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annual report of a Polish research project entitled "Studies on Variability and Genetics of Rhizobium" being conducted at the M Curie - Sklodowska University located in Lublin. Jadwiga Ziemiecka and Zbigniew Lorkiewicz are the principal investigators of this research. The period covered is 1 Jan-31 Dec 62. OFFICIAL USE ONLY

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3. Studies on Variability and Genetics of Rhizobium

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Summary.

Our general purpose is to carry out fundamental studies on the genetics and variability of Rhizobium, which in these bacteria is exceptionally high. On the basis of our results we shall try to obtain by different means strains of Rhizobium with a high nitrogen fixing power.

We have started our experiments on October 1, 1960. From 10.1.1960 to 12.31.1962 we have performed complex investigations on the cytological, physiological, serological and genetical properties of about 150 strains of nodule bacteria.

Besides that we have collected phage strains and isolated bacterial and phage mutants.

Cytological studies.

Strains of Rhizobia were grown on sap from leaves or from roots of homologous plants. We have obtained in these cultures transient bacteroid-like forms. Using continuous microscopic observations we found, that these bacteroid-like cells had still the ability of division and some of them were motile. Besides this, intensive cytochemical studies of nodule bacteria in normal cultures or treated with gamma rays or radiomimetic substances were carried out. After irradiation surviving cells had a specific shape and internal structure.

Physiological studies. We have determined the ability of various strains of Rhizobium to ferment sugars and alcohols. Besides that investigations were carried out in order to identify the aminoacids, vitamins and organic bases requirements of

Rhizobium. Studies were also carried out in order to determine the influence of the composition of bacteriological medium on the symbiotic activity of slow growing Rhizobia. Recently, the formation of indoleacetic acid by different strains of Rhizobium was studied.

Serological studies.

Investigations were performed on cross agglutination between H antigens as well as O antigens of various strains of Rhizobium. Using agglutination test with flagellar or somatic antigen a relationship was noted among strains of nodule bacteria for clover, peas and vetch.

Recently we have started to examine the antigenic structure of Rhizobium by means of agglutination test of absorbed sera. In these studies strains for clover, vetch and peas are included.

Studies on rhizobiophages.

For our virological studies we have isolated about 200 strains of Rhizobium in pure culture and 100 strains of bacteriophages. The influence of some physical and chemical agents on bacteriophages was investigated. Some differences were noted between phage groups in respect of their sensitivity to particular factors. A preliminary classification of 50 strains of Rhizobium by means of phages was performed. On the basis of typing with phages our strains may be divided into 8 different groups. Besides phages isolated from nodules and soil, about 30 45 spontaneous mutants of phages were obtained for lysotypia.

On the basis of their plaque morphology these mutants can be divided into 3 groups.
Our Rhizobium strains for alfa-alfa were tested for their lysogeny. Using the routine procedure we obtained 75 phages. Some of them are probably temperate.

Genetical studies. In order to study genetical properties of nodule bacteria we isolated about 70 mutants of Rhizobia. Besides antibiotic resistant strains, morphological mutants were also studied. From smooth and mucoid forms /SM/, several rough strains producing a small amount of gum /RF/ were isolated. Furthermore 2 rough, non mucoid /R/ mutants were obtained. Physiological and serological properties of morphological mutants were studied. The antibiotic resistant or rough mutants were used in our investigations on genetical transformation. By means of DNA isolated from streptomycin resistant strains it was possible to transfer this resistancy to the streptomycin sensitive strains. Morphological mutants were especially suitable for these studies. The highest frequency of transformation was 0,01 - 0,08%. We are trying to determine the properties of our rhizobial strains by means of lysotypia, physiological or serological methods. Some of the used tests may appear suitable for a quick differentiation of active and inactive strains. The plant test, which is at present the most commonly used, requires at least 2 months for estimation of the ability of nitrogen fixation of Rhizobium strains. We are looking for more convenient methods for the

determination of strains effectiveness. Besides that we are trying to obtain very effective strains either using mutagenic agents /Ultraviolet light, radiomimetic substances/ or genetical methods.

Physiological properties of Rhizobium.

Early investigators reported the reduced efficiency when Rhizobium strains were cultured on media containing aminoacids. In 1956 H o l d i n g /1/ showed, that the effectiveness of Rh.melilcti and Rh.trifolii strains were reduced by serial transfers on a medium added with glycine. Similar investigations were carried out by T i l o /1/, who studied the effect of alanine, valine, leucine and glycine on symbiotical activity of Rh.leguminosarum, Rh.trifolii and Rh.meliloti. W o l f and B a l d w i n /2/ observed, that strains of Rh.trifolii cultivated in glycine medium lost their ability to incite nodule production.

In order to study the influence of different aminoacids in laboratory cultures of Rhizobium, on the effectiveness of their symbiotical activity in plants, pot experiments were carried out during the year 1962. The plants used were - Soybeans and yellow Lupine.

The plants were inoculated with active strains of Rh.lupini and Rh.japonicum, which were cultured beforehand during 1 year on media containing:

1. yeast extract, 2. casein hydrolysate, 3. a mixture of aminoacids such as contained in the casein hydrolysate 4. a mixture of glycine, alanine and tryptophane, 5,6,7 - aminoacids as in 4, but added separately. Although none of the strains lost the ability to incite nodule production, strains from culture on alanine show reduced infectiveness. At the flowering stage plants were harvested and weight.

The N-content of whole plants was analysed by Kjeldahl method.

The results are presented in figure 1 .

Main results with yellow Lupine.

The best growth and Nitrogen content of this plant was obtained in the series inoculated with Rh.lupini /strains "Cz" and No 271/ cultured on caseine hydrolysate or on tryptophane. Cultures derived from yeast extract media /ser.1/ influenced the plants also well, but to a smaller degree. Cultures on mixtures or on single aminoacids /ser. 3,4,5,6/ gave no satisfactory results. A specially high decrease in the effectiveness of Rh.lupini was noted in series inoculated with its strains grown on alanine or on glycine.

Soybeans.

The best growth and N-content were found in series inoculated with Rh.japonicum /strains 94p, 102c/, cultured on yeast extract /ser.1/. Cultures on caseine hydrolysate or on tryptophane /ser.2/and /ser.7/ were next in their effectiveness. Other aminoacids had a depressive effect, especially marked in case of the alanine series /ser.6/.

It seems therefore, that the best effectiveness of slow growing Rhizobium strains can be obtained through their culture, either on caseine hydrolysate or on yeast extract. Their culture on a mixture or on single aminoacids is not useful in that respect, with the exception of tryptophane.

Our observations agree with the earlier ones: rhizobial ineffectiveness and effectiveness may be affected by the kind of culture media. It is known that Leguminous nodules are reservoirs of various aminoacids /H u n t 1951/3/ S e n

and B u r m a 1953/4/. It seems that during the culture of Rhizobium on media containing amino acids the nitrogen fixing process may be inhibited /H o l d i n g, T i l o and A l l e n 1960/1/. In our experiments it was observed that some aminoacids which stimulated the multiplication of slow growing strains in their cultures, are not suitable for preparing the plants inoculants.

The auxin production by different strains of Rhizobium was reported by many authors R o b e r t s /5/, T h i m a n n/6/, K e f f o r d /7/, G e o r g i and B e g u i n /8/, T h i m a n /6/ emphasize the role of auxin in nodule formation and growth. As the presence of beta-indoleacetic acid in Rhizobium can influence the effect of this microorganism on leguminous plants our investigations were carried out with respect of auxin formation in differently treated cultures of Rhizobium. Following strains of Rhizobium: 3 strains of Rh. trifolii, 3 strains of Rh. melliloti and 3 strains of Rh. japonicum were grown in liquid media containing various nitrogen compounds: yeast extract, caseine hydrolysate, mixture of aminoacids: glycine, alanine, valine, leucine, proline, phenyloalanine, aspartic acid, glutamic acid, tyrosine and cysteine, tryptophane or serine being added separately. Beta-indoleacetic acid /I.A.A./ in cultures was detected by means of paper chromatography, according to V a n u r a and M a c u r a's /9/ modification /1960/.

The spots were identified by comparing them with standarts with various concentrations of I.A.A. It was observed, that the presence of tryptophane was necessary for I.A.A. formation.

Beta-indoleacetic acid was detected not only in liquid cultures of two effective strains of Rh.trifolii but also in the culture of one parasitic strain. From among 3 strains of Rh.meliloti only 1 strain gave on chromatograms a detectable spot of I.A.A. Cultures of Rh.japonicum /3 strains/ did not convert tryptophane to I.A.A. and did not produce this auxin in our experimental conditions.

