have a lower molecular weight. r was always measured at a concentration of free ε-DNP-lysine of 1×10^{-5} M and at a temperature of +4°C. The binding capacity of nonspecific bovine gamma globulin and its subunits corresponded under the given conditions to r = 0.1. The omission of nonspecific binding capacity on the basis of weight was found to be most suitable since the binding capacity of the L chains or from that of their mixture, the nonspecific binding of ε-DNP-lysine on bovine gamma globulin antibody and its subunits was therefore eliminated from the results of measurements by deducting the [illegible]

units expressed as r.

We first determined the value of r for native antibody and for S-sulphonated antibody without separation of the chains. Table 1 shows that S-sulphonated antibody which was subjected to the action of the separation medium has its activity, expressed as r, decreased to about one half of that of its original activity. In further work we investigated whether there was any residual binding activity in the individual subunits arising from S-sulphonation at pH 8.6 and 5.7. Higher subunits which do not differentiate one from another on the Sephadex column were taken together for analysis. In Fig.6 the r of the individual subunits is denoted over the corresponding fractions. They represent the average of at least three experiments and are corrected for nonspecific adsorption. Among the subunits of S-sulphonated antibodies, it is only the higher subunits whose binding activity is preserved after separation in considerable degree. Free H and L chains taken without mutual contamination have no significant binding activity demonstrable by equilibrium dialysis.

In several pilot tests we confirmed that activity was covered by mixing H and L chains, in the same way as in equine antitoxin where it was first observed by us. We therefore undertook a study on the conditions and quantitative relationship of the recovery of antibody activity. We sought the conditions promoting the recombination of the subunits. We have found that the final activity was not influenced by whether H and L chains were mixed in acid separating solution, after neutralization or even after transferring the separate chains into borate buffer. Recombination of the chains thus occurs in a neutral medium in

the absence of urea and the history of the subunits before this time has no significant effect on recombination.

The average of 10 experiments showed that the recovered activity corresponded after deducting nonspecific adsorption to r = 0.68. If we wish to know the yield of recombination of chains we must compare the recovered activity with the activity of S-sulpho antibodies which were not subjected to separated chains.

Table 1 shows that preparation corresponding to this requirement possesses r = 0.86. The recovery of binding activity after separation and recombination of the chains was thus almost 80%. If, however, we wish to compare the activity of recombined complex with the activity of native antibody, the recovery of activity is about 40%.

We mixed H and L chains in different weight ratios keeping the total weight concentration constant. If the chains recombined in stoichiometric relationship and united with a bond whose firmness was comparable with the covalent bond with which they were bound in the native molecule, the activity found in our experimental set-up would follow the broken curve shown in the upper part of Fig.7 with a sharp maximum corresponding to the composition by weight of H and L chains. In actual fact, the activity of the mixture was different as shown in the lower part of the Fig.7. The activity has no sharp maximum but is almost constant in a wide range of ratios of H : L. We shall attempt to give an explanation of this parabolic shape of the curve on Fig.7 when explaining other findings.

In our next experiments we wanted to elucidate the role of hapten in the formation of complexes of H and L chains, i.e. the determine whether the active complex of chains is formed ub

in borate buffer in absence of hapten or only after its addition. It is obvious that equilibrium dialysis cannot provide an answer to this question. The measurement of increase in the binding of hapten by dialysis stopped after given time intervals gave results which were not reproducible although we tried to standadize all procedures. Polarography proved much more suitable here, because instantaneous states of decrease of free hapten can be followed. We have investigated the course of the decrease of free hapten which was added in a concentration of 1.4×10^{-5}M on the one hand to the native antibody, on the other hand to the mixture of H and L chains in borate buffer prepared fresh and to a mixture which had been left to stand for 20 hours. We confirmed that native antibody binds hapten so quickly that at the moment of recording the first data, i.e. several minutes after the addition of hapten, equilibrium has already been reached and the height of the wave of free hapten shows no further change. The curve on Fig.8 shows that ε-DNP-lysine passes into bound form very slowly with recombined complexes of H and L chains. In our conditions it took at least 50 hours. Hapten reacts with a greater initial velocity with preparations in which the chains were mixed and incubated for a long period before the addition of hapten. We assume that this finding suggests that H and L complex with a specific active site is formed in the mixture of chains before the addition of hapten.

The determination of specificity can also be studied on antihapten antibodies. We combined chains from bovine antibody on the one hand with chains of nonspecific bovine gamma-globulin, on the other hand with chains of pig antibody to the same determinant group. No hybrid of bovine and pig antibodies, i.e. neither

the mixture of bovine H and pig L, nor the mixture of pig H and bovine L, bound ε-DNP-lysine specifically. No aspect other than this binding was investigated so that we cannot say whether the chains recombined at all or whether possible recombined complex was inactive. The activity of hybrids of specific and nonspecific chains are given in Table 2. Hybrids of specific H chains with nonspecific L chains also attain a certain activity, even though lower than the complex with specific L chain, whereas in the hybrid of specific L chain with nonspecific H chain activity is not significantly increased.

In order to appreciate the role of the individual chains in determining specificity and in the formation of the active site, we elaborated an auxiliary concept, in which we considered alternately one chain as a sort of "inactive form" of the active site carrier and the second chain as its "activator". This concept empowers us to evaluate the activity by the quantities r_H and r_L which represent the number of moles of ε-DNP-lysine bound to a "mole" of chain considered as the inactive form. It is 107,000 g. for H and 53,000 g. for L chains in this ratio the mole of antibody, i.e. 160,000 g. is composed. In cases where H and L chains are mixed in the same proportions as present in the native molecule, the quantities r_H and r_L are equal to the quantity r calculated in relation to total protein.

The quantities r_H and r_L calculated for the data in Fig. 7 increase in direct proportion to the presence of excess of the component considered as the "activator" in the mixture. An adequate explanation for this finding is evidently the fact that the active complex of H and L chains cannot be compared in its firmness with native antibody since it shows considerable dis-

sociation. It follows from this, of course, that the quantity r is not suitable for expressing recovered activity since it will be dependent on the concentrations of protein components and that it will be necessary to choose a different way of evaluation in further experiments. A confirmation of this explanation can only be given when data on the binding of hapten on complex H and L chains in a wide range of protein concentrations will be available. The data from such an experiment would also finally decide how much of the decrease of activity found in S-sulpho antibodies or in complex of antibody chains in comparison with native molecule /see Table 1/ should be ascribed to irreversible inactivation and how much is due to the fact that in the case of the complex we are dealing with a multicomponent dissociating system. It will also be necessary to obtain experimental data on the basis of which a standpoint could be taken up towards the alternative explanation of the parabolic shape of the curve in Fig. 7, that various active complexes of H and L chains are formed in which the ratio of H : L is not in all cases the same as in the native molecule.

The different role of the two chains in determining specificity cannot be displayed examining the combination of chains originating from the same antibody. This question can only be decided by determining whether and to what degree some chain can be substituted by a chain from nonspecific gamma-globulin. The different role was best displayed where excess nonspecific chains were added /see Table 2/. These experiments show that the H chain, considered to be the "inactive form" can be activated by excess nonspecific L chains to a greater degree than by an adequate quantity of specific chains. The reverse conception, however, fails: L chain as the "inactive form" is not "activated" by nonspecific H chains. Increased activity could not be demonstra-

creation of a concept of H subunits as "inactive forms" and L chains as "ativator" for the illustration of the unequal importance of H and L chains in the active complex does not mean that we consider the synthesis of antibody to take place via an inactive form. The significance of H chains can be compared with the significance of inactive forms of other biologically active substances. The significance of the L chain is best understood by the term "activator", the effect of its addition can best be compared with the activation of enzymes by the addition of various co-enzymes, metal ions etc. It remains to explain why the effects of L chains of nonspecific gamma-globulin are not quantitatively identical with the effect of chains of antibody. L chains are undoubtedly heterogeneous. It appears that the extent of the heterogeneity of the L chains of antibody is less than that of the L chains of nonspecific gamma-globulin. Let us assume that the ability to "activate" specific H chains is a property of only certain L chains from a whole set which is found in nonspecific gamma-globulin or that this ability is not present in all L chains in the same degree. At the same time it need not be assumed that the ability to "activate" the H chain of a certain specificity is necessarily related to specificity to hapten. The "activating" effect of L chains of nonspecific gamma-globulin is therefore less than that of L chains of antibody. If added in excess, however, "activation" takes place to the same or to a higher degree.

Using the model of antibody to the dinitrophenyl group we succeeded in confirming all the findings which we were the first to establish on the model of equine antitoxins. We confirmed that isolated subunits H and L do not display any appreciable binding activity, and that residual binding activity is only attributable to

incompletely split antibodies. The fact that the binding activity is recovered by mixing H and L chains although restitution of the covalent bond between them did not occur, was convincingly confirmed. Finally, it was confirmed the H chain plays the decisive role in determining the specificity of the recombined complex.

The model working with two easily soluble high molecular components /H and L chains/ and a low molecular hapten made it possible to study interactions by physico-chemical methods. We found that the interaction of H and a L chains takes place in a neutral medium even without the presence of hapten and so slowly that the reaching of equilibrium is a question of days. Findings on the role of the chains in determining specificity were supplemented by a number of findings. L chains obtained from nonspecific gamma globulin can produce the same "activation" of the antibody H chain as L chain from antibody, if the nonspecific L chain is added in excess. On the other hand, the L chain from antibody cannot "activate" nonspecific H chains even when added in excess. Nor did we succeed in obtaining active hybrids from the chains of bull and pig antibodies even when the antibodies were directed against the same determinant group. The new data led us to the hypothesis of the mechanism of interaction of H chains carrying potential specific binding activity with heterogeneous mixtures of L chains from which only some provide an active complex. It would appear at the same time that this complex is considerably dissociated. Sufficient variants of the experiments have not yet been made to confirm this hypothesis but the results obtained to date show that S-sulpho subunits of antibody to the dinitrophenyl group are very suitable

for further study and we hope that we shall succeed in detecting a series of further properties of antibody subunits on this model.

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Legendes to Figures

Fig.1. Starch gel electrophoresis of bovine and pig antibody and gamma-globulins and their derivatives.
Composition of buffer: 0.05 M formic acid, 6 M urea. 1 - bovine gamma globulin, 2 - S-sulpho bovine anti-DNP antibody, 3 - S-sulpho bovine gamma globulin, 4 - S-sulpho pig gamma globulin, 5 - S-sulpho pig anti-DNP antibodies, 6 - pig gamma-globulin.

Fig.2. Starch gel electrophoresis of bovine gamma-globulin and anti-DNP antibodies S-sulphonated at pH 8.6.
Composition of buffer: 0.05 M formic acid, 6 M urea, 1 - gamma-globulin, 2 - antibody, 3 - antibody S-sulphonated in excess dinitrophenol, 4 - antibody S-sulphonated in excess ε-DNP-lysine.

Fig.3. Starch gel electrophoresis of bovine gamma-globulin and anti-DNP antibodies S-sulphonated at pH 5.7.
Composition of buffer: 0.05 M formic acid, 6 M urea. 1 - gamma-globulin, 2 - antibody, 3 - antibody S-sulphonated in excess dinitrophenol, 4 - antibody S-sulphonated in excess ε-DNP-lysine.

Fig.4. Starch gel electrophoresis of L chains of gamma-globulin and antibody. Composition of buffer: 0.035 M glycine buffer, pH 8.8, 8 M urea. 1 - chains of nonspecific bovine gamma globulin, 2, 3, 4 - chains of isolated anti-DNP antibody from three individual bulls.

Fig.5. Chromatography of S-sulpho bovine gamma globulin and anti-DNP antibody on Sephadex G-100.
Medium: 0.05 M formic acid, 6 M urea. Abscissa: volume of eluate

Ordinate: optical density at 2537 Å /registered by Uvicord LKB/. Crosshatched area denotes fractions pooled. Different subunits denoted by letters.