The age of Rhizobium cultures plays a considerable role in I.A.A. production. It was noted, that for the estimation of beta-indoleacetic acid production 10 to 15 days old cultures of Rhizobium were most suitable. In older cultures a degradation of I.A.A. was observed. Quantitative determinations of I.A.A. could not be accomplished in Rhizobium cultures by paper chromatography. Further experiments are planned in that respect.

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Serological studies.

In further serological studies on Rhizobium 4 antisera against Rh.lupini were obtained. The antigens of Rh.lupini /strains isolated from lupine or serradella/ were agglutinated only by homologous immune sera. Heterologous antigens of Rh.trifolii, Rh.leguminosarum, Rh.melliloti, Rh.phaseoli, Rh.japonicum, Rh. sp. from Arachis and Rh.sp. from Anthyllis vuln. did not produce any cross agglutination with antisera of Rh.lupini. The results are given in table 1.

In our earlier investigations a serological relationship was shown among strains of Rh.trifolii and Rh.leguminosarum for peas and vetch. The purpose of the present research was to investigate thoroughly serological properties of somatic antigens of these Rhizobia.

For this purpose somatic antigens were prepared from 42 strains of Rh.trifolii and from 18 strains for Rh.leguminosarum. For preparation of O antigens overnight cultures of Rhizobium strains in liquid Thornton medium were washed 3x and heated for 2,5 hrs at 100°C.

Cross agglutination tests were carried out using 12 different O-antisera, which were obtained from rabbits immunized with O antigen from 9 strains of Rh.trifolii and 3 strains for Rh.leguminosarum. The results are presented in tables 2a, 2b and 2c.

The results presented in these tables show, that some of the studied strains are serologically related with heterologous strains belonging to other physiological groups

of Rhizobium. Nevertheless, important antigenic differences exist not only among the strains of diverse groups but also within the same physiological group.

Recently we started to study antigenic properties of Rh.trifolii and Rh.leguminosarum using for this purpose cross agglutination tests and antigens prepared by various methods. In order to examine antigenic structure absorption tests of immune sera were also applied.

The preliminary results of the absorption tests o. Rh.leguminosarum from vetch antisera by somatic antigens either of Rh.trifolii /8 strains/ or of Rh.leguminosarum /3 strains/ are given in table 3.

A modification of K a u f f m a n n's /1954/ method was used for this purpose. Antisera diluted to 1:5 with a saline containing 0,5 p.c. of phenol were mixed with an equal volume of a bacterial suspension. These mixtures were incubated at 37°C for 6 hours and transferred to refrigerator for 12 hr /4°C/. The absorption of antiserum was repeated twice.

The results of the absorption tests of the serum against vetch strain by heterologous strains are given in table . The serum absorbed by an antigen of peas strains agglutinated notonly the homologous strain from vetch but also some strains from clover. However, every of the tested clover strains absorbed all antibodies in the studied serum against peas strains; but the absorbed serum agglutinated still homologous vetch strain. On basis of these results it may be assumed that Rhizobium strains for vetch have besides the specific antigen

a common one for vetch, peas and clover strains.

The results obtained in the present research are similar to these of S t e v e n s /1923/, P u r c h a s e, V i n c e n t and W a r d /1951/, V i n c e n t /1953/, V i n c e n t /1954/. It was shown in studies of these authors that the group of Rhizobium trifolii strains is heterologous in respect to the structure of somatic antigen. F r e d, B a l d w i n, M c C o y /1932/, V i n c e n t /1942/, R e a d /1953/, V i n c e n t and W o t e r s /1953/, K l e c z k o w s k i and T h o r n t o n /1954/ demonstrated, that strains isolated from various nodules of the same plant might be different in some respect or even not related at all in agglutination tests. P u r c h a s e, V i n c e n t /1949/, P u r c h a s e, V i n c e n t and W a r d /1951/, V i n c e n t /1954/ concluded on basis of their studies, that the somatic antigens of Rh. trifolii were characterized by higher specifity than their flagellar antigen.

At present a detailed analysis of the somatic antigens of the bacteria of this serological group is being carried out, based on cross agglutination with somatic antigens obtained in various ways, and on the saturation of antibodies in the sera of those strains which give a positive reaction in the cross agglutination test.

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Genetical studies.

First reports on the nodule bacteria dissociation are due to I s r a i l s k y et al. /1930, 1933/ A l m o n and B a l d w i n /1933/ obtained some divergent types of Rhizobium from filtrates of Rhizobium and filtered phage lysates. K l e c z k o w s k a /1950/ isolated morphological mutants from the phage resistant cultures of Rh.trifolii. J o r d a n /1952/ used for this purpose X-rays, Ultraviolet irradiation and diazomethan. Balassa and G a b o r /1961/ obtained an X R-mutant using UV-light irradiation.

Numerous papers on the antigenicity of bacterial fractions were published. Some of them were concerned with the antigenicity of DNA. There is a controversy among the investigators in respect of its antigenicity /B r a u n 1958, O l i t s k i 1959, T a r n o k 1960, H i l g e r t 1961, H e r m a n 1960, B l i x 1954, C z a m o v a 1958, J a b ł o ń s k i 1961/.

Recently the workers at the Brandeis University demonstrated, that thermally denatured DNAs of T-even phages of E.coli - had antigenic properties.

The antigenic structure of morphological mutants of Rhizobium and of their nucleic acid fractions remain still obscure.

During the reported period about thirty streptomycin resistant and morphological mutants of Rhizobium were obtained.

Furthermore we are trying to isolate mutants requiring for their growth aminoacids, vitamins or organic bases.

For this purpose Lederberg's method is applied.

In addition investigations on antigenic structure of morphological mutants of Rhizobium trifolii were carried out.

For these studies following strains were chosen: a smooth, mucoid strain /SM/, a rough, nonmucoid mutant /R/, and rough strain producing a small amount of gum /RF/.

The antigens of these mutants were prepared from rhizobial cultures grown in WW1 medium. Of the following composition: H_2HPO_4 - 5,0g; KNO_3 - 2,0; $MgSO_4 \cdot 7H_2O$ - 2,0g; $CaSO_4$ -1,0g; $FeCl_3$ - 0,1 g; casein hydrolysate - 50 ml, glucose - 5,0 g; H_2O up to 1000 ml, pH 7,0.

In the studies on the morphological mutants following antigens were used:

1. W-antigen. To 3x washed rhizobial suspension equal volume of aceton was added. The precipitated bacteria were repeatedly washed with aceton and collected on filter paper. The bacteria were dried, and a subsequent extraction of antigen took place. 1g dry-weight of bacteria was

suspended in 50 ml H_2O dist. with subsequent shaking at 4° for 24 hr. After dialysis and centrifugation, the supernatant was used as W-antigen.

2. H-antigen - was obtained by the treatment of Rhizobium with 0,1N NaOH.

3. P-antigen was obtained by the Campbell method.

In studies on the antigenicity of nucleic acid fractions a preparation of DNA was obtained by lysis of Rhizobium suspension using 1 p.c. sodium deoxycholate or 3 p.c. sodium laureate at 70° for 1 hr.
The precipitated DNA was deproteinized using chloroform abd butanol./Sevag procedure/. This was repeated many times /18x/ in order to remove proteins. In some experiments the phenol method of Gieorgisw was used with subsequent deproteinization by Sevag procedure. These preparations were marked as:"native DNA". The homogenate was obtained by disintegration of rhizobial suspension with ultrasonic vibrations followed with sodium deoxycholate treatment. Rabbits were immunized by administration 20 ml of either antigen.

Besides native DNA and ultrasonic homogenate, following antigens were used in the diffusion tests in agar:

DNA-distreptase= native DNA treated with distreptase.

Single stranded DNA=native DNA heated at 98° for 20 min. and rapidly chilled.

Glucose-DNA= native DNA with addition of 100 γ glucose/ ml DNA, heated at 98° for 20 min. and cooled slowly /8 - 10 hr/.

Somatic antigen was isolated using the Campbell method /1954/.

The rhizobial slime was obtained by centrifugation of the culture at - 12000 rpm. for 1 hr.

Proteins were determined by the Lowry method; DNA and RNA contents were measured using Burton and Cerriotti's procedure respectively. Polysaccharides were estimated by the enthrone method.

The precipitation reactions with DNAs, their derivatives and homogenate were carried out using double diffusion tests on Petri dishes.

Serological studies on morphological mutants of Rhizobium trifolii.

Almost all rough mutants /R,RF/ of Rhizobium trifolii were isolated during the last reported period. Growing Rhizobium in a medium containing erythromycin 20 γ /ml resulted in the selection of rough mutants. We have observed that R/rough - nonmucoid/ strains, contrary to RF ones /rough, producing a small amount of gum/, tend to revert to typical SM microorganisms.

The frequency of reversion was influenced by the composition of the medium. For example on the minimal Davis medium supplemented with casaminoacids 1 SM revertant appeared per 10^8-10^9 of R cells. The back mutants did not differ from the typical culture in respect to their morphology. No difference was observed among R or RF mutants and SM forms in their ability ~~of ability~~ of carbohydrate fermentation.

In preliminary vegetation experiments we have observed a lower ability of nodulation and also a slighty higher sensitivity of R and RF strains to the lytic activity of phages in comparison with SM strains was noted.

The results of precipitation test in tubes are shown in table 4 and datas concerning absorption of immune sera are given in table 5. Some essential differences were observed in the antigenic structure of various forms. W-antigen of SM culture reacted with homologous as well with heterologous antisera, while rough forms did not contain this antigen. Opposite results were obtained with P-antigens. The weakest reaction was obtained with the antigen of SM, a stronger one with RF and the strongest with the antigen of R strain. P-antigens were used in the antibody absorption tests. SM anti-serum was completely absorbed by antigens of R or RF forms, while in the reversed system RF or R- antisera were absorbed only partially.

These results indicate that our rough /R/ mutants contrary to those obtained by K l e c z k o w s k a or J o r d a n, reverted spontaneously on artificial medium to smooth and mucoid forms. R-mutants obtained by K l e c z k o w s k a or J o r d a n required a plant passage to obtain their reversion. Morphological mutants may be easily detected by serological methods. Presumably the antigenic differences between SM and R or RF strains are connected in some respect with the lack of gum in rough cultures.

Conclusions.

1. The transfer of smooth and mucoid-into rough forms may occur spontaneously or following the UV irradiation or

after erythromycin treatment.

2. R-mutants, on the contrary to RF forms, tend to revert to typical SM microorganisms.
3. SM cultures contained large amounts of W-antigen, whereas R-forms were deprived of this antigen.
4. P-antigens of rough cells absorbed completely antibodies of SM-antiserum, while the antigen of SM bacteria only partially absorbed the R or RF antisera.

Studies on the antigenic properties of nucleic acid fraction of Rhizobium.

In the first series of experiments it was found that the "native DNA" had antigenic properties. Moreover this fraction retained its antigenicity after treatment with distreptase, containing DNase. These experiments implied that the antigenic activity of the tested fraction was not related to DNA. The treatment of the native DNA with trypsin did not influence its antigenic properties.

Chemical analysis had shown that the nucleic acid fraction of Rhizobium obtained by the A v e r y et al. method comprised besides DNA and RNA a polysaccharide containing glucose /cf. fig. 2/. "Native-DNA", DNA-distreptase, DNA-trypsin, and single stranded DNA formed identical two bands of precipitate. With glucose-DNA only a single precipitin band was shaped. After the treatment of homogenate by trypsin instead of four only three bands were obtained with the homologous serum /tabl. 6/.

In serological studies on the nucleic acid fractions it was found, that antigenic properties were not due to DNA or proteins but to a polysaccharide. This polysaccharide seemed to be identical with the somatic polysaccharide. One of the specific groups of the polysaccharide in question was found to be glucose. In the ultrasonic disintegrate of Rhizobium one of the antigenic components had a protein character.

Conclusions.

1. In the nucleic acid fractions of Rhizobium antigenic component appeared to be a polysaccharide.
2. This polysaccharide, containing glucose, was identical in the precipitation test in agar with the somatic antigen.
3. One of the antigenic components in the rhizobial ultrasonic homogenate had a protein character.

Transformations.

In spite of a considerable number of studies on transformation, the problem was thoroughly investigated in regard to a few bacteria. These include Pneumococcus /G r i f f i t h /1928/ , A v e r y et al./1944/, H o t c h k i s s /1951,1954 i 1958/; E p h r u s s i - T a y l o r /1951/; P a k u l a /1958 1961/. Transformations of Haemophilus influenzae were studied by /A l e x a n d e r et al /1951,1953,1954/; Z a m e r h o f et al./1957/ and V o l l with G o o d g a l l /1961/. Studies of Neisseria in this respect were carried out by A l e x a n d e r with R e d m a n /1953/, and C a t l i n /1958, 1960,1961,1962/ Recently S p i z i z e n /1958,1961/ published

his studies on transformation of Bac.subtilis. C o r e y and S t a r r /1957/ obtained transformation of Xanthomonas phaseoli. Reports were also made on transformation of Staphylococcus /B r a c c o 1957, P a k u l a et al. 1958/.

Transformation of Rhizobium were studied by B a l a s s a. The author used following genetical markers: antibiotic resistance, production of nodules, synthesis of anvigen and of cysteine. Better results were achieved when B a l a s s a /1961/ applied rough mutants as donors of DNA.

In our studies transformation of Rhizobium was studied using streptomycin resistance as a genetic marker.

Experiments were carried out using 60 Rhizobium strains of various physiological groups. Besides that, one rough /R/ and one semicapsular mutant /RF/ were used.

DNA was obtained by lysis of rhizobial suspension in citrate saline with sodium deoxycholate at 55° for 1 hr. The preparation was deproteinized 2-5 times by the Sevag procedure using chloroform and butanol. Afterwards DNA was precipitated with ethanol and dissolved in citrate saline. This process was repeated 2 - 3 times. About 130 DNA preparations were obtained from 22 streptomycin -resistant mutants. The composition of DNA preparations of the SM strain and mutants are given in table 7 .

The concentration of DNA was determined by the B u r t o n method, of protein- by that of L o w r y, of ribose- by that of C e r r i o t t i and of polysaccharides - by the anthrone method. The process of transformation was studied in liquid Thornton medium. The overnight culture of Rhizobium was suspended in fresh medium and supplemented with DNA /0,5 -

40 µg/ml/. After 90 min. of incubation the transorming culture was diluted with a 4-fold volume of fresh medium and grown for 5 hr. at 28° with shaking. The results of transformation were checked by planing the bacteria on medium with 500 µg/ml of streptomycin.

The influence of various factors which stimulate the transformation in other species were also studied in the present research. The following factors were tested: human, horse and rabbit serum /1 - 5%/, charcoal /0,1 - 1%/ egg albumin and bovine albumin digested with trypsin /0,1 - 1%/, DL-histidine, casaminoacids /0,1 - 1%/ and versene /50, 100, 200, 300 µg/ml/. Out of the compared media, Thornton medium proved most useful.

The majority of investigations were carried out with Rhizobium trifolii. Transformation within 9 of the studied strains of Rhizobium trifolii was dependent on the donor and recipient strains /c.f. table 8/. DNA of strain 325a transformed all recipient cultures, the frequency being 0.0009 - 0.0012%. Other strains transformed 8/b_2, SM/ or 4 /b_1/ out of 9 tested recipients. The highest frequency of transformation was obtained with the rough /R/ and /RF/ strains /0,082 and 0,012% respectively/. In numerous experiments transformation in the group of Rh. leguminosarum for peas strains was found. DNAs of 2 strains of Rh. leguminosarum for Pisum transformed homologous strains with the frequency of 10^{-6}. These DNA preparations appeared to be active also with one out of 9 tested strains of Rh. trifolii and one out of 8 studied strains of Rh. meliloti. In all these experiments the transformation frequency was of the order of 10^{-7}.

However, DNAs of the studied Rh. meliloti and Rh. lupini did not transform the streptomycin resistance marker.

In all the experiments colonies developing from transformed cells did not differ morphologically from recipient colonies. It was also found, that the resistance of transformants to streptomycin was the same as that of donor cells /e.g.40000 - - 100 000 µg/ml/.

The highest competence of the recipient smooth and mucoid strains was obtained after 6 hr. of growth.

Similarities and differences are noticeable in comparison of the results of our experiments with those achieved by B a l a s s a /1961/. Our alfa-alfa and lupine strains contrary to those of B a l a s s a gave no transformation. In B a - l a s s a's studies /1961/ with Rh.trifolii the transformation frequendy within the same strain was higher than that between heterologous strains. In our experiments transformations showed approximately equal frequencies. It was also observed, that there exists a correlation between the production of capsular polysaccharides and the competence of recipient cells. On the whole mucoid strains were less easily transformed, which seems to be compatible with R a v i n's view /1961a/, according to which transformations are dependent on the quantity of capsular polysaccharides.

Frequency of transformation was also dependent on the property of the recipient strain itself. For instance in case of strains 325a and b_1.

In the case of SM capsular strain a periodical competence of recipient cells to DNA was observed after 3,6 and 9 hours of growth.

Until now we failed to receive a simultaneous transformations of

of two markers, namely of streptomycin resistance and morphology of colonies.

Plan for future work. For the year 1963 we are planning to continue our genetical studies esp. on mutagenicity and transformations. We should like to undertake more detailed investigations on the physiological properties /including modulation / of the isolated mutants and to take special interest in the isolation of aminoacid- and vitamin - requiring mutants. Our studies on transformation would be performed using new genetical markers including the effectiveness of N_2 fixation.