Fig.6. Activity of subunits of bovine anti-DNP antibody S-sulphonated at different pH.
Medium for separating subunits: 0.05 M formic acid, 6 urea. Abscissa: volume of eluate. Ordinate left: optical density at 2537 Å. Ordinate right: value of r. Upper part - Preparation S-sulphonated at pH 8.6, lower part - Preparation S-sulphonated at pH 5.7. Activity expressed by quantities of r are depicted as crosshatched columns and given in numerical values appended to the separate subunits.

Fig.7. Activity of different mixtures of H and L subunits of bovine anti-DNP antibody. Abscissa: percentage of subunit in mixture. Ordinate: activity expressed as quantity r. Upper part - hypothetical curves corresponding to ideal case of very firmly bound H and L chains in stoichiometric complex. Lower part - experimental curve.

Fig.8. Polarographic registration of reaction of ε-DNP-lysine with complex of H and L chains of bovine anti-DNP antibody. Abscissa: time interval from mixing of protein with hapten. Ordinate: concentration of free ε-DNP-lysine. Total amount of protein in experiment 2.5 mg. 1 - hapten added immediately after mixing H and L chains, 2 - hapten added 20 hours after mixing H and L chains.

Table 1

Activity of bovine anti-DNP antibodies and their derivatives

No.	Method of modification	r^+	Relative activity %
1	Native	1.77	100
2	S-sulphonated /pH 8.6, 20 hour./		
	a/ dissolved in borate buffer	1.40	79
	b/ dissolved in 0.05 M formic acid with 6 M urea and transferred after 20 hrs. in borate buffer	0.86	49

$^+$After reaching equilibrium with 1 x 10^{-5} M ε-DNP-lysine

Table 2

Activity of mixtures of bovine anti-DNP antibody and bovine gamma-globulin chains

Type of + chain	Concentration in mixture mg/ml	Total concentration of protein mg/ml	r^{++}	r_H^{+++}	r_L^{+++}
H_{sp}	0.50	0.50	0.00	0.00	-
L_{sp}	0.50	0.50	0.09	-	0.09
H_{sp}+	0.33				
+ L_{sp}	0.17	0.50	0.58	0.58	0.58
H_{sp}+	0.33				
+L_g	0.17	0.50	0.34	0.34	-
H_{sp}+	0.33				
+L_g	0.85	1.18	0.36	0.86	-
H_g+	0.33				
+L_{sp}	0.17	0.50	0.11	-	0.11
H_g+	1.00				
+L_{sp}	0.17	1.17	0.06	-	0.14

+ H_{sp}, L_{sp} - chains of antibody H_gL_g - chains of nonspecific gamma-globulin

++ corrected for non-specific adsorption

+++ r_H and r_L - represent moles of ε-DNP-lysine bound to 107,000 g of H_{sp} chain and to 53,000 g of L_{sp} chain respectively.

For discussion in the topic IV.

Possible Relation between Some Results of the Study of Delayed Type Hypersensitivity in Tissue Culture and the Mechanism of Antibody Formation

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Antibody formation and delayed type hypersensitivity are usually considered as two principally different immunological mechanisms, although potential relations between them are often discussed. Most frequently the possibility is considered that delayed type hypersensitivity represents a certain stage in the development of the mechanism of antibody formation or that the state of delayed hypersensitivity is evoked by the binding of special, unconvential antibodies onto cells. Both phenomena /mechanism of antibody formation and delayed type hypersensitivity/ are mostly studied separately, without paying sufficient attention to the fact that both these mechanisms usually exist in the sensitized organism simultaneously and that they both can participate in its immunological reactions.

In the study of delayed type hypersensitivity various methods were intensively developed in the past few years for the demonstration of this type hypersensitivity in vitro, mostly by means of migration inhibition by specific antigen of cells from a sensitized animal. The importance of this reaction, its specificity and reproducibility are beyond any doubt nowadays.

Nevertheless, one basic fact should be kept in mind. In the experiments of this type the reaction of the cellular populat-

is obvious that only a small percentage of the cells present can be really sensitive to the given antigen. It is, after all, unthinkable that in an organism hypersensitive to several different antigens each of its mesenchymal cells could be specifically hypersensitive to each of these antigens. This conclusion logically ensues from all we know at present on the basis and the manner of the development of immunological reactions.

Recently direct evidence has been brought forward for the correctness of this view. David et al. /1/ have demonstrated that mere 2,5 per cent from a sensitized animal mixed with 97,5 per cent cells from a normal animal are capable of eliciting a state in which the whole cellular population reacts on the addition of specific antigen as though it were taken from a sensitized animal. It is evident that the migration of normal, nonsensitized cells was influenced as a result of the reaction between a small percentage of hypersensitive cells and the anigen; the most probable explanation is that in the course of the specific reaction of hypersensitive cells with the antigen substances possessing pharmacological activity are released which influence the behaviour of the remaining normal cells. We too arrived at similar conclusions on using another experimental approach, i.e. when cultivating simultaneously two spleen fragments, one from a sensitized and one from a normal guinea pig, in a single cultivation chamber in liquid medium /13/. Our results indicated that on adding specific antigen identical migration changes occur in a part of the experiments in the normal fragment taken from a hypersensitive donor. The only possible explanation of this phenomenon is again the presumption that some pharmacologically active substances penetrate through the liquid medium from the hypersensitive to the normal fragment. Analogous con-

[illegible] et al. /9/.

The mentioned results are in conformity with the general hypothesis presuming that the reaction of hypersensitive cells with antigen induces the formation of pharmacologically active substances /mediator/ which elicit, through an irritation of cells, the actual manifestations of delayed type hypersensitivity /5/.

The second important point is the question, whether the reaction of hypersensitive cells with antigen in vitro always leads only to the inhibition of their function /as it is generally believed since the experiments by Rich and Lewis/, or whether it can manifest itself sometimes, on the contrary, as a stimulation. Evidence can be found here and there in the literature on the stimulatory action of antigen on cell suspension from hypersensitive animals /2, 6, 14, 15/. Also recent studies on the induction of mitotic division in the tissue culture of leukocytes from tuberculin-sensitive subjects following the addition of this antigen /7, 8/ may be a certain reflection of this fact. Nevertheless, most authors still consider cell injury as the only form of the response of hypersensitive cells to the antigen.

At our laboratory this problem has been thoroughly analyzed. We used two experimental approaches, viz. the determination of the migration of hypersensitive guinea pig cells in the presence of graded doses of specific antigen on the one hand /11/, and the study of the dynamics of migration at various time intervals on the other hand /12/. The experiments were performed using our standard technique of spleen fragment cultivation in liquid medium with objective photographic recording of the increasing size of several parallelly cultivated fragments /10/. The results are

expressed as cytotoxic indices /CI/, i.e. as the ratio between the growth in experimental conditions and that in control cultivations.

In the first series of experiments with normal and hypersensitive spleen fragments graded doses of antigen /PPD tuberculin ranging between 50 gamma to 0,02 gamma and of Old Tuberculin from 1 : 100 to 1: 250 000/ were used. Higher antigen doses usually used by most authors studying similar problems regularly result in an inhibition of the migration of hypersensitive cells. The relationship between the quantity of antigen used and the degree of migration inhibition is statistically significant. On using very small antigen doses, i.e. PPD 0,5 to 0,1 gamma, OT 1 : 2 000 to 1 : 50 000 we observe in some cases /in 23 out of 56 experiments with sensitive cells/ a marked stimulation of the migration; this result is statistically significant. Similar stimulation was never observed in control cultivations using nonsensitized cells or on using nonspecifically toxic antigen /purified Salmonella paratyphi B endotoxin/, both in sensitized as in normal cells.

In the second series of experiments antigen doses used were 10 gamma PPD and 1 : 100 OT and migration dynamic was followed at regular time intervals during the incubation of spleen fragments. Cells of normal fragments with or without antigen and cells of fragments from sensitized animals without antigen migrate relatively evenly thoughout the experiment. On the other hand in hypersensitive cells incubated with antigen a stimulation of the migration activity is regularly observed in the first hours, as compared with the control groups, while later cells migration is slowed down or ceases altogether, thus leading to the final result observed after the end of the experiment as the inhibition of

ration stimulation in the first hours of the experiment as well as t the final inhibition are statistically significant. In control experiments the effect of purified endotoxin was studied which in low doses causes a moderate stimulation of the migration activity for the duration of the whole experiment, while in higher doses it leads to a permanent inhibition from the beginning up to the end of the experiment, both in normal and in sensitized cells.

The above mentioned results as well as the quoted experiments of other authors permit to conclude that the response of cells from a hypersensitive organism to specific antigen can appear not only in the form of an inhibition, but also of a stimulation of their activity. The result obviously depends on the conditions of the experiment, antigen dose, degree of hypersensitivity, etc. On the basis of single literary data as well as of our own preliminary experiments we believe that the basis of the observed reactions of hypersensitive cells incubated with antigen should be seen in the changes of their metabolic activity.

The above results and conclusions must be brough into connection with the previously discussed hypothesis that a small percentage of hypersensitive cells react specifically with antigen and influence /probably as a result of the release of some mediator or mediators/ the behaviour of the other principally non-sensitized cells. In other words, we have to admit that the reaction of the cellular population to the proceeding reaction of delayed type hypersensitivity can manifest itself by a stimulation of the function of these cells, probably as a consequence of their increased metabolic activity.

This hypothetical conclusion is the very reason for discussing the results of the study of delayed type hypersensitivity

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at the Symposium on the Mechanism of Antibody Formation. The exact relationship between delayed type hypersensitivity reactions and the mechanism of antibody formation is not known yet; there is, at least, one fact we must keep in mind, i.e. that in immunized animals usually both mechanisms exist simultaneously. It is known at the same time that the process of antibody formation in vitro /especially the secondary response/ is accompanied by an increased activity of the cellular suspension present, which manifests itself e.g. as a stimulation of DNA synthesis /3, 4/. We might perhaps consider the possibility that in the metabolic changes observed at the beginning of antibody formation in vitro participates the simultaneously proceeding reaction of delayed hypersensitivity. We could even presume that delayed type hypersensitivity reaction connected with the general metabolic stimulation of the cell population constitute the physiological basis for antibody formation; all this is naturally merely a hypothesis.

The main interest of our studies lies exclusively in the field of delayed type hypersensitivity. We think though that some of the reported results can be interesting also for those studying the mechanism of antibody formation.

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Immunological Competence of Different Stages of the Lymphoid Cell

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The ontogenetic scheme of the development of mammal lymphoid cells which are the carriers of immunological reactivity, is now regarded to be probably as follows: The first lymphocytes develop from the epithelial base of the thymus or analogous organs under the humoral influence of the mesenchyme /2, 19, 1/. These lymphocytes disseminate into the circulation and secondary lymphatic tissues. There, they are either reutilized and provide the stimulus for the proliferation of reticulum cells and lymphocytopoiesis /21/ or themselves become full value lymphoid cells /22/. In any case, at the end of the neonatal period there is a supply of small lymphocytes at the periphery, and in the secondary lymphatic organs. Small lymphocytes here are capable of transition to large lymphoid cells /4/. These possibly develop also from reserve of primitive cells /of the character of reticulum cells/ in which lymphocytopoiesis can also be stimulated by the humoral effect of the thymic stroma /12, 15, 7/.

The lymphocytopoiesis enhancing function of the thymic stroma and the medium of the thymus seems to be contradictory to the immunological reactivity of the lymphoid cells /13/.

Small lymphocytes in the circulation and in the secondary organs, like most lymphoid cells, have the power of differentiation and modulation, into histiocytes and also possess

immunological competence /6/.

In this work we have attempted to compare the immunological competence of lymphoid cells on their route from the thymus via secondary lymphatic tissue to the lymph and during their transformation from large and small lymphocytes to histiocytes.

Material and Methods

1. Source of cells: All cells were obtained from young adult Chincilla rabbits. Thymus cells and mesenteric or mediastinal lymph node cells were isolated from the organs by teasing into Hank's or Earle's solution. Lymph cells were obtained by direct puncture of the cisterna chyli of donors immediately after they had been sacrificed and were resuspended in Hank's or Earle's solution with heparin. Histiocytes were washed out of the lungs in Hank's or Earle's solution according to the method of Myrwick et al. /14/.