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Bacteriophages of Rhizobium.

A. Properties of Rhizobium phages.

For phage classification A d a m s /1952/ underlines the importance of physiological properties of phages and of the effect of various physical and chemical factors on their activity

B u r n e t /1953/, was the first to apply chemical agents for classifying 11 serological groups of dysentery-coli phages. A n d e r s o n /1953/ and Y a m a m o t o /1957/ found, that phages of the T series react differently to osmotic shock. S t e n t /1958/ believes that the sensitivity to UV irradiation may be utilized for phages differentiation. K l e c z k o w s k a and K l e c z k o w s k i /1953/ determined the lethal effect of UV irradiation on bacteriophages of Rhizobium. Studies on adsorption of a bacteriophage on some Rhizobium strains were also carried out in the same laboratory /K l e c z k o w s k a, 1945/.

As the properties of Rhizobium phages were till present not much studied, we started to investigate their serological relationship, their inactivation by physical and chemical factors and their adsorption to bacterial cells.

25 phages belonging to 5 physiological Rhizobium groups were chosen by us.

In all our experiments a liquid medium "5" /L a i r d 1932/ and an agar medium "Th1" were used, the composition of the latter being: glucose - 10,0 g; K_2HPO_4 - 0.5 g; $MgSO_4$ - 0.2 g; NaCl - 0.1 g; $FeSO_4$ - 0.02 g; $MnSO_4$ - trace; $CaCl_2$ - 0.01 g; calcium glycerophosphate - 1.0 g; yeast extract - 100 ml; casamino acid - 0.1 g; dist.water up to 1 l ; agar - 7.0 or 12.0 g; pH of medium 7.2 - 7.4.

Influence of physical and chemical factors.

Osmotic shock of phages was studied by the Yamamoto method /1957/. The result of studies on sensitivity in that respect of 25 phages belonging to 5 physiological Rhizobium groups are presented on diagram /fig.3/. Phages of Rh.trifolii and Rh.lupini for lupine proved to be the most sensitive - 0.05 - 1.0 p.c. active particles remaining after osmotic shock.
Phages of Rh.leguminosarum for vetch and peas and Rh.meliloti apparently were less sensitive.

Sensitivity to UV irradiation. Phages were diluted 150 x in a 0.15 M phosphate buffer and poured in Petri dishes of 5 cm diameter to form a layer of 2 mm. The irradiation was carried out with the Hanau UV lamp at a distance of 20 cm.

Different sensitivities were established for two phages of Rh.leguminosarum for vetch / fig.4 /. Phages of Rh.lupini for lupine were the most sensitive. Their inactivation curve showed an initial shoulder lasting up to 1 min. of irradiation (fig.5).

Photodynamic action of dyes. Phages of titre of 1 x 10^8 were mixed with an equal volume of dyes diluted in distilled water to 1:25000. The mixture was poured on plates of 10 cm diameter to form 2 mm layers. The irradiation was carried out with two 20 W Tungsram fluorescent lamps at the distance of 20 cm. The activity of phages was checked after 0, 5, 15, 30 and 60 minutes . Readings of the results were taken after 48 hours of incubation.

The sensitivity of 25 phages , belonging to 5 physiological Rhizobium groups, to the photodynamic action of 7 dyes was studied. It was found, that the titre of the phages declines already after 5 minutes of irradiation, though considerable decline was noted only after 30 and 60 minutes of irradiation.

Differences in the degree of inactivation were found among the strains of phages, phages of Rh.meliloti being the most sensitive. The strongest photodynamic effect was noted in case of azur I and toluidine blue, the weakest being with methylene blue (tab1. 9).

For a more detailed study of the kinetics of inactivation 4 phages of Rh.trifolii differing in sensitivity to azur I and to toluidine blue, were selected. No considerable differences were found among these 4 strains in respect of their

sensitiveness to photodynamic action of either azur I or toluidine blue. The largest difference in case of toluidine blue amounted to K= 0.15 /min.$^{-1}$/ for phages 1 K 22 and to K=0.22/min.$^{-1}$/ for phage 4 K 27 . In case of azur I no differences were found.

Inactivation by urea was studied by Burnet method /1933/.

The sensitivity of 25 phages of 5 physiological Rhizobium groups, to the action of urea was also studied. The K values calculated after 50 minutes of inactivation process are presented in diagram (fig. 6). Here no conspicuous differences in the sensitivity of these phages were found.

Influence of sodium citrate was studied by the method of Burnet /1933/, whereby various quantities of this agent were added to agar "Th1". It was found, that all the 25 phages studied lysed sensitive bacteria still in the presence of 1.5 per cent of sodium citrate. As much as its 2.0 per cent concentration was necessary for inhibition of the lytic activity of these phages.

Neutralization of Rhizobium phages by antiphage sera /diagram 7/.

The aim of these studies was to determine serological relationship among Rhizobium phages. For this purpose 2 immune sera and 17 strains of Rhizobium phages were used. Antiphage sera were obtained by immunizing rabbits with phage 21K 21 of Rh.trifolii or phage 4L 39 of Rh.lupini for lupine. The phages stocks of titres 6 x 10^{11} and 9 x 10^{11} for Rh.trifolii and Rh.lupini for lupine respectively were administered

subcutaneously by the Adams method /1959/. The K value of immune sera for homological phages amounted to 295 in case of phage 21K 21 and 362 for phage 4L 39. The adsorption rate of other phages strains was determined by means of the two obtained antiphage sera. The following serum dilutions were used: 10^{-2}, 10^{-3}, 10^{-4}. The neutralization process was examined after 5, 10, 15, 30 and 60 minutes.

The phages studied by us were neutralized by these sera with various rates. Our diagram presents the constants K of the neutralization rate for sera diluted to 10^{-3}, measured after 5 minutes.

Serum 21K 21 neutralized other phages of Rh.trifolii to a similar degree, the only exception being phage 1K 22, for which the constant K was the lowest. Phages of lupine bacteria were neutralized by this serum to a much higher degree. Out of other studied heterological groups, 3 phages, namely 3L 11, 5W 36 and 10G 1, were neutralized rapidly /see diagramme 7/.

Antiphage serum 4L 39 neutralized homological phages /except the phage 2L 39/ to a similar degree. A few phages of clover bacteria /e.g. 1K 22 for which K=1130/ were neutralized to a much higher degree by this serum than Rh.lupini phages. Phages of the remaining heterological groups were neutralized by this serum only to a slight degree.

The results of cross reactions with antiphage sera may show, that there exists a close serological relationship between phages of Rh.trifolii and Rh.lupini for lupine.

No evident relationship between phages of clover and lupine bacteria on the one hand, and phages of peas and vetch bacteria on the other, was established.

Adsorption rate of phages to cells of Rhizobium.

The adsorption rate of phage 4L 39 of Rh.lupini for lupine and of phage 3L 11 of Rh.meliloti to homologica1 cells of Rhizobium was examined. The adsorption rate was determined by 2 methods /acc. to A d a m s /:

1. assay of unadsorbed phages.
2. assay of infected bacteria.

For studies of the adsorption rate, young 5-hour cultures of Rhizobium were used. In the case of the first method phages were added up to the ratio 10:1 of phage to bacteria. Mixtures of bacteria and phages were centrifuged/10.000rmp./ in a centrifuge head, previously cooled to -10°C. In the case of method 2, the ratio of phage to bacteria was 1:10. For the inactivation of unadsorbed particlesw of both phages specific serum for phage 4L 39 of Rh.lupini for lupine was used. The value ~~xxxxxxxx~~ constant of this serum for heterologous phage 3L 11 K = 240. The adsorption process was checked after 1,3,5,15,30, 40 and 60 minutes. The results of studies are presented on figures 8 and 9. Figure 8 gives a comparison of the adsorption rate of phage 4L 39, while Figure 9 - that of 3L 11. In both figures the results of both methods are shown. Studies of the adsorption rate of phages

4L 39 and 3L 11 to homological strains of Rh.lupini and Rh.meliloti allowed to find out, that these bacteria adsorbed 81,5 - 95,5% of phage particles. After 1 minute 81 - 83% of phages were adsorbed. Afterwards the number of adsorbed phages was increasing progressively and reached a peak of 92 - 95,5% after 15 minutes. After that time a decline of the adsorption rate was observed, the mean percentage of adsorbed phages being 84 - 87% after 60 minutes.

Both methods brought similar results, the difference not exceeding 2%.

From the above experiments it follows that Rhizobium phages are greatly differenciated serologically and also in their sensitivity to osmotic shock and to UV irradiation.