2. Fractionation of cell suspensions. The lymph node, thymus and lymph cell suspensions were separated into a fraction with a complete predominance of small lymphocytes and fractions with a higher content of larger cells /other than small lymphocytes/ by gradient centrifugation on layers of sucrose at 25, 30 and 35% concentration in Hank's or Earle's solution with 20% autologous rabbit serum. Centrifugation was continued for 6 - 9 min. at 25 - 30 g. and sometimes repeated, particularly in nodes. After centrifuging, the cells were washed twice with Hank's or Earle's solution, resuspended in the same medium and their absolute number and differential count determined.

3. Antigen. With the exception of the control samples, BSA antigen was added to the cell suspension in amounts of 0.01 mg. per 10-20 million cells.

4. Cultivation. Suspensions of 10-100 million cells were placed in diffusion chambers of 0.8 ml. volume, with filters of Czech production /VUFS, 0.1 - 0.3 /u porosity, exceptionally HUFS 0.3 - 0.5 /u porosity; in these filters the great majority of pores are smaller than 0.45 /u and as distinct from the filters Millipore FA they were found to be impermeable for cells and in most cases with Membranfilter-Gesellschäft Göttingen filters with a porosity of 0.15 - 0.275 /u. Carefully sealed chambers were introduced into the peritoneal cavity of 3-6 day-old Chinchilla rabbits treated with antibiotics. 1 - 3 chambers were placed in each infant rabbit. The rabbits are not themselves capable of an antibody reaction to the amount of antigen used. The chambers were removed at various time intervals from 7 - 13 days, exceptionally after longer cultivation.

5. Morphological analysis. Smears of the initial suspension and smears and imprints of the inner surface of the chamber filter after cultivation were fixed wet with Carnoy and stained with methylene green-pyronine Y and also examined without treatment in phase contrast. In some cultivations filter imprints were also rapidly dried, fixed with ethanol and stained by the one-stage method for antigen and the two-stage method for antibody using conjugates of anti-BSA serum with fluorescein-isothiocyanate according to a modification of the method of Marshall et al. /11/. Differential counts were made from 10,000 cells of the initial suspension and from 1000-5000 cells after cultivation.

6. Serological determinations: The fluid from the chambers was used to determine the anti-BSA antibody content by the method of passive microhaemagglutination with tanned and benzidine-treated

sheep erythrocytes.

7. Types of experiments. Antibody production and the number of antibody-containing cells in the different cell suspensions were determined after cultivation in the separate chambers according to the method described previously /5/.

The effect of thymus stroma on the immunological properties of cultivated lymphoid cells was determined in two further modified cultivation methods: a. In chambers where thymus stroma, obtained by washing out the small cells from 3 - 6 small fragments of the organ /2 x 2 mm./, was cultivated for 12 and 43 days. Chambers filled with Earle's solution were placed into the recipients at the same time. After 12 and 43 days all the chambers were taken out and the fluid inside replaced through a hole in the wall of the plexiglass ring with a suspension of lymphoid cells freshly isolated from lymph nodes or lymph with antigen /BSA/.

b. Double chambers were made of two rings of plexiglass and closed by three filters, the centre one /of 0.25 - 0.4 /u porosity/ being common to both assembled chambers. The outer filters had a porosity of 0.15 - 0.275 /u. Fragments of thymus /3 - 6 fragments 2 x 2 mm/ were enclosed in one of the assembled chambers, or as a control, only Earle's solution or fragments of lymph nodes. In the second of the assembled chambers lymphoid cells were cultivated together with antigen /BSA/.

The total number of chambers used in the experiments can be seen from the tables. Recipients from one litter were always used for the cultivation of different types of cells so that the influence of individual factors of the recipient on the cell suspensions should be minimal. In some experiments the chambers

intervals.

Results

From tables 1 and 2 it is seen that all cultures containing more than $10 \cdot 10^6$ lymph or lymph node cells produce antibodies inside the chambers in titres up to 1 : 256. The suspensions of pure small lymphocytes from these sources are somewhat inferior in this respect to suspensions which include larger cells. The production is both number and time dependent: higher numbers of cells in starting suspensions in most cases give higher titres of antibodies; on the 7th day of cultivation the production is negligible, the highest titres appear between the 8th and 10th day of cultivation.

Lung histiocytes /table 4/ seem at least to contribute to antibody formation, because the admixed amount of lymphoid cells gave demonstrable antibodies if cultivated alone.

On the other hand, thymus cells /table 3/ do not give any relaible titres with the exception of separated small thymus lymphocytes which produce demonstrable titres in more than 80% of cultivations. Their production is again time and number dependent. In comparison with small lymphocytes from secondary lymphoid tissues their production is lower and a little delayed.

The same picture is provided by the count of antibody containing cells traced by the immunofluorescent method /Fig.1/. 1.5 - 2% of cells from lymph node and lymph are stained specifically on the 9th day of cultivation, thymus small lymphocytes are a little inferior and thymus cell suspensions including larger cells range from zero to 0.7%.

In lymph node and lymph cultivations plasma or immature plasma cells could be identified among the antibody containing

cells, whereas in small lymph and thymus lymphocytes cultures there was a remarkable prevalence of reactive forms of small lymphocytes among the positive cells. Large lymphoid cells and even histiocytic forms were occasionally found to contain antibodies both in the cytoplasm and sometimes /on day 8 or 9/ even inside the nucleus. In some histiocyte cultivations only histiocytes were found to contain antibodies, indicating that they might have the capacity to produce antibodies on their own.

In routine cytological analysis, the thymus cell cultivations differed from secondary lymphoid organ cell cultures in their tendency to histiocytic and epithelial outgrowth which was pronounced especially in the samples including higher proportions of large cells in starting suspensions.

In histiocyte cultures there was a development of either heavily phagocytosing cells or large pyroninophil histiocytes with activated nucleoli. The proportion of macrophages to pyroninophil histiocytes is about 4 : 1.

The morphological details of cellular differentiation during cultivation and antibody formation have been described in previous work /5, 6/.

The idea that thymic stroma and thymic reticulum cells might exercise some depressive action on the immunological capacities of lymphocytes was tested in cultivations of thymic stroma plus lymphocytes from secondary lymphoid organs. As shown by tables 5 and 6, both in double chambers and in chambers containing precultivated thymic stroma, which forms a typical epithelial sheet after 10-12 days, lymph or lymph node lymphocytes cultivated together with thymus tissue produced lower titres of antibodies if any. On the other hand, in such joined cultivation of thymus reticulum plus lymphoid cells from secondary organs, a good

8 days of cultivation in double chambers, 24 . 10^6 cells have been counted in chambers containing Earle solution and target lymphoid cells, and 35 - 51 . 10^6 cells were found in chamber containing thymus fragments and target cells. Over 90% from these surviving cells were small lymphocytes.

Discussion

Both antibody formation inside the diffusion chambers and counts of antibody containing cells show that immunological capacity decreases in the sequence: 1. lymph node and lymph cells with higher proportions of larger cells /large lymphocytes, blasts, recticulum cells/; 2. small lymph node and lymph lymphocytes which enter the immunologically competent stage either by differentiation towards larger lymphoid cells /4/ or by direct modulation /6/; 3. small thymic lymphocytes /with the same morphological changes accompanying their immunological function/; 4. thymus cells indlucing large lymphoid cells and thymus reticulum, which appears to depress the immunological reactivity of lymphocytes in enhancing their proliferation.

Even lung histiocytes /which might at least in part be descendants of lymphoid cells, see ref.16/ appear to have the capacity of differentiating into heavily pyroninophil forms and elaborating antibodies.

It is likely that the lymphoid cell from the blast stage on is immunologically competent and preserves this quality throughout its life up to the stage of the small lymphocyte and even nonphagocytic histiocyte. In the passage of the cell from primary to secondary lymphatic organs and into the circulation this quality might be influenced by local environmental factors among which the proliferation stimulating factors - such as the

thymic Metcalf /12/ factor - are at variance with intracellular events connected with adaptive proteosynthesis. As to the immunological competence, thymic lymphocytes appear not to be different from lymphocytes resident in secondary lymphatic tissues and in circulation. Thymus might ensure the nonspecific differentiation and multiplication of lymphoid cells not because it is antigen proof /10/, but because steadily proliferating lymphoid cells do not enter the inductive phase of antibody formation. The immunological competence of thymus lymphoid cells /chiefly small lymphocytes/ can be detected even by transfer of cellular suspensions into whole recipient where thy might be separated from the depressive action of the reticulum /20,19/.

The term "immunologically competent cell" can thus hardly be attributed to some morphological character of the lymphoid cells: as a morphological phenomenon, the lymphoid cell seems to be nonrestricted in its functional capacities in either stage of differentiation. A speculative attempt /e.g.3/, to correlate the small lymphocyte with the functional concept of incompetence cannot be justified. There might be only quantitative differences in the ability of the lymphoid cell to undergo the intracellular changes required and to multiply as a committed cell.

A high proportion of cells has been found to contain antibodies. The percentage of positive cells is comparable to the results of Makinodan and Albright /9/. It is in marked variance with the results obtained by the plaque technique /18/. This difference might be due to absorpiton of antibodies in diffusion chembers. It is, however, hard to believe that this could be the case even in cells which do contain intranuclear antibodies. In the first days of antibody formation these cells form considerable

antibody containing but antibody secreting or liberating cells are revealed by the plaque technique, and this might be the main source of discrepancy between the two methods.

Summary

Lymphoid cells from normal rabbits were cultivated in diffusion chambers with BSA antigen, the chambers being implanted into newborn rabbits. Antibody titres from the chamber fluids and counts of antibody containing cells /detected by immunofluorescence/ show that lymph node and lymph cellular systems including higher proportions of larger cells are better producers than lymph node and lymph pure small lymphotic suspensions; in thymus the relation is the reverse: only small thymic lymphocytes give reliable antibody production. The idea that thymic reticulum might have an enhancing effect on lymphocytopoiesis and in the same way a depressive action on the antibody forming capacity of lymphoid cells has been proved by cultivations of thymic stroma with lymphoid cells from secondary lymphatic organs. Antibody formation was heavily depressed by thymic tissue in the chambers. It is likely that even lung histiocytic cells are antibody producers.

It is concluded that a lymphoid cell, from its blast stage to the small lymphocyte or histiocyte, preserves its antibody forming capacity and only its localization and local effects /such as the effect of thymic stroma/ might influence its specific changes towards antibody containing types.

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Table 1.

Log_2 titers of anti-BSA antibodies formed in diffusion chambers by lymph node cells with different proportions of small lymphocytes and larger cells, cultivated with BSA antigen

Total number of cells	Days of cultivation	% of small lymphocytes in the starting suspension: 99-100	mean	95-99	mean	90-95	mean	85-90	mean	80	mean
100											
75-100	7					0;3;0;0;0;0	0,5				
	8					8;3	8				
	9			5	6	5;5;4	4,7				
	11			5	5	6;0	3				
50-75	9	5;5	5	6;7;6;6	6,3	8;8;4;5;5	6				
	10			6;7	6,5						
	11					7;7;6;2;4	5,2				
25-50	8	4;3	3,5	4;3;5;4	4			8;8	8	8;8	8
	10	8	8	6;6	6						
10-25	10							5;6	5,5		
10											

Table 2.

Total number of cells	Days of cultivation	% of small lymphocytes in the starting suspensions: 99-100	mean	95-99	mean	90-95	mean	85-90	mean	85	mean
100	10							7	7	7	7
75-100	7							0	0		
	9	6;3;5	4,7					4;6	5		
50-75	7	5;0	2,5			0	0				
	8	7	7								
	9									5	5
	10	6	6	6	6	7;8;7;7;8;6	7,2			7	7
	11	2	2			4	4				
25-50	8	4	4	4;4	4	3;4;5	4	5	5	6	3
	9	4;4;5	4,3	3;5	4			6	6	7;6	6,5
	10	4;4	4	6	6	5	5	7	7	5	5
	11					4	4	5	5	5	5
10-25	8	1;2;2	1,7								
	9	3;3	3	3;3	3			4;5;3	4	4;3;4;4	4,5
	10	3;4	3,5	3	3	4	4				
10	8									0	0
	10			0	0			0;2	1		

Log_2 titers of anti-BSA antibodies formed in diffusion chambers by lymph cells with different proportions of the small lymphocytes and larger cells, cultivated with BSA antigen.