Serologically related groups of phages of Rh.trifolii and Rh.lupini for lupine showed also a similar reactions to osmotic shock. The nature of this analogy is not yet explained. However, no correlation between serological properties and sensitivity to photodynamic action of dyes was established, though such interdependence was found by B u r n e t /1953/ W e l s h and A d a m s /1954/ and Y a m a m o t o /1958/. It may be possible that this incompatibility results from the difference of methods used.

In our experiments phages resistant to osmotic shock were not found to be specially sensitive to the photodynamic action of dyes, though such a suggestion was made by Y a m a m o t o /1958/ in reference to phages of the T series.

The results of our present studies seem to point to the usefulness of serological tests and of the tests of sensitivity

to osmotic shock and UV irradiation - to differenciating Rhizobium phages.

The tested phages did not show significant differences in respect to the sensitivity to urea and sodium citrate as well as in photodynamic reactions.

Typing of Rhizobium strains by means of bacteriophages.

During the year 1962 rhizobial phages were isolated, propagated and selected for the purpose of typing. 80 phages for Rhizobia of various physiological groups were selected out of 100 virus strains. The titres of these phages ranged from 10^5 to 10^{10}. Phages with a low titre were propagated or adapted to strains belonging to other physiological groups of Rhizobium.
Pure cultures of phage strains were obtained by serial repeated isolations ~~of various~~ from single plaques. In our experiments on plates, mutants or variants of phages with different plaque morphology were observed. Until now 45 morphological mutants were isolated from among phage strains of Rhizobium: Rh.trifolii, Rh.leguminosarum for peas, Rh.meliloti, Rh.lupini for serradella and Rh.phaseoli. It can be assumed that some of these mutants may appear suitable in research on lysotypia.
We are planning to use selected phages with adequate spectra of activity for typing of ab. 200 strains of Rhizobium belonging to various physiological groups.

Lysogeny of Rhizobium.

For the study of lysogeny 50 strains of Rh.meliloti were used. Out of them 32 were recently isolated and the remaining strains were the laboratory ones, derived from different countries /Poland, France, Belgium, England, Soviet Union and U.S.A./.

The supernatant fluid of 48 hour cultures was studied on the presence of phages by the method described in report for 1961. All 50 Rh.meliloti strains were investigated for release of phages into cultural medium with the use of 23 indicator strains.

Out of 50 Rh. meliloti strains - 27 liberated phages in the presence of one or more indicator strains. In all except one of the laboratory strains the presence of phages was established, while in recently isolated strains of Rh.meliloti the release of phages was found only in 10 out of 32 tested strains.

These studies are still in progress.

In the supernatant of cultures of 9 Rhizobium strains different morphological forms /kind of plaques/ of phages were found. On this basis these phages could be divided into 3 followingtypes:

1. Phages forming turbid plaques of a diameter 1 -2,5mm.
2. Viruses forming plaques of similar size but with more clear centers and with sharp edges.
3. Phages forming clear plaques with diameter 4 - 5 mm.

Phages forming turbid plaques gave no evident lysis, while with those forming clear big plaques a complete lysis of Rhizobium was achieved. Phages of the type 2 were intermediate in this respect.

50X1-HUM

Cross-agglutination type with somatic antigens of Rhizobium

Antigens			Immune sera for Rh. lupini strains			
			from serradella		from lupini	
			0g	S	271	R
Rh. trifolii		2p	0	0	0	0
		b1				
		b2				
		x				
		3p				
		F				
		325a				
		N				
Rh. leguminosarum	from peas	1	0	0	0	0
		5				
		222				
	from horse-beans	0g	0	0	0	0
		73				
Rh. lupini	from serradella	4p	+ + +	+ + +	0	0
		5	+ + +	+ + +		
		0g	+ + +	+ + +		
		K	+ + +	+ + +		
		B	+ + +	+ + +		
		S	+ + +	+ + +		
	from lupine	Cz	0	0	+ +	+ +
		271			+ + +	+ + +
		K			+ + +	+ + +
		R			+ + +	+ + +
		359			+ + +	+ + +
Rh. meliloti		Thp	0	0	0	0
		3p				
		413a				
		N 94				
		Ch				
		Belg.				
Rh phaseoli		1	0	0	0	0
		3				
Rh.sp.from Arachis		R	0	0	0	0
		Belg				
Rh. japonicum from soy-beans		94p	0	0	0	0
		L				
		101c				
		102c				
Rh.sp from Anthyllis		P74	0	0	0	0

Notes:
++ agglutination titer 640
+++ " " 1280 - 2560

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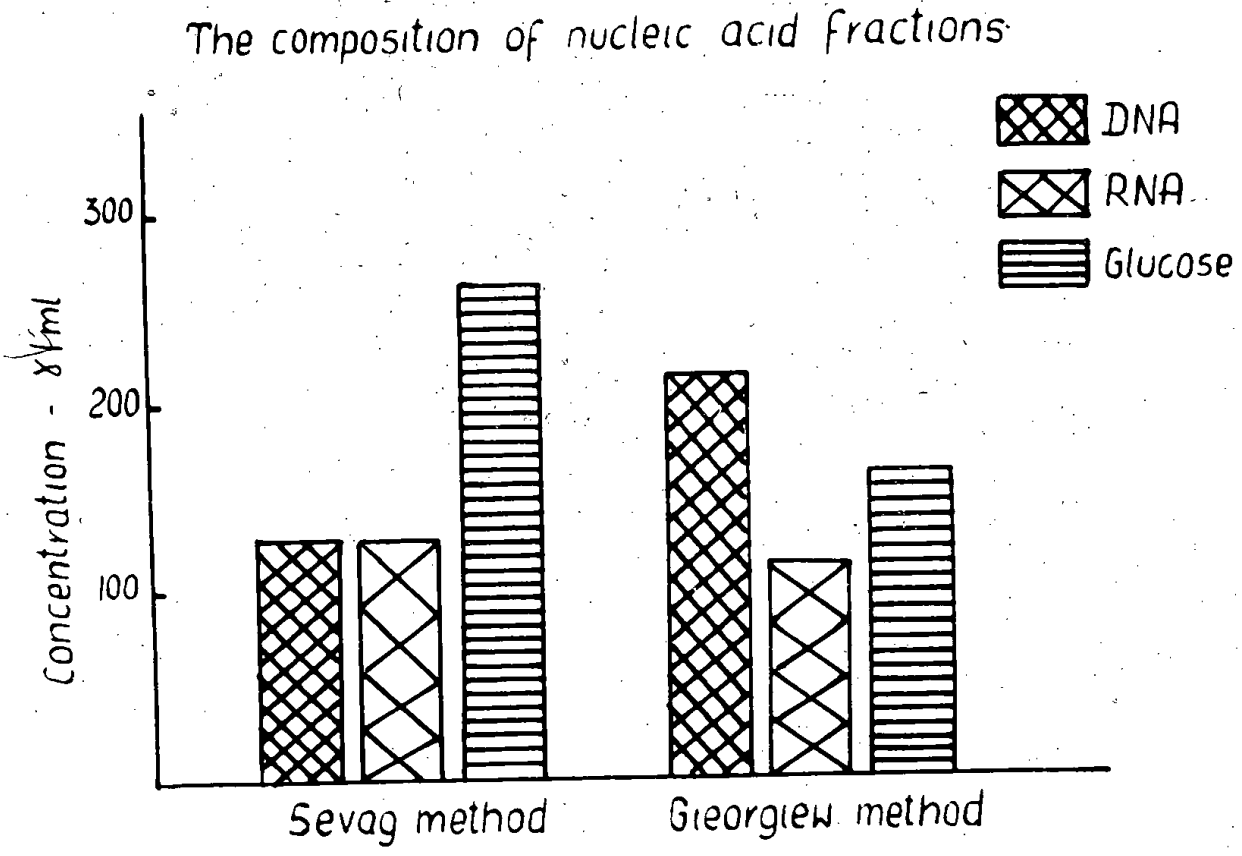