Table 3.

Log 2 titers of anti BSA antibodies formed in diffusion chambers by thymus cells with different proportions of the small lymphocytes and larger cells, cultivated with BSA antigen

Total number of cells	Days of culti-vation	% of small lymphocytes in the starting suspension: 99-100	mean	95-99	mean	90-95	mean	85-90	mean	<85	mean
100	10					1	1	0;1;0		0,3	
	13			6	6	2;2	2				
75-100	7							0;0;0		0	
	8			0	0			0		0	
	9	6;5;5	5,3					1;1		1	
	10			2	2			2		2	
	11			2	2			1		1	0
	13	3	3								0
50-75	8	3;0	1,5	1;1;0;0	0,5	0;2	1				
	9	3;3;4;6	4	2;2;0;0	1						0
	10	3;6;1;4;3;3;3	3,3	4;4;4;2;2;2;1;1;1	2,3	0;0;0;1;1;1;0	0,4	0;1;0;0		0,3	
	11	3;2;6;4;6	4,2	2;2	2	0;0;0;0;0;0	0				1
25-50	8					1	1				
	9							0;2	1		
	10	3	3			1	1	2;2;1;0	1,3		
	13	8;3	7,5	4	4						
10-25	7							0		0	
	10	3;2;4;1	2,5								

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Table 4.

Log_2 titers of anti-BSA antibodies formed in diffusion chambers by lung histiocytes with 1-10 % admixture of lymphoid cells, cultivated with BSA antigen.

Total-number of cells	Days of cultivation	% of histiocytes in the starting suspension					
		95-99	mean	90-95	mean	85-90	mean
100							
75-100							
50-75	8			2	2		
	13			4	4		
	15	5;5;8	6				
25-50	9	5;5	5				
	10			0;2	1		
10-25	8					0	0
	9					2	2
	10					1	1
	11					4	4
	22	1;2	1,5				

Table 5.

Log_2 titers of anti-BSA antibodies formed in diffusion chambers by lymphoid cells cultivated with BSA antigen; experiments with double chambers: in one of them the target lymphoid cells were grown, the other was filled either with Earle solution, with lymph node fragments or with thymus fragments.

Lymphoid cells +Earle sol.	Lymphoid cells +lymph node	Lymphoid cells + thymic stroma	Source of lymphoid cells, % of small lymphocytes	No of lymphoid cells $.10^6$	Time of cultivation of lymphoid cells in days
4;4	3;3	0;0;0;0;0;1	l. node, 94%	50-55	11

Table 6.

Log_2 titers of anti BSA antibodies formed in diffusion chambers by lymphoid cells cultivated with BSA antigen either alone or on thymic reticulum which has been precultivated in the same chamber for the time indicated

Precultivation of thymic stroma in days	Lymphoid cells only	Lymphoid cells+ thymic stroma	Source of lymphoid cells, % of small lymphocytes	No of lymphoid cells $.10^6$	Time of cultivation of lymphoid cells in days
43	7;7;8	3;6;1	l. node + lymph, 85%	67	11
12	3;4;4;5	0;0;0;1			
	+1,26; 1,01; 0,43; 0,47	+0,14; 0,2	l. node, 95%	34	8

+% of cells containing antibodies revealed by immunofloroscence

Formation of 19 S and 7 S Type Viral Antibodies. The Role and Nature of "Antibody Cofactor".

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In recent years data have been published on the formation of 19 S and 7 S type antibodies in response to various viruses /6, 7, 1 et al./. In our contribution, findings will be presented mainly concerning the nature of antibodies to influenza viruses. A further difference in the character of anti-influenza antibodies will be described in addition to the 19 S - 7 S type, i.e. the change in their ability to be potentiated by the so-called "antibody cofactor". We will show that misleading results in the detection of 19 S and especially of 7 S influenza antibodies can be obtained if the additive effect of cofactor is disregarded. Some characteristics of this serum component will be therefore presented first.

We found /4, 5/ that the titres of specific influenza anti-haemagglutinins decreased after heating of antisera /56 °C/30 mins./, if a "non-avid" A2 influenza strain /i.e. a strain of low antibody sensitivity, also designated A2- /cf 2/ was used as antigen in the haemagglutination inhibition test /HI/. The decrease was much more marked in sera taken after first virus administrations than in hyperimmune sera /Fig.1/. It was possible to restore the titre of HIT antibodies to their original, or even to a higher, level /Fig.1/ by adding normal unheated serum containing no inhibitors to A2-virus.

The fact that the activity of heated or stored immune sera increases after the addition of normal unheated serum, is not a new finding in virology /Gordon, Mueller, Meyer, Morgan, Whitman, Leymaster and Ward, Chanock, Casals and others/. Our experiments, however, offered evidence that, at least with influenza viruses, the potentiating factor is not complement, but an entirely different thermolabile serum component. We proposed the name "serum cofactor" or "antibody cofactor" for this component.

Several approaches were used to prove that the cofactor is not identical with complement or its components /C'1 - C'4 /. Clear evidence was obtained when comparing the titres of cofactor and C' components in sera freed of complement by binding on the antigen-antibody complex. Further, substances with anticomplementary activity, such as dextran sulphates, heparin, pelentan and trypsin, or the removal of the C'3 component of complement by zymozan treatment, do not substantially affect the cofactor titre. The presence of EDTA, which blocks the activity of C'1 component of complement, doeshot inhibit the cofactor activity in human, mouse, pig, piglet and horse sera.

With some animal sera we were successful in separating the cofactor from the components of complements, using gel filtration. Separation of guinea pig serum on Sephadex G-200 is illustrated in Fig.2. Cofactor activity occurred mostly in the first peak containing the macroglobulins, whereas the individual complement components were mainly present in the 7 S peak. It must be added that with many other sera the first C' component was also detected in the macroglobulin peak. /C' components were detected using reagents R1, R2, R3 and R4, deficient in the respective C' component/

On paper electrophoresis, the cofactor from mouse serum moved along with β-globulins. On immunoelectrophoresis, the cofactor activity of bovine serum was bound to the β_2-macroglobulins. The macroglobulin character of cofactor from various animal sera was also demonstrated by density gradient zonal centrifugation /3/. In Fig.3 the macroglobulin nature of mouse, bovine and pig cofactor is evident. However, the cofactor from serum of newborn unsuckled piglets is of lower molecular weight showing an $s_{20,w}$ value of 3.0 to 4.0 S. The smaller molecular size of piglet cofactor was also confirmed by chromatography of piglet serum on Sephadex G-200 /Fig.4/.

Now some remarks on the 19 S and 7 S influenza antibody formation. The character of antibodies in rabbit serum taken 10 days after administration of non-avid A2-influenza virus is illustrated in Fig.5. Both 19 S and 7 S antibodies can be detected in unheated samples against the homologous antigen. The titres of these anti-haemagglutinins decrease after heating /56°C/30 mins/ but can be restored by adding cofactor in the form of normal mouse serum. - Using the A2+ virus as antigen in the HI test, the 7 S globulins can also be detected in this antiserum, though in a low titre, which is rather surprising. We cannot say, however, in what quantity 19 S anti-A2+ antibodies are present in this antiserum. The samples from the macroglobulin peak show quite high HI activity, but we found that the nonspecific γ inhibitor /against A2 + strains/ is present in this peak after Sephadex chromatography of normal sera.

The Sephadex separation of another rabbit antiserum is illustrated in Fig.6. The serum was taken 10 days after A2+ the

administration of influenza virus. The formation of 7 S antibodies against the homologous antigen is obvious. The 19 S antibodies are also probably present, but - as in the previous case - they cannot be differentiated from the non-specific γ inhibitor. - Both 19 S /in a small quantity/ and 7 S antibodies can be detected against a non-avid /-/A 2 strain, but only after addition of the serum cofactor, the importance of which thus becomes clearly evident.

Extensive immunisation experiments on white mice revealed great variability as far as 19 S and 7 S antibody response is concerned. There were cases where 19 S antibody formation preceded that of 7 S antibody, as expected. However, in other experiments, 7 S antibody appeared simultaneously with, and in equal or even higher amounts than 19 S antibody. This occured on the 3rd - 5th day. Detection of antibodies - or their titre - depended on whether serum cofactor was present in the respective fractions. This is especially true of 7 S antibody, because owing to the macroglobulin character of cofactor, the later was not present in fractions from the 7 S globulin peak. A similar case is shown in Fig.7 which illustrates the separation of mouse serum taken 5 days after virus administration. Judging from results which samples tested in saline, a small amount of 19 S and 7 S antibody should be present. However, if the same samples are tested with cofactor added /i.e. using normal mouse serum 1: 15 as diluent/, the detected amount of 7 S antibody increases considerably. This is also true for virus neutralizing /VN/ antibodies. /VN activity was tested only in fractions from the top of the first and second protein peaks/. In many cases the activity of 7 S antibody was much more enhanced by the cofactor than that of 19 S antibody.

On summary, we would like to point out that in addition to the replacement of 19 S by 7 S antibodies, a further change in the character of antibodies from "early" and "late" influenza antisera can be observed, i.e. their ability to be potentiated by the "antibody cofactor". The former change /19 S - 7 S/ is usually more rapid and does not coincide with the latter because antibody which is and that which is not potentiated by the cofactor can be both of the 7 S type.

Serum cofactor does not play any role in the activity of antibody /either 19 S or 7 S/ to bacteriophage ΦX 174. Our results of 19 S and 7 S antibody formation were similar to those in experiments of Uhr and Finkelstein /7/. However, in addition to the expected sensitivity and insensitivity of 19 S and of 7 S antibody, respectively, to mercaptoethanol, we observed the formation in rabbit of 7 S type anti-phage antibody which was sensitive to 2-mercaptoethanol treatment.

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The Inductive Phase of Antibody Formation Studied with Isolated Cells

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At the Symposium in 1959 we summed up experimental results which made it possible to reach the conclusion that the first phase of antibody formation can be considered to be functionally distinct /processes taking place during that phase/ from the later phase - the actual production of antibodies /15/. This conclusion was based on observations that the initial phase of antibody formation is much more sensitive to certain forms of interference /X-irradiation /6, 23/, vitamin deficiency /1/, the action of hormones /3/, and particularly on data obtained in experiments with the transfer of isolated spleen cells to young homologous recipients /13//. We showed that if spleen cells are cultivated with the antigen in vitro antibody formation does not occur; cells potentially capable of antibody formation /immunologically competent/ only survive in tissue culture and the process of differentiation for antibody formation starts only when they are transferred from tissue culture to tissue culture in vivo in a newborn recipient /14/. Using this method of transfer we showed that the smallest number of spleen cells capable of forming a sufficient level of antibodies in young recipients for detection by the agglutination reaction /i.e. about 0.1 /ug N antibody/ml./ is 10^6. We further found that of this number probably only in the order of 10^3 cells participate

in actual production, i.e. about 0.1%. On the basis of these quantitative findings we were able to exclude the simplest version of the clonal selection theory, by which antigen acts directly by the proliferation of cells already producing the given type of antibody. Our calculations showed that on this assumption and with our methods of detecting antibodies, they should have been detectable sooner than they actually were experimentally /20/. This was the basis which led to our arriving at the conclusion that actual production is preceded by a qualitatively different, inductive phase of antibody formation whose basis is a change in biochemical and morphological properties during the process of differentiation /15/.

In the most recent years we have made further observations in support of these conclusions. With a model of transfer of isolated cells we showed that the duration of the inductive phase can only be little shortened but that it cannot be eliminated /17/ if an increasing number of spleen cells is used for transfer. We used a whole spectrum of inhibitors of nucleic acids and found that only a few inhibit antibody formation even if those which do not affect antibody formation have marked antimitotic activity /18/. The nucleic acid inhibitors, e.g. 6-mercaptopurine and 6-thioguanine, act only during the inductive phase of antibody formation./16/. On a similar model, the transfer of spleen cells to lethally irradiated isologous recipients, Makinodan et al. /9/ reached the same conclusion, i.e. that the first phase of antibody formation /the latent phase/ actually exists and can be explained in terms of disorganization and reorganization of the germinal centre.

There are, however, certain data, obtained for the most part

with phage and virus antigens /12, 24, 5/, which are at variance with these results and conclusions. These authors found increasing titres of antibodies in the serum very soon after giving antigen and by the interpolation of the results almost eliminated the possibility of the existence of a period during which antigens were not formed. However, since in our experiments, particularly with the immunization of young animals, we continue to reach the opposite conclusion /19/ we attempt in the experiments submitted to provide further evidence.

Direct proof of the existence of an inductive phase would be provided if, using a population of a sufficient number of cells /i.e. at least 10^7 - 10^8/ and a sensitive method, we could demonstrate directly on the cellular level that antibody producing cells do not appear for a short period after the administration of antigen, or if such cells are already present in a given number, that this number does not increase during a short period after antigen injection. We selected the plaque technique of determining antibodies which was introduced by Jerne/8/ as most suitable in resolving our problem and modified it by using agarose, a substance forming a gel medium without an anticomplementary effect /4/. The test was further modified to permit morphological and autoradiographic observations of antibody producing cells /22/.

Method: Cells isolated from the spleen or lymph nodes were washed three times and diluted to a given concentration in Parker solution with 0.5% HSA and immediately before mixing temperated at 42°C. One part of cells was added to two parts of agarose containing washed sheep erythrocytes /3 parts 1% agarose in Parker solution with HSA + 1 part 6% erythrocytes in Parker

ture of cells, erythrocytes and agarose was pipetted drop by drop from a height of about 60 cm. into a Petri dish or onto a slide. Drops with a diameter of 18 mm. and single layers of erythrocytes and lymphatic cells are formed. If the drop is dried it has a thickness of about 20 /u. The drops are incubated at 37°C in a moist atmosphere with 5% carbon dioxide for 6 - 20 hours. After incubation drops are overlayered with guinea pig complement absorbed with sheep erythrocytes. One ml. of complement used contained 20 units. The plaques are counted after incubation at 37°C for 1/2 hour, and the complement then removed by veronal buffer and the drops fixed in formalin vapour. After fixation they are washed in distilled water and dried. The preparations were stained with Giemsa-Romanowsky diluted 1 : 20 for 3 - 5 min. for the morphological determination of the lymphatic cells. For autoradiographic determinations they were covered with stripping film Kodak AR10.

We first determined the dynamics of the increase in the number of antibody producing cells after i.v. immunization of mice of a noninbred H strain /weight approximately 20 g./ with 0.5 ml. 1% suspension of sheep erythrocytes. Before the addition of antigen we find an average of 65 antibody forming cells from the total number of 10^8 cells /minimal 10, maximum 140/; 24 hours after the addition of antigen we find, on an average, double the number. This increase, however, is only found in some individuals; in others the number remains within the limits of the initial value after 24 hours and may even approximate to the minimal number of cells determined before immunization /17 to 10 cells/ 10^8/. In other animals after 24 hours we found the highest values which were found in non-immunized animals /110, 129, 159/10^8/,

in some, however, the number was double the highest values before immunization /377, 315/10^8/. A uniform increase in the number of antibody-producing cells of an average of 1,741 occurs only after 48 hours and the highest values are obtained after 4 and 5 days and after that the number of cells producing haematolytic antibodies in the spleen decreases. Since the largest number of cells forming haematolytic antibody was found on the fourth day we investigated the morphological characteristics of antibody producing cells at this time. The cells were classfied according to the usual convention /10/ and we found that 25% of antibody forming cells were small lymphocytes, 47% were medium-sized lymphocytes and 27.5% large lymphocytes. Cells which would probably gradually be transformed into typical plasmocytes were seen only rarely. The greatest increase in the number of antibody producing cells was between the third and fourth day; we wished to determine whether the increase in the cell population at this time was due mainly to mitotic activity. It would also be possible for the quantitative increase to be due to unequal contact with antigen and variations in the time during which cells develop into antibody producing cells under the influence of antigen. If the first theory were correct most of the cells would incorporate labeled thymidine between the third and fourth day. We first used thymidine-H^3 but it was found that most of the cells remain deeper that 2 u below the surface in the thin layer of agarose so that labeled cells were not detected. We were more successful on using thymidine-C^{14} whose effect penetrates to a distance of 90 μ /2/. In the first experiment thymidine-H^3 in amounts of 10 μC was injected i.p. into mice three times at intervals of 6 hours between the third and fourth day after immunization. Only

were labeled with thymidine. In the subsequent experiment we therefore administered thymidine from the injection of antigen every 8 hours to 72 hours, i.e. a total of 10 doses /one mouse received a total of 50 μC/. In these experiments we found that the total number of labeled small, medium-sized and large lymphocytes was 25%, the large lymphocytes being labeled 100%. The same percentage, i.e. 25% was labeled in smears and in agarose if the drops were fixed immediately without incubation. Of a total of 50 examined centric cells only 40% had incorporated thymidine. If we add 10% of cells which could have acquired labelling between the third and fourth day, during which thymidine was not given in this experiment /on the basis of the preceding experiment/, we consider it to be definitely established that not all cells detected as antibody producing cells, arise by mitotic division. The experiments determining the time course of the increase in the number of antibody producing cells in mice make it clear that we are dealing with a most heterogeneous population with a diverse individual history. In nonimmunized adult mice there is not only variation in the number of cells in different dindividuals before immunization, but after giving antigen the rate of onset of antibody formation which is very marked already after 24 hours, shows individual differences. We consider that the individual heterogencity is the result of the diverse immunization history in adult animals and it is probably the reason for the diverse results obtained when immunizing adult animals.

On the other hand our conclusions on the time course of the onset of antibody formation are mainly based on developmental studies during ontogenesis. We have repeatedly confirmed /21/ that a real primary reaction can be expected with most antigens only in newborn animals. We therefore determined the number of

antibody forming cells in newborn rabbits from the first to the 30th day of life, both in nonimmunized animals and in newborn animals injected i.p. at different ages with 1 ml. 10% sheep erythrocytes. Shortly after birth we did not find a single cell in lymphatic tissue which formed antibodies. Only from the 20th day did we find very small numbers of cells producing antibody to sheep erythrocytes /Tab.2/ in nonimmunized rabbits. On the other hand, as found previously by Říha /11/, if sheep erythrocytes are injected into newborn rabbits, antibodies are already formed on about the fifth day of life. In accord with this, antibody producing cells are detected at this time by the plaque method /Fig.3/. It is also evident from the experiments on rabbits that the number of cells detected at the same hours after antigen injection increases continuously with age. Whether this was due to the spontaneous maturation of lymphatic tissue or to antigens of the intestinal microflora or food antigens which have chemical groups in common with the antigens of sheep erythrocytes /7/, we attempted to decide by further experiments.

To solve this question we used once again the model of sterile piglets which were fed on a nonantigenic diet, as described previously. If we determine the appearance of producing cells in normally reared piglets /which received colostrum and were reared with the mother under normal conditions/ we do not find antibody forming cells immediately after birth or on the seventh day of life, but already on the 14th day there are 9 antibody forming cells per 10^8 spleen cells,/i.e. 63 cells in the whole spleen/, in another animal of the same age we found 277 cells calculated to the whole weight of the spleen. These results stand in sharp contrast with those obtained in piglets

reared under sterile conditions on a nonantigenic diet. Up to one month, i.e. for the whole period sterile artificially fed piglets were reared, we did not detect one antibody-forming cell to sheep erythrocytes if antigen had not been given. On the other hand, if the piglets are given an injection of sheep erythrocytes /10 ml. 20% suspension of sheep erythrocytes i.p./ on the first day of life, the first antibodies can be detected after 72 hours /Tab.3, Fig.4/. Whether the number of cells detected at first /72 hours after immunization/ changes with age is difficult to decide on account of the small number of results. It would seem, however, that it is more affected by individual factors than by maturation, as we thought originally /18/.

The results show explicitly, that antibody formation does not start spontaneously during individual development if the individual is protected from antigenic stimuli. The proof that the so-called "spontaneous" development of antibody forming cells in normally reared piglets is due to antigens encountered by the animal, appears to be given by the experiment in which the number of antibody forming cells was determined in sterile piglets fed on an antigenic diet - degraded cow's milk. On the 30th day of life 7 plaques were detected in one animal and 10 in another. In both cases, however, not a single antibody forming cell was present in the lymph nodes /Tab. 4 /. The difference between the onset of antibody formation in normally reared rabbits and sterile piglets /Fig.5/ is probably that in the first case antigen acts during development while in the second andantigenic stimulus is not present.

However, there are several conclusions that are common both for rabbits and sterile piglets. On the basis of the data obtained in 8 - 10-day rabbits it can be excluded that a previously exist-

ing cell in the organism at the time of immunization could give rise by proliferation to the number of cells determined after the negative period. If we assumed that the number of producing cells arose by proliferation, then at zero time in the rabbit there would have to be only 1/250 antibody producing cell for the total 10^8 spleen cells. One antibody-forming cell would only be present in about 10^{11} lymphatic cells, which is much more than the young organism actually contains /this amount would correspond to about 10 - 100 kg. rabbit/. The situation in piglets is similar to that in infant rabbit. In this case the onset of antibody formation is a little later and therefore in the zero hour, only 1/1000 of a cell from the entire population of 10^8 lymphatic cells would be able to take part in antibody formation. This would correspond to the presence of one cell in 10^{11} lymphatic cells, thus again a number greater than that present in a newborn piglet. The second possibility that at zero hour one cell is present which reaches the number determined in 72 hours by the very slow process of proliferation in untenable since at 24 and 48 hours the producing cells could actually be detected in both models /piglets and newborn rabbits/. If an estimation is made of the doubling time in both models the basis of experimental data obtained between 72 and 96 hours, it comes to 5 hours. On the basis of the above calculation and the very short doubling time we are of the opinion that producing cells do not arise by a process of proliferation of preformed cells. It would certainly be correct if we could provide direct proof in the newborn that the production cell which is first detected does not arise by division and does not incorporate thymidine-C^{14}. We have no such proof at present for technical reasons. In order

to detect the very small number of cells appearing in the newborn it is necessary to use concentrated cell suspension, 10^8 cells per 1 ml. If cells are added in this concentration to agarose, the area of the plaque of Ø 1 mm. contains approximately 4,000 lymphatic cells. It is therefore impossible in such a concentration to estimate what cells are centric, i.e. productive.

The second more probable conclusion which is also in keeping with the previous findings with the isolated cell transfer method assumes that for a certain time after antigen injection during the primary reaction the cell passes through the inductive phase and does not produce antibodies and that processes take place during this time which are distinct from the later process of actual antibody production. It is possible that part of the cells which are capable of responding to antigen /competent cells/ divide already in the course of functional transformation when antibody is not yet produced. However, on the basis of experiments with the incorporation of thymidine into antibody producing cells it must be accepted that at least a part of competent cells is transformed into producing cells without mitotic division.

We will attempt to treat the experimental results in the light of the basic unresolved questions of the origin of antibodies. If we consider the theory of the existence of multipotent stem cells capable of reacting with different antigens, then at the time of administration of the antigen only an unprobably small amount of stem cells would be present in a physiological condition allowing differentiation according to the type of stimulus. If we find that out of a total of 10^7 lymphatic cells only one cell would be just in the

state permitting it to react to any antigen, then this hypothesis seems highly unprobable. In adult animals the ratio of the total number of cells to the producing cells shifts to the side of the producing cell. In our experiments, we have shown that this occurs under the influence of antigen. It cannot, therefore, be doubted that there is a certain form of branching process in cell population capable of responding to a corresponding antigen. It would seem, therefore, that the quantitative results obtained in newborn developing animals point to the hypothesis of the selection type in which antigen would act either on certain cells genetically preformed or arising by a mutation process and selected by the antigen. These immunologically competent cells, differentiate biochemically and morphologically during the inductive phase into antibody producing cells.

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Table 1.