Fig. 3

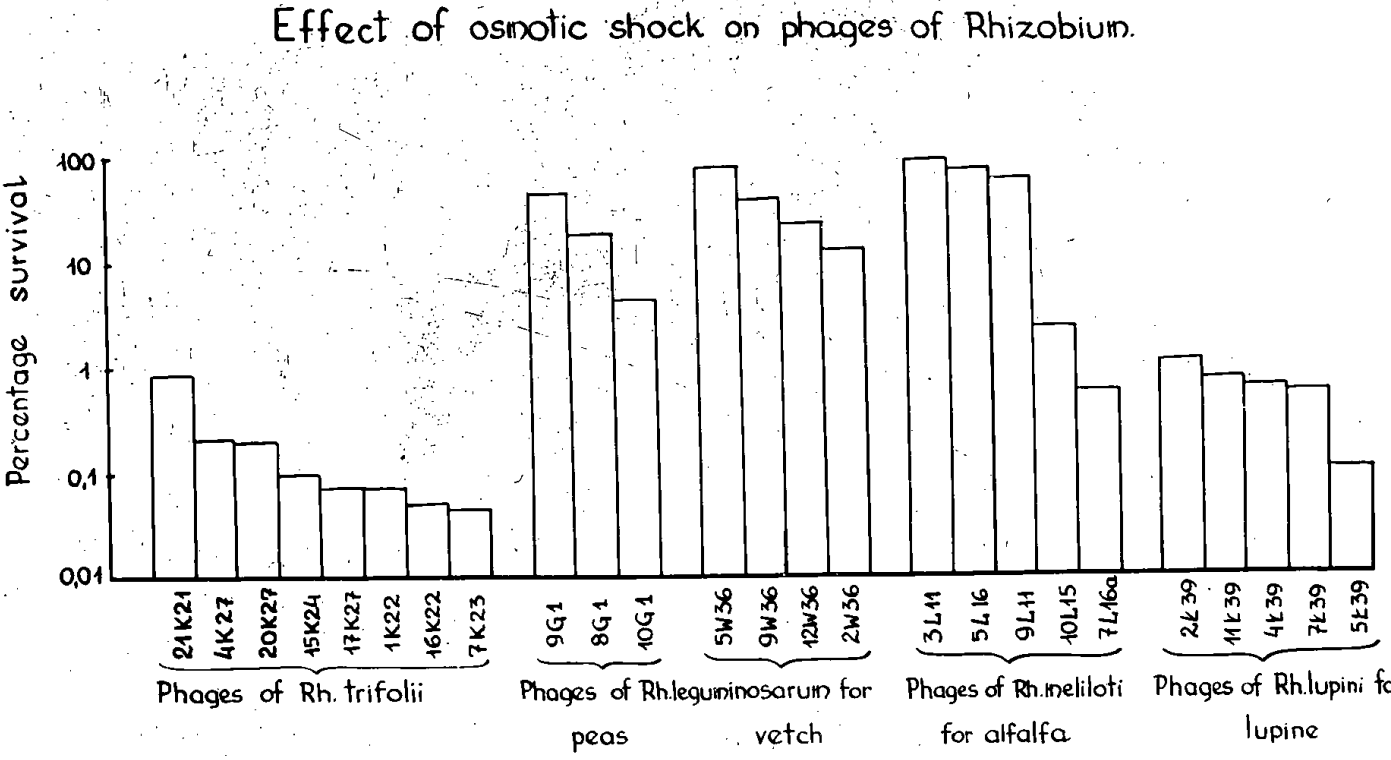

Fig. 4

Survival curves of phages of Rh. legum. for vetch after ultraviolet irradiation

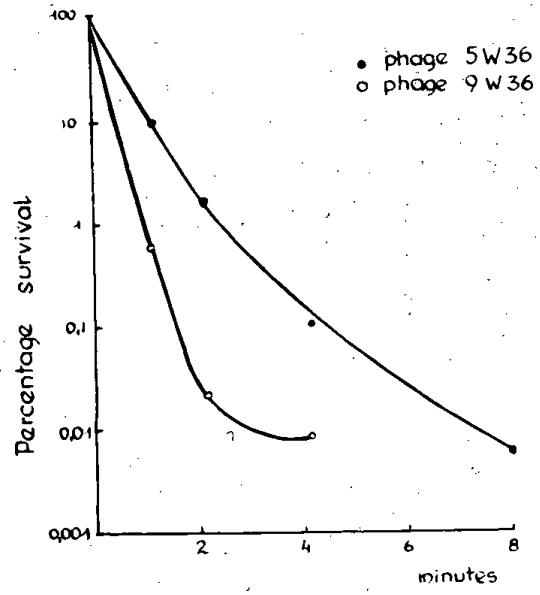

Fig. 5

Survival curves of phages of Rh. lupini for lupine after ultraviolet irradiation

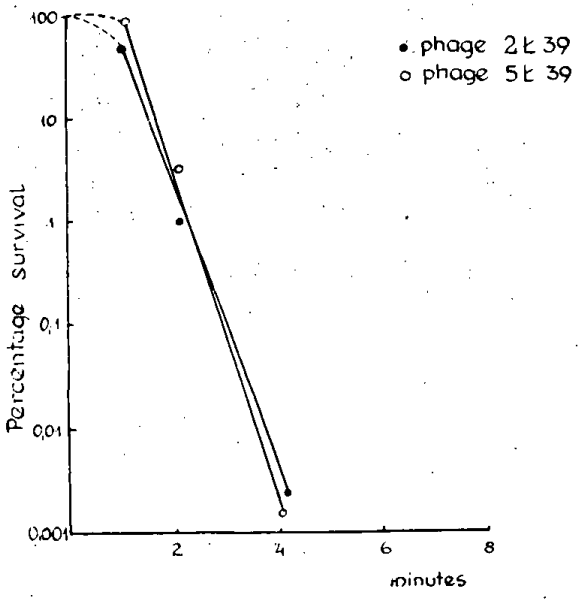

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Table 2a

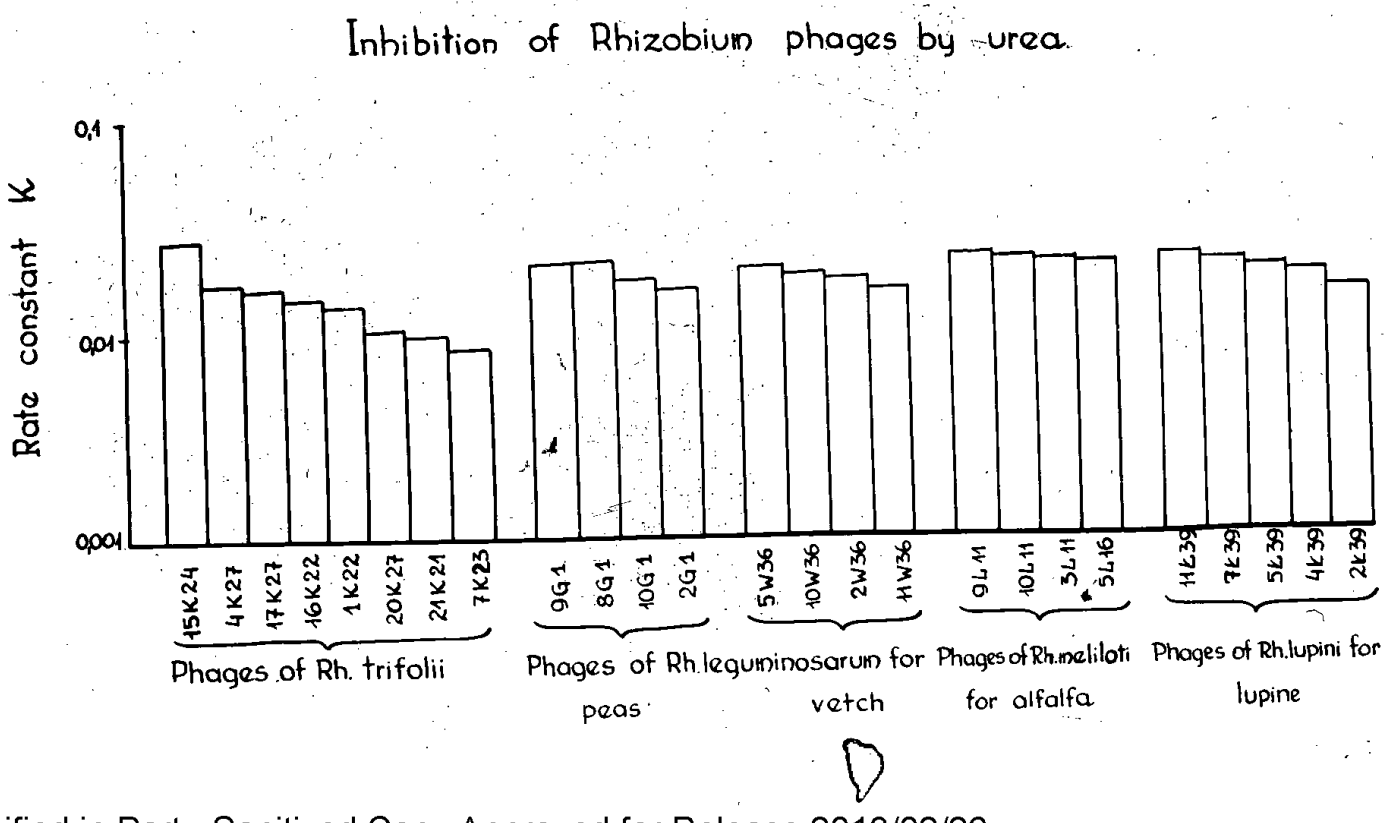
Cross-agglutination between strains of Rh. trifolii and Rh. leguminosarum