Number of antibody producing cells in individual mice immunized with sheep erythrocytes

Days after immunization	Number of plaques per 10^8 spleen cells	Average number of antibody producing cells per 10^8 spleen cells	Percentage of antibody producing cells per 10^8 spleen cells
Non-immunized	10, 10, 23, 23, 59, 60, 65, 107, 140	55	0,00005
1 day	10, 17, 110, 129, 159, 315, 377	159	0,00016
2 days	356,1237, 2530, 2843	1741	0,0017
3 days	2131,5747,7833,9750	6.115	0,0061
4 days,	21300, 32600, 35500, 46200	33 900	0,034
5 days	16000, 25976, 38192	26723	0,026
7 days	9667, 16580	13 084	0,013
8 days	10050, 12870	11 460	0,011
9 days	4920, 16117	10 519	0,01
10 days	4557, 8687	6 622	0,0066
11 days	3617	3 617	0,0036
13 days	833, 3793	2 313	0,0023

Table 2.

Number of antibody producing cells /per 10^8 spleen cells/ in newborn rabbits immunized with sheep erythrocytes

Age of rabbits at the time of immunization days	Non-immunized	After immunization				
		24 hrs.	48 hrs.	72 hrs.	96 hrs.	120 hrs.
3				0		
4			0 0			
5		0 0			203	
6	0			234		
7			0 0			192
8		0 0			1773	
9	0		0	335		
10		0	0 4	11		
11	0		9			
12	0	3				
13	0 0			444		
16	0					
20	8 1					
26				336, 302		

Table 3

Number of antibody producing cells /per 10^8 spleen cells/ in newborn precolostral sterile piglets reared on non-antigenic diet

Age of piglets at the time of immunization days	Non-immunized	After immunization			
		48 hrs.	72 hrs.	96 hrs	120 hrs.
1	0 0	0,0,0,0,	0,0,37,37,50	18,797,1450	719
6		0	6,53		
8	0				
13			283		
17	0				
18		0	16		
19	0,0,0,0,0				
29		0	2		

Table 4

Number of Antibody Producing Cells in One-month Old Piglets reared Sterile and Fed on Conventional Diet

		weight in g	total number of cells	total number plaques	percentage of productive cells
Piglet 3	spleen	5,17	$6,1 \times 10^8$	10	0,00000164
	lymph node	3,29	$1,5 \times 10^8$	0	0
Piglet 5	spleen	7,43	$9,5 \times 10^8$	7	0,00000073
	lymph node	3,07	$2,34 \times 10^8$	0	0

The Change in the Molecular Weight of Antibodies Synthesized in Tissue Culture after Second Immunization

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The change in properties of antibodies in the course of immunization is of great theoretical and practical interest. Such a phenomenon has been reported by many authors. It was found in particular that antibodies in sera of hyperimmune rabbits subjected to second immunization with serum albumin exhibit immunochemical properties that are on the fourth day from those displayed by antibodies on the seventh to tenth day after immunization. The former /"earlier"/ antibodies require considerably more antigen for their precipitation than do the latter ones. /7/.

Recently, a number of laboratories have succeeded in showing that after the injection of antigen, sera contain antibodies of a high molecular weight /19 S antibodies/, then their number decreases with increasing number of antibodies of a molecular weight about 160,000 /7 S antibodies/. This has been particularly clearly demonstrated in the case of primary response /1, 3, 13/.

This work deals with possibility of 19 S antibody formation in hyperimmune rabbits by investigating antibodies formed by cells of spleen and lymph nodes on separate cultivation in vitro rather than serum antibodies that are mixtures of antibodies synthesized in different organs. Cells were isolated from rabbit organs on the third day after second immunization, i.e. on the day when one would expect sythesis of antibodies with abnormal

precipitating properties. A comparative study was made of antibodies synthesized on the fifth day after the second immunization. "Heavy" 19 S antibodies were distinguished from "light" 7 S antibodies by filtering through gel Sephadex G-200.

Methods

Rabbits were immunized with a human serum albumin /HSA/ according to the following scheme /6/. They were given increasing amounts of albumin intravenously for three weeks, i.e. one, two and three mg /9 injections/. After two-three months rabbits were injected with 50 mg of HSA each /second immunization/. On the third or fifth day after reimmunization rabbits were exsanguinated and the spleen and inguinal and popliteal lymph nodes removed. These were minced with scissors and passed through sterile capron mesh. The cells were then washed and suspended in Eagle's medium. The final volume of the spleen cell suspension was 6 to 8 ml, that of the lymph node cells 3 ml. Up to 20 % of the serum of a normal or experimental rabbit was added to the suspension.

Cells were cultured in special small vessels comprising two parts /Fig.1/. A small cup /Fig.1/ was filled with a mixture of Eagles's medium and rabbit serum /about 6 ml. in all/ with a small glass covered magnetic bar /Fig.4/ placed on the bottom. A ring /Fig.2/ with a cellophane membrane /Fig.3/ stretched on it was placed on the top of the cup, both being tightly fastened with rubber rings from outside. 1.5 ml. of cell suspension with 3 /uc of algae C^{14}-protein hydrolysate, was poured onto the membrane and the asembled vessels were placed in crystalliser /Fig.6/. All vessels were placed under a hood. The chamber was continuously fed with a mixture of O_2 and CO_2 /95% and 5%/.

The medium was mixed in the cups by special magnetic mixers that were switched in at intervals by relay /for further particulars see 10/.

After incubation for 18 or 40 hours at 37°C the cells were collected together with the nutritional medium and homogenized. The homogenate was centrifuged, first at 4,000, then at 40,000 rpm and passed through a column with Sephadex G-25. Cellulose-fixed bovine globulin /as a non-specific immunosorbent/ was added liquid. After its removal by centrifugation cellulose-fixed HSA was added as a specific immunosorbent. Both immunosorbents were washed eight times and then bound antibodies eluated at pH 2.5. The eluate from fixed globulin usually failed to exhibit any radioactivity or the number of impulses did not amount to more than 3 to 5% as compared to that in the eluate from fixed HSA.

To estimate the size of molecules of antibodies synthesized in the cell culture the eluates were filtered through Sephadex G-22 together with the serum of the experimental rabbit /11/ in accordance with the procedure described by Flodin and Killander /5/ using a column 46 x 2.3 cm and Tris-HCl buffer /0.04 M- with 0.28 M NaCl/. The fractions leaving the column were analysed, their protein concentrations was determined by Lowry method and the radioactivity was counted in a gas flow counter for 500 seconds.

Results and discussion

The cells of the spleen and lymph nodes of hyperimmune rabbits under the conditions of cultivation applied in our experiments had synthesized fairly large amounts of antibodies. By means of immunosorbents in 13 experiments it was found that

that 40 hours' cultivation gave rise to 60 to 360 /ug antibody protein per one gram wet weight of the spleen.

The physico-chemical study of antibodies synthesized in the cell culture meets with considerable difficulties because there are soluble substances and fragments of cells disintegrated during cultivation in nutritional medium together with antibodies. This was, in particular, pointed out by Pospišil and Franěk /12/. The task is, however, facilitated if the synthesized antibodies are isolated by means of specific immunosorbents. This enables one to obtain preparations of high purity. In accordance with this procedure the radioactive antibodies synthesized in vitro were isolated in the experiments below by means of fixed HSA.

The size of the molecules of antibodies formed in the cell culture was determined by filtration through a column filled with Sephadex G-200. According to Flodin and Killander /5/ such a filtration leads to the division of all serum proteins into three main fractions with sedimentation constants 19 S, 7 S and 4 S. Therefore, by labelling a protein, say, with radioactive aminoacids and passing it with serum through Sephadex G-200, it would be possible to estimate approximately the molecular weight of the protein through localization of the tracer in a particular fraction.

In our experiments 1.0 ml. of the solution of radioactive purified antibodies synthesized in vitro was mixed with 0.5 ml. rabbit serum, passed through Sephadex G-200 column and the protein concentration and radioactivity of the fractions measured. The results of a representative experiment with antibodies synthesized by spleen cells on the third day following second immunization are given in Fig.2. It will be seen that the serum proteins diagler

three clear peaks. The greater part of radioactivity was accounted for by the first peak. This shows that radioactive antibodies had a higher sedimentation constant than that of the proteins of the second peak that are still held by the gel, or higher than 7 S. The rabbit is known to have antibodies with sedimentation constants of 7 S and 19 S only. Hence under conditions of this experiment most synthesized antibodies had a high molecular weight and their sedimentation constant was 19 S.

It was not alwys, however, that the first protein peak accounted for most radioactivity. In a number of experiments the ratio of radioactivity between the first and second peaks was different from above experiment /Fig.3 and 4/. This appears to be due to the fact that the amount of "heavy" antibodies synthesized depends on the individual reaction of the rabbit to the injection of antigen.

The second experimental series dealt with antibodies synthetized by spleen cells taken on the fifth day after the second immunization. In this case practically all radioactivity was accounted for by the second protein peak /Fig.5/. This means that only antibodies of the 7 S type were synthetized, as was to be expected.

The results obtained in the first experimental series were not due to artefact. This is substantiated by the following consideration. The radioactivity accounted for by the first protein peak did not result from any particles that would have been mechanically sorbed on cellulose during the isolation of antibodies by immunosorbents. As we had already mentioned, non-specific adsorbtion was either absent or very weak. Neither was the tracer in the first protein peak connected with aggregates

of 7 S antibodies that could appear in the process of their isolation. This is shown by sedimentation investigations of rabbit antibodies isolated by immunosorbents from sera on the seventh to tenth days after second immunization. These sedimentation experiments gave only a single peak with a constant 6.1 S /7/. Furthermore, if aggregates of antibodies had appeared on isolation, they would have also been observed in experiments with antibodies synthesized on the fifth day after the second immunization. We have, however, seen from Fig.5 that in these experiments practically all radioactivity was accounted for by the second protein peak, the sedimentation constant being essentially 7 S.

It can therefore be considered as established that on the third day after the second immunization of hyperimmune rabbits the cells of spleen and lymph nodes are capable of synthesizing antibodies of a high molecular weight /about one million/. On the fifth day, however, they cannot yet be found, only antibodies of 7 S type then being synthesized. It cannot be excluded that it is these "heavy" antibodies that exhibit the abnormal precipitation properties mentioned above.

It was suggested that 19 S antibodies were synthesized after second immunization by these cells had not yet come into contact with antigen and for which the secondary introduction of antigen was to be regarded as the primary one /2/.

This point of view however, is contradicted by the quick start followed by just as quick a depression of the synthesis of "heavy" antibodies in our experiments. According to Dixon et al. /4/ antibodies in rabbit serum after the first introduction of serum albumin appear not earlier than on the seventh day,

second day.

It is, therefore, more likely that "heavy" antibodies after second immunization are synthesized by cells that had already been in contact with antigen. From the third day after second immunization the amount of antibodies formed in vitro is fairly large and it was shown in our experiments that the percentage of "heavy" antibodies may, thus, be rather high. It would be reasonable to suggest that cells that synthesize "heavy" antibodies should have a specific structure similar to that of cells from the early stages of plasmatic series. It has been shown that many large pyronophylic cells resembling plasmablasts in structure can be found among cells of the spleen and lymph nodes of hyperimmune rabbits on the third day after the second immunization with HSA /8/.

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Legends to figures

Fig.1. The chamber and vessels for cell incubation.

Fig.2. Filtration through gel Sephadex G-200 rabbit serum and radioactive antibodies synthesized by spleen cells on third day after second immunization.

1-protein concentration, 2-radioactivity.

Fraction volume 0.9 ml.

Fig.3. Idem

Fig.4. Filtration through Sephadex G-200 rabbit serum and radioactive antibodies synthesized by cells of lymph nodes on the third day after second immunization.

Fraction volume 0.9 ml.

Fig.5. Filtration through Sephadex G-200 rabbit serum and radioactive antibodies synthesized by spleen cells on fifth day after second immunization.

Fraction volume 2.2 ml.

Development of Immune Reactions in the Absence or Presence of an Antigenic Stimulus

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The aim of this work was to differentiate serum factors with some immune activites, which develop spontaneously without dependence on an antigenic stimulus, from antibodies which arise in response to the injection of antigen. Under normal conditions(conventionally reared animals) antigenic stimuli are practically uncontrollable; they act in the organism, such as bacteria colonizing the intestine, respiratory tract and surface of the body. Moreover, food contains antigenic substances which are also a source of antigenic stimuli. Antibodies arising in response to them, without interference and control, are called natural antibodies. On the other hand, natural factors are present in the serum which have the capacity of interacting with macromolecular substances (e.g. alpha macroglobulin reacting with insulin, natural conglutinin with zymosan (1)). In this communication, the role of antigen in developing of the serum factors above mentioned is studied.

Experimental model. The newborn animals contain antibodies transferred from the mother and a great number of antigenic substances stimulating the maturing of its own immune mechanisms. The level of transferred antibodies does not usually permit detection of antibodies formed by the young animal itself. It is, therefore, of advantage to use a type of animal whose placental barrier does not permit the transfer of antibodies into the circulation of the foetus. If such animals (e.g. piglets) are artificially fed and do not obtain maternal colostrum, we do not find antibodies

passively transferred from the mother in their serum. In order further to exclude contact of the newborn animal with microbial antigens, the foetus is removed from the mother before birth under sterile conditions and put into an incubator where it is excluded from bacterial contamination.(Figs 1, 2, 3). However, the rearing of animals without microorganisms does not fully guarantee that antigenic stimuli are completely eliminated, because of contents of dead bacteria and other antigenic components in food. We have attempted to reduce this source of antigenic stimuli by using a nonantigenic diet (mixture of amino acids and vitamins) and also by observing the development of immune factors in the early postnatal period, when immune response to small doses of antigen is reduced. We consider that studies of natural and immune factors in animals which have not obtained antibodies by transfer from the mother and are reared under sterile conditions in the early stages of ontogenesis, is the best model for solving these questions at the present time.

The characterization of some serum fractions of sterile precolostral piglets. The electrophoretic pattern of piglet sera shows, in agreement with published results, that the newborn do not possess serum γ-globulin. After concentration, however, protein has been detected in the region corresponding to γ-globulin (2). Sera of precolostral piglets contain approximately 40 µg per ml. of this protein. The incorporation of methionine into the γ-globulin fraction of the newborn was demonstrated by the use of labeled methionine S^{35}. The rate of increase in radioactivity indicated the typical process of synthesis (3). The nature of γ-globulin in the newborn was studied by physico-chemical and immunochemical methods. Two fractions were separated on DEAE cellulose. A fraction (I) of the neonatal γ-globulin is not precipitated and has a sedimentation coefficient of 2.7 S. The other fraction (II) is precipitated

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with antiserum against the γ-globulin of adult pigs and has a sedimentation coefficient of 5.1 S (4). This fraction bears no relation to Bence Jones protein but a relation between 5 S neonatal γ-globulin and H chains of adult pig γ-globulin was determined by the fingerprint technique (5).

The evidence that antibodies are not present in precolostral piglet. Antibodies have not hitherto been demonstrated by any serological reaction to any bacterial, virus, phage or tissue antigens. No antibodies were detected by the agglutination reaction to the O and H antigens of various Gram-negative bacteria, nor by passive haemagglutination with antigens isolated from bacterial strains and bound on to sheep erythrocytes (6).

Passive haemolysis was carried out with erythrocytes, to which different antigens were adsorbed, not only with usual erythrocyte concentration (1%) but with progressively decreasing concentrations of erythrocytes (0.001%) (Fig.4). In this way the amount of antibodies necessary to produce haemolysis was greatly decreased, to the level 10^{-6} µg N/ml (7). The bactericidal reaction for which strains in the S-form were selected, has the same degree of sensitivity (8). Even detection of antibodies by opsonization of strains in the S-form (at the level of 10^{-5} µg N/ml) with sera of newborn precolostral piglets was negative. The sera do not display neutralization activity to phage (the experiments made by Dr.Trnka) neither neutralization activity against poliomyelitis viruses (Dr.Slonim). Antibodies were not only not found in the serum but also not in the concentrated γ-globulin fraction even on using passive haemagglutination and passive haemolysis with adsorbed diphtheria toxoid as in the experiments of Segre (9). We conclude, therefore, that the protein of the γ-globulin type in precolostral newborn piglets has not any detectable antibody activity.

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Although antibodies were not detected in newborn piglet sera, a certain amount of complement was demonstrated immediately after birth by the haemolytic reaction (about 2 $C'H_{50}$ units). The amount of complement increased gradually in animals reared under sterile conditions; 20-day old piglet had 15 - 20 C'H units/ml. (9).

The immunological properties of piglet sera without the presence of antibodies. Some changes in antigen suspension similar to antigen-antibody reaction can be observed without the presence of antibody: bacterial agglutination at low pH, haemolysis by complement of erythrocytes previously treated with tannic acid, etc. The data will be presented that complement can act on certain bacterial surfaces even in the absence of antibodies.

a. Bactericidal reaction: In experiments using strains in the typical S-form, e.g. strain of Salmonella paratyphi B, we never detected bactericidal activity in precolostral piglet sera. On testing a larger group of Gram-negative bacteria, however, we found that these sera exert a bactericidal effect on some bacterial strains. These strains, which were sensitive to piglet complement, were in the typical R-form. On absorbing sera at 0^{o} with R strains the bactericidal effect of serum was not abolished; it was abolished, however, if any components of complement was inactivated. In selected strains it was found that there is a direct relationship between the character of their surfaces (R-form) and the degree of dilution of test serum still capable of bactericidal activity (Table 1). We further demonstrated that there is a dependence of the amount of piglet complement, the character of the bacterial surface and the bactericidal effect: sera with a small amount of complement only have an effect on strains in the R-form (Table 2). Higher complement levels (5 $C'H_{50}$ units) act against strain 346 which has the characteristics of bacteria in the S-form and which is not sensitive to sera with

a lower complement level. Bactericidal activity is thus determined by the surface of the bacteria and not by its specific antigenic structure (the series included strains of coli, S.typhi, Shigella shigae, etc.). At the same time as using precolostral piglet serum we also worked with precolostral calf sera. It was shown that their bactericidal effect against some strains, e.g. Shigella shigae, is higher than would have been expected from the relationship between calf and piglet serum (Fig. 5). We therefore used different serological methods to determine whether antibodies to the Shigella strain were not present in the calf serum. We found that with O antigen adsorbed to erythrocytes (antibodies to this antigen are responsible for the bactericidal reaction) and passive haemolysis done with minimal numbers of erythrocytes (0,001%), the demonstration of antibodies by this method is more sensitive than the bactericidal reaction (T.3). We conclude that if antibodies are demonstrated in newborn serum by the bactericidal reaction only and not by the more sensitive passive haemolysis with minimal amounts of erythrocytes, precolostral serum probably does not contain antibodies. We further found that the bactericidal activity of precolostral serum can only be abolished if it is absorbed with zymosan in the presence of complement. If native calf serum is treated with zymosan, the bactericidal action disappears; after adding piglet complement to the absorbed calf serum in a concentration which does not itself have a bactericidal effect, bactericidal activity was not changed and the bactericidal effect was not present. On the contrary, in calf serum first inactivated at 56°C and treated with (Fig.6) zymosan to which the same amount of piglet complement had been added after inactivation, bactericidal activity was almost fully restored. Our contemporary programme is to establish whether absorption of sera does not affect some components of complement which were restored on adding neonatal complement or whether precolostral sera contain

a substance with properties such as properdin or natural conglutinin.

b. Opsonizing effect of precolostral piglet serum:

A study was made of the opsonizing activity of neonatal precolostral piglet serum to strains in the S- and R-form. Phagocytosis was determined by perfusion of the isolated liver 10) and by the method of determining the time course of bacterial clearance from the circulation in neonatal piglets. Strains, which are in the typical S- and R-form were selected for the study of opsonization (Table 1). The concentration of the bacterial suspension for opsonization was 5×10^4 bacteria/ml. 0.1 ml of this suspension was mixed with 0.9 ml. test serum and left for one hour in the refrigerator. Bacteria were introduced into the afferent cannila leading to vena portae and the number of bacteria not taken up by the liver was determined in samples taken off from the inferior vena cava. Opsonization was carried out in the same way in clearance tests in vivo and 10^5 bacteria/kg. body weight were injected into the blood stream of piglets. The bacterial count was determined in blood samples obtained by cardiac puncture at different time intervals after injection.

In the first experiments on the isolated rat liver it was found that the uptake of E.coli suspended in Ringer solution is dependent on the character of the bacterial surfaces. An average of only 10% of the S-form are retained, whereas in the R-form the number retained amounts to 55 - 64%. In experiments in which E.coli (S-form) were opsonized with neonatal serum containing 2 - 3 units of $C'H_{50}$ complement, it was found that complement has an opsonizing effect and increases the number of bacteria retained from 10 to 48 %. Any inactivation of complement components abolished the opsonizing activity (Table 4) (11).

In further experiments phagocytosis was determined by the method of clearance of bacteria from the blood stream in neonatal precolostral piglets. Evidence was provided that

E.coli in the S-form is not phagocytosed in the newborn (Fig.7). On the other hand if the R-form of the strain is injected into neonatal piglets it is effectively cleared from the blood stream (Fig.8). In order to determine the minimal amount of antibodies for opsonization and phagocytosis of coli in the S-form, we mixed a bacterial suspension in the S-form with different amounts of rabbit antibody. It was found that dilutions of 10^{-8} - 10^{-9} of antiserum still produce a full opsonizing effect displayed by the complete clearance of the injected bacteria from the blood stream. Dilutions of 10^{-10} and 10^{-11} lead to only a partial and transient decrease of the number of circulating bacteria (Fig.9). Since the same strain mixed with neonatal serum containing a small amount of complement did not have an opsonizing effect it shows that the precolostral sera of neonatal piglets does not contain antibodies detectable by this very sensitive test (by which antibodies are determined in a concentration of about 10^{-6} µg N/ml). This experiment, therefore, shows that the sera of animals in which no antibodies have been demonstrated by any method, contain complement which acts on some bacterial surfaces and is thus able to imitate immune processes for which the presence of antibodies has hitherto been considered to be necessary.

The development of antibody formation in sterile animals after immunization with different antigens:
The study of antibody formation in sterile precolostral piglets has a number of advantages. Since there is no transfer of γ-globulin from the mother and there is no level of natural and passively acquired antibodies, the first antibodies detected are formed by the infant animal itself. Since the serum contains only a small amount of neonatal γ-globulin of low molecular weight, the character of the [illegible] antibodies can be well determined.

a. Time course of onset of [illegible] formation after immunization with different antigens: Newborn precolostral piglets were given an injection of S.paratyphi B and antibodies detected mainly using the bactericidal reaction permitting detection of 10^{-3} mg of antibody N/ml. The newborn animals were given i.p. injections of the maximum dose of antigen that they tolerate, i.e. 2.5×10^{10} bacteria in 5 ml. The first antibodies were but rarely detected 5 days after immunization. The amount of antibody increased to the 10 - 15th day after the injection of antigen. If sheep erythrocytes are injected (20% suspension in amounts of 10 or 20 ml) antibodies appear earlier, in most animals on the fifth day after the injection of antigen. However, we did not succeed in demonstrating antibody on the third day after injection, even using very sensitive tests. For demonstrating antibodies by the haemagglutination reaction we use a 0.1% suspension of erythrocytes, for demonstrating antibodies by the haemolytic reaction a 0.001% suspension. Very similar results - i.e. negative demonstration of antibodies on the third day after the injection of antigen and reliable demonstration on the fifth day - were obtained after immunization with the corpuscular antigen of phage T 2 and virus (Sabin attenuated vaccine strain). A comparison of the results after immunization with the different antigens is given in the table 5, Fig. 10. It is evident that the smallest immunizing effect was displayed by the bacterial antigen S.paratyphi B. If it cannot be objected that the different results in the formation of antibodies express different sensitivity of the bactericidal reactions to all antigens (the used test [illegible] was at the level of 10^{-6} mg/ml) [illegible] to consider the quality and quantity

It is [illegible] the amount of antigenic substance [illegible] the [illegible] of corpuscular antigens [illegible] haemagglutinating and virus and phage neutralizing [illegible] with a certain

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part of the whole injected corpuscle. We, therefore, wished to know whether the previously observed finding that increasing the amount of antigen speeded up the onset of antibody formation in young animals (12) would be valid for neonatal piglets. From the results (Fig.11) it is evident that increasing the amount of injected erythrocytes and phage (Fig.10) led to the earlier onset of antibody formation and also to a higher resulting titre.