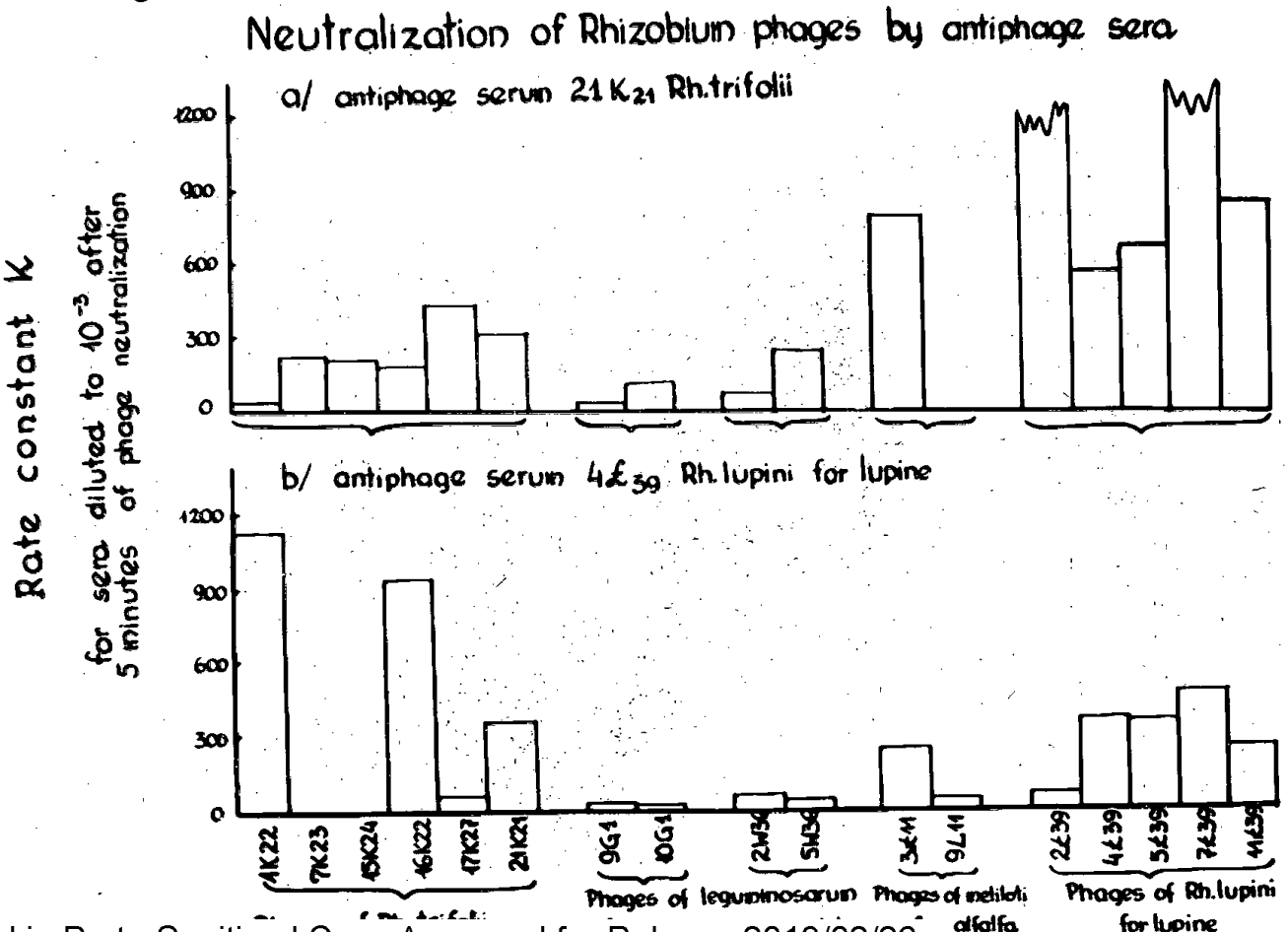
Somatic antigens of strains Rh. trifolii	Immune sera anti-O for the following strains											
	Rh. trifolii									Rh. leguminosarum		
										from peas		vetch
	b_2	3p	x	325a	205	209	243	C-33	C-90	5	PRE	67
2p	++	+++	+++	+++	+	+	0	0	0	+++	0	+++
3p	+++	++	+++	+++	+	+	0	0	0	+++	0	+++
b_2	+++	+++	+++	+++	+	+	0	0	0	+++	0	+++
x	++	+++	+++	+++	+	+	0	0	0	+++	0	+++
b_1	+++	+++	+++	+++	+	+	0	0	0	+++	0	++
F	+++	+++	+++	+++	+	+	0	0	0	+++	0	++
325a	++	++	++	+++	+	+	0	0	0	+++	0	+++
N	+++	++	+++	+++	+	+	0	0	0	+++	0	++
200	++	++	+	+	+++	+++	+++	0	0	0	0	0
201	+	++	+	+	+++	+++	++	0	0	0	0	0
205	0	0	0	0	+++	+	+	0	0	0	0	0
209	+	+	+	+	++	+++	+	0	0	0	0	0
210	0	0	0	0	+++	+++	+++	0	0	0	0	0
211	0	0	0	0	+++	+++	++	0	0	0	0	0
219	0	0	0	0	+++	++	++	0	0	0	0	0
226	0	0	0	0	+++	++	++	0	0	0	0	0
237	0	0	0	0	++	++	++	0	0	0	0	0
243	0	0	0	0	++	++	+++	0	0	0	0	0

Notes:

+	agglutination titer	40 - 300
++	" "	600 - 1000
+++	" "	1200 - 2600

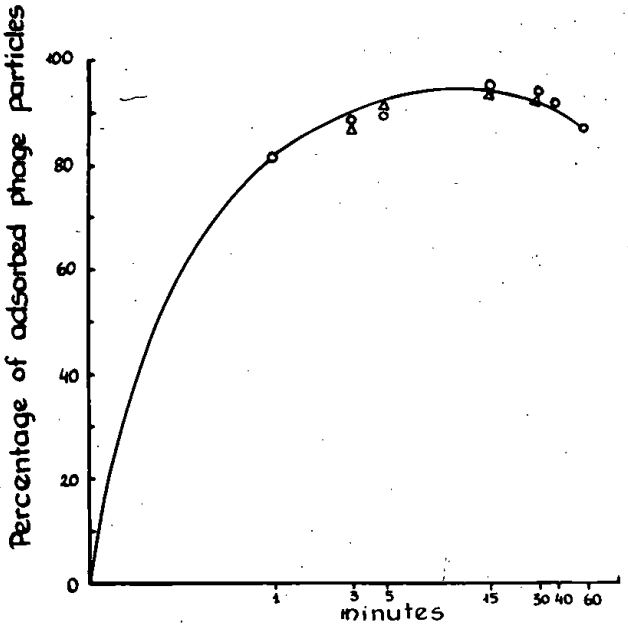
Fig. 6





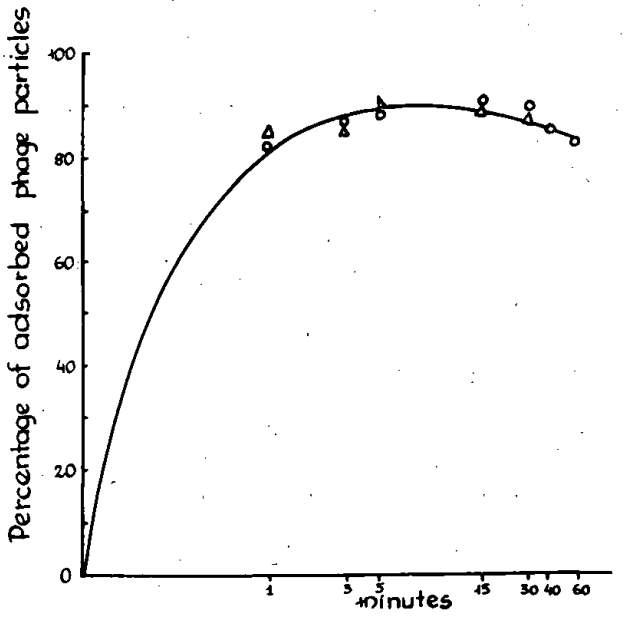
Adsorption rate of phages to cells of Rhizobium