The effect of the passive transfer of antibodies for the onset of antibody formation in infant animals has often been discussed and investigated in a number of works. Most work has shown that the transfer of antibodies delays the onset of immunization processes. It was found only rarely that the passive transfer of antibodies has a stimulating effect. In the work of Segre (13) on the same model of precolostral piglets, the passive transfer of antibodies was considered to be the basis of a good onset of an immunity response in infant animals. In all the antigens used we obtained a good response in precolostral piglets. We made a comparison of the immunizing effect of the same amount of erythrodyte antigen in piglets reared conventionally with the mother and in sterile precolostral piglets. The table shows that the passive transfer of antibodies inhibits to some extent the onset of the actual immunizing process in infant animals (Table 5, Fig. 12).

We further wished to find out the earliest onset of antibody formation and whether there is an increased immune response to different antigenic stimuli during the maturing of the individual in infant animals reared non exposed to antigens. We were unable to detect antibody formation after immunizing the foetus in utero one month before term (gestation lasts three months in pigs) using S.paratyphi B and Brucella suis as antigens; these results were published in 1960 (6). In recent years we have used sheep erythrocytes for intrauterine immunization and have demonstrated antibody formation immediately after birth in piglets immunized one month before term. In some animals, however,

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antibodies were not detected at birth after intrauterine immunization. This could have been because the level of antibodies formed was not high enough or because antibodies which could have been detected during the month disappeared before birth. If such an animal is given the same injection of antigen immediately after birth as is given to newborn animals not immunized in utero, the onset, course and level of antibody formation has the typical character of a secondary reaction (table 7). If the same dose of antigen as the first immunizing dose (10 ml. of 20% sheep erythrocytes) is injected into young animals of various ages reared under sterile conditions, we do not observe a significant increase in the immunizing effect which would show that marked changes in the ability to respond to antigen during ontogenesis occur without an antigenic stimulus. This finding has obviously a parallel in the number of antibody producing cells detected, as will be reported later.

b. Characterization of the first antibodies formed: A determination was made of the molecular weight of the first antibodies in the sera of precolostral sterile piglets. Antibodies to erythrocytes, phage and strain S.paratyphi B were centrifuged in a saccharose gradient in a Spinco rotor 40 centrifuge at 35,000 r.p.m. for 16 hours. Six layers were successively separated in which the bactericidal, haemolytic and neutralizing activity of antibodies were tested. In all samples, antibodies were found in the last, i.e. the fifth and sixth fractions at the bottom of the tube. These tests confirmed a whole series of similar work that the first formed antibodies in newborn animals are of the macromolecular type (14,15).

The first detectable antibodies, however, are not characterized by this property only, but also by serological properties. If antibodies to erythrocytes are determined by the test described, in which the sensitivity is increased

by lowering the erythrocyte concentration, we do not find that the increased sensitivity of the serological reaction has a linear dependence as in the sera of hyperimmune adult animals (Fig.13). The results show that infant antibodies have a different binding capacity from that of the antibodies of hyperimmune adult animals. This could be due to the fact that they are mainly 19 S antibodies. We therefore isolated antibodies of the 19 S character from adult hyperimmunized animals on Sephadex G-200 (16) and determined the dependence of the amount of antibody essential for 50% haemolysis at different concentrations of erythrocytes. In 19 S antibodies of adult animals we found a linear dependence, i.e. within the class of one molecular type of antibody the binding capacity changes. This was also confirmed for 7 S antibodies. The antibodies formed immediately after birth during the secondary reaction, according to their sedimentation characteristics in a sacharose gradient, are antibodies of the 7 S type. These antibodies however, when tested for binding capacity by the test using a decreased number of erythrocytes, also failed to show a linear dependence. Again the reverse to 7 S antibodies of adult animals.

Differences in the binding capacity in the first antibodies of immunized piglets were found with anti-phage sera. It was found that the antibodies have a very small neutralizing capacity, which, however, is considerably(?) increased if complement is added to the test. The source of complement was the serum from precolostral non immunized piglets which itself has no neutralizing activity to phage. Here complement evidently acts as a cofactor which strengthens the binding of easily dissociable antibodes on the phage particle similarly as in the first antiviral antibodies described by Dulbecco (17).

A further characteristic property of the first antibodies in newborns is their different sensitivity to mercaptoethanol (ME). Hyperimmune sera were tested and it was found that different concentrations of ME used by some authors (18, 19), for the inactivation of 19 S antibodies (1 M, 0.1 M, 0.05 M) do not exert a different effect. After the action of these concentrations we found the same titres, i.e. the same decrease in the initial haemagglutination reaction. If, however, we investigate the first antibodies of infant animals, the serological activity of antibodies disappeared after treatment with the given concentrations of mercaptoethanol. If sera obtained from infant animals reacting after birth with a secondary reaction (i.e. those which were immunized in utero) are treated with 0.05 M ME, the titre of antibodies is only partly decreased. However, 1 M concentration of ME, which has the same effect on hyperimmune sera as 0.05 M ME completely abolishes the serological activity of infant 7 S antibodies.

The results obtained with immunization of the foetus and newborn with different antigens provide evidence that the character of the antigen is of importance for the stimulation of the antibody response as well as the amount of antigen injected. Unlike the proteins found in the serum of nonimmunized newborn piglets in the γ-globulin zone, which have a low molecular weight of about 5 S, the first antibodies formed are macromolecular antibodies with different binding capacities from those of adult animals. Newborn piglets are able to react with a secondary reaction immediately after birth if the first dose is given in utero. These antibodies appear to be of the 7 S type. But their properties still show some characteristics of primitive antibodies (low binding capacity, sensitivity to mercaptoethanol). It can therefore be assumed that the change in molecular type, like increased resistance to ME and changes in binding capacity, will not occur at once

but that there will evidently be further antibodies of intermediate molecular characteristics.

The present results permit a more exact distinction between the antibody and nonantibody natural components of sera. Antibody is not only substance bound to a certain group of proteins of sera (according to present knowledge to γ-globulin) but is the result of adaptation processes in the organism after contact with antigen. The adaptation process is characterized by the formation of molecules of increasing fitness and effectiveness in their reaction with antigen. It is just these properties that distinguish antibody from the nonantibody components of the serum which are present and have a certain binding capacity with some macromolecular substances - even imitating the reaction of antigen with antibody - but which are formed spontaneously and their properties do not undergo any change after antigen injection.

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Table 1

	Coli strains in S-form				Coli strains in R-form		
	378	346	055	322	16	3A	283
Unstability in suspension heated 100°C - 1 hr	-	-	-	-	+	+	+
Agglutination in acriflavine solution 1 : 500	-	-	-	-	+	+	+
Electrophoretic migration velocity mm/3 min.	24	20	20	61	55	62	80
Phagocytosis: % of bacteria removed by liver perfusion	11	16	30	14	64	55	89
Dilution of serum for 50% bactericidal effect — precolostral piglet	0	0	0	0	1:128	1:128	1:0
Dilution of serum for 50% bactericidal effect — precolostral calf	1:1	1:2	1:1	0	1:256	1:256	1:5

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Table 2

Dependence of bactericidal activity of piglet sera containing different amounts of complement on the surface character of bacterial strains

1/50 units/ml of piglet sera	S-form →					R-form
	coli 127	coli 346	coli 378	Shig	coli13A	coli
1	0	0	0	1:1	1:8	1:8
1,5	0	0	0	1:1	1:8	1:16
2,2	0	0	0	1:2	1:8	1:16
3,1	0	0	0	1:4	1:32	1:32
5	0	1:1	1:1	1:6	1:64	1:64

Table 3

Demonstration of antibodies to Shigella by different methods

	reaction	dilution of serum
anti-Shigella rabbit serum	bacterial agglutination	1 : 1280
	passive hemagglutination 0,1%	650.000
	passive hemolysis 0,25%	65.000
	passive hemolysis 0,1 %	280.000
	passive hemolysis 0,001 %	1,500.000
	bactericidal reaction	500.000
newborn calf sera	bactericidal titer	32, 16, 8, 64
	passive hemagglutination 0,1%	0
	passive hemolysis 0,001 %	0
newborn piglet sera	bactericidal titer	1, 2, 1, 1, 1
	passive hemagglutination 0,1%	0
	passive hemolysis 0,001 %	0

Table 4

Uptake of E.coli 346 by the perfused rat liver suspended in Ringer-Locke and after opsonization by complement of newborn colostrum free piglets

Experimental conditions	Total number of experiment	% of bacterial uptake average	% of bacterial uptake maximum - minimum values
0,1 ml. E.coli 346 + 0,9 ml. Ringer-Locke	14	10	(28 - 0)
0,1 ml. E.coli + 0,9 ml. native piglet serum (p.s.)	8	48	(63 - 37)
0,1 ml. E.coli + 0,9 ml. p.s. inact.30 min. at 56°C	5	9	(24 - 2)
0,1 ml. E.coli 0,9 ml. p.s. + EDTA	5	12	(21 - 7)
0,1 ml. E.coli + 0,9 ml. p.s. absorbed by zymosan	5	10	(18 - 0)
0,1 ml. E. coli + 0,9 ml. p.s.treated with 2ME	3	12	(23 - 2)

Table 5

Antibody formation in precolostral sterile piglets immunized with different antigens immediately after birth

The type and amount of antigen		Days of life 3	5	7	10	
Salmonella paratyphi B type 10^9 heat inact. bacteria		0	0	0	1:1	*
		0	0	0	1:1	
		0	0	0	2	
		0	0	0	8	
		0	0	1:1	10	
Sheep red blood cells 20 % v/v	10 ml	0	0	16	256	**
		0	2	16	64	
	20 ml	0	4	16	256	
		0	2	16	64	
T 2 phage particles	10^7	0	40	320	320	***
	10^9	0	80	640	1280	

* 50 % bactericidal reaction with S. paratyphi B estimated at 3 hours after incubation at 37°C

** Hemagglutination with inactivated sera (for 30 min. at 56°C) with 0,1 % suspension of sheep red cells

*** 50% inhibition test in the presence of precolostral calf serum complement diluted 1 : 5

Table 6

Comparison of formation of antibodies to sheep erythrocytes in precolostral and colostrum-fed piglets

	1.0% suspension of sheep erythrocytes	Days after immunization				
		0	3	5	7	9
Pre-	10 ml	0	0	0	1:16	1:256
colost-	10 ml	0	0	1:16	1:16	1:256
ral	20 ml	0	0	0	1:16	1:64
	20 ml	0	0	1:2	1:16	1:64
	10 ml	1:16	1:16	1:8	1:16	1:32
Colost-	10 ml	1:128	1:8	1:8	1:16	1:8
rum-	20 ml	1:32	1:16	1:8	1:8	1:8
fed	20 ml	1:32	1:16	1:16	1:8	1:8

Table 7

Primary and secondary response to sheep red cells in newborn piglets

Primary				Secondary			
Age in days	serum	serum treated with ME 0,05 M	1 M	Age in days	serum	serum treated with ME 0,05 M	1 M
0	0	-	-	RBC 0	0	-	-
5	0	-	-	→5	0	-	-
9	2	-	-	9	16	0	0
10	2	-	-	10	1024	512	0
11	4	-	-	11	8192	2048	0
13	8	-	-	13	8192	1024	0
15	128	0	0	15	8192	2048	0
17	256	0	0	17	4096	512	8
19	128	0	0	19	2048	-	-
21	256	0	0	21	4096	-	-

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Table 8

Effect of complement on 50 % phage neutralization

Days after immuniz. with 10		serum dilution									
		1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2
	5	11%	15%	28%	34%	82%					
	10	14%	17%	15%	22%	28%	38%	50%	67%	67%	71
	15	6%	17%	20%	21%	20%	22%	42%	62%	82%	92
	5	33%	75%								
	10	30%	58%	69%	103%						
	15	33%	69%	112%							

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