Figure 8



Phage 4k 39 of Rh.lupini from lupine

Figure 9



Phage 3L 11 of Rh. meliloti from luceme

Notes: o-assay of unadsorbed phage

Table 20

Cross-agglutination between strains of Rh. trifolii and Rh. leguminosarum

Somatic antigens of strains Rh. trifolii	Immune sera anti-O for the following strains											
	Rh. trifolii									Rh. leguminosarum		
										from peas		vetch
	b_2	3p	x	325a	205	209	243	C-33	C-90	5	PRE	67
C-1	0	0	0	0	0	0	0	0	0	0	0	0
C-2	0	0	0	0	0	0	0	0	0	0	0	0
C-3	0	0	0	0	0	0	0	0	0	0	0	0
C-4	0	0	0	0	0	0	0	0	0	0	0	0
C-5	0	0	0	0	0	0	0	0	0	0	0	0
C-6	0	0	0	0	0	0	0	0	0	0	0	0
C-7	0	0	0	0	0	0	0	0	0	0	0	0
C-8	0	0	0	0	0	0	0	0	0	0	0	0
C-9	0	0	0	0	0	0	0	0	0	0	0	0
C-10	0	0	0	0	0	0	0	0	0	0	0	0
C-33	+	+	+	+	+	+	0	+++	++	0	0	0
C-39	0	0	0	0	0	0	0	0	0	0	0	0
C-45	+	+	++	+	0	0	0	0	0	0	0	0
C-61	+	+	+	+	0	0	0	0	++	0	0	0
C-69	0	0	0	0	0	+	0	+++	++	0	0	0
C-75	0	0	0	0	0	0	0	0	0	0	0	0
C-79	0	0	0	0	0	0	0	0	0	0	0	0
C-80	0	0	0	0	0	+	0	0	+	0	0	0
C-90	0	0	0	0	++	0	0	++	+++	0	0	0
C-92	0	0	0	0	0	0	0	0	0	0	0	0
C-94	0	0	0	0	0	0	0	0	0	0	0	0
C-99	0	+	+	+	0	+	0	++	++	0	0	0
C-38	0	0	0	0	0	+	0	0	++	0	0	0
K-4	+	+	+	+	0	0	0	0	0	0	0	0

Notes:
+ agglutination titer 40-300
++ " " 600-1000
+++ " " 1200-2600

Table 2c

Cross-agglutination between strains of Rh. trifolii and Rh. leguminosarum

Somatic antigens of strains Rh. leguminosarum		Immune sera anti-O for the following strains											
		Rh. trifolii									Rh. leguminosarum		
											f. vetch	from peas	
		b_2	3p	x	325a	205	209	243	C33	C-90	67	5	PRE
strains from peas	1	+++	+++	++	++	+	++	0	0	0	+++	+++	0
	5	+++	+++	++	+++	+	++	0	0	0	+++	+++	0
	222	0	0	0	0	0	0	0	0	0	0	0	0
	301	0	0	0	0	0	0	0	0	0	0	+	+
	302	0	0	0	++	+	+	0	0	0	0	+	0
	312	0	+	0	0	+	+	0	0	0	0	0	0
	402	0	0	0	0	0	0	0	0	0	0	0	+
	404	0	0	0	0	0	0	0	0	0	0	0	+
	PRE	0	0	0	0	0	0	0	0	0	0	0	+++
	P8	0	0	0	0	0	0	0	0	0	0	0	++
strains from vetch	67	+++	+++	0	+++	0	0	0	0	0	+++	+++	0
	W6	0	0	0	0	0	0	0	0	0	+	0	0
	W8	0	0	0	0	0	0	0	0	0	+	0	0
	W9	0	0	0	0	0	0	0	0	0	+	0	0
	W10	0	0	0	0	0	0	0	0	0	+	0	0
	W12	0	0	0	0	0	0	0	0	0	++	0	0
	W14	0	0	0	0	0	0	0	0	0	+	0	0
	W15	0	0	0	0	0	0	0	0	0	+	0	0

Notes:

\+ agglutination titer 40 - 300
++ " " 600 - 1000
+++ " " 1200 - 2600

Table 3

Agglutination with absorbed sera

Antisera absorbed with heterologous antigens	Somatic antigens										
	Rh. leguminosarum			Rh. trifolii							
	from vetch	from peas									
	67	1	5	2p	b_1	F	N	x	325a	b_2	3p
67/1	1500	0	0	320	640	160	640	1280	1280	640	320
67/5	1800	0	0	640	640	320	640	1280	1280	1280	320
67/$2p_1$	1280	0	0	0	80	40	160	320	640	160	320
67/b_1	1280	0	0	80	0	40	80	320	1280	0	640
67/F	640	0	0	0	0	0	80	320	320	640	640
67/N	1280	0	0	0	0	0	0	160	40	80	320
67/x	1280	0	0	0	0	80	80	0	80	0	160
67/325a	320	0	0	0	0	0	0	0	0	160	320
67/b_2	320	0	0	0	0	0	0	0	320	0	320
67/3p	160	0	0	0	0	0	0	0	0	0	0
67/67	0	0	0	0	0	0	0	0	0	0	0
antiserum not absorbed	2560	640	640	1280	1280	640	1280	2560	1500	1280	640

Table 4

Precipitation of Rhizobium antigens

Antisera	Antigens								
	SM			RF			R		
	W	H	P	W	H	P	W	H	P
SM	16	8	8	2	2	8	—	8	16
RF	16	4	4	—	8	16	—	8	16
R	8	8	4	—		8	—	16	32

W – W-antigen
H – alkaline hydrolizate of bacteria
P – P-antigen
Figures denote the titre of precipitation
— no precipitation

Table 5

The absorption of antisera by means of heterologous antigens

Antigens used in precipitation tests with absorbed antisera	Antisera					
	$\frac{SM}{RF}$	$\frac{SM}{R}$	$\frac{RF}{SM}$	$\frac{RF}{R}$	$\frac{R}{SM}$	$\frac{R}{RF}$
SM	100%	100%	100%	100%	100%	100%
RF	100%	100%	75%	100%	50%	100%
R	100%	100%	50%	100%	75%	100%

Results are given in percent of the serum absorption.
$\frac{SM}{RF}$ - SM serum absorbed with P antigen of RF culture.

Table 6

Diffusion test in agar

	Antigens	Immune serum against native DNA	Immune serum against Homogenizate		Antigens	Immune serum against native DNA	Immune serum against Homogenizate
1	Native DNA	○ II	○ II	7	Homogenate	○ II	○ IIII
2	Distreptase DNA	○ II	○ II	8	Tripsin-homogenate		○ III
3	Tripsin DNA	○ II		9	Distreptase + homogenate	○ II	○ II
4	Glucose DNA	○ I	○ I	10	Slime	○ II	○ II
5	UV - DNA	○ II	○ II	11	Somatic antigen (acc. Compbell)	○ II	○ II
6	Single-stranded DNA	○ II	○ II				

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Table 7

Composition of DNA preparations of the xSM strain of Rh. trifolii and its mutants /µg/ml/

Type of growth of donor strains	DNA	ribose	protein	glucose
SM	120-200	118	81	125
RF	250	175	40	136
R	340	190	85	68

Table 8

Frequency of transformation in the Rh. trifolii group.

Donor strains	Reciplent strains											
	2p	3p	b_1	b_2	F	325a	N	T/f	X-SM	X-RF	X-R	C-92
b_1	0,000013	0	0,00032	0,00013	0	0	0	0	0,000027	—	—	—
b_2	0	0,000035	0,00012	0,000018	0,00024	0,000025	0,0004	0,00027	0,000013	—	—	—
325a	0,00014	0,00055	0,0012	0,00009	0,00085	0,00012	0,00011	0,00016	0,000046	—	—	—
X-SM	0,0018	0,00023	0,0036	0,0054	0,00027	0	0,0045	0,00012	0,0028	—	—	—
X-RF	—	—	0,0022	—	0,0023	—	0,0115	—	0,00035	0,00032	0,008	0,012
X-R	—	—	—	—	—	—	—	—	0,001	0,08	—	—

The percent of the transformants was calculated in relation to the final number of cells in the transformed culture.

Notes: 0 - no transformation

— - no tested

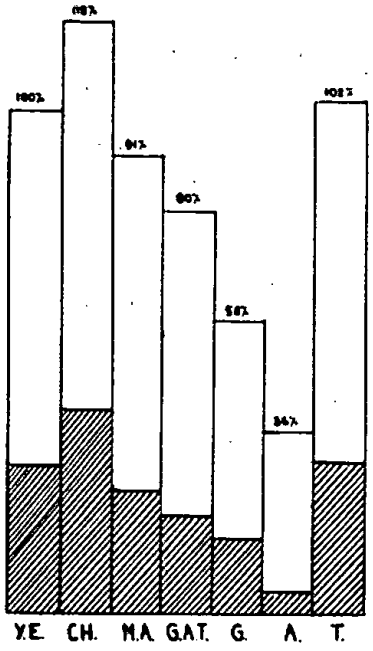
Table 9

Photodynamic action of dyes on phages of Rhizobium.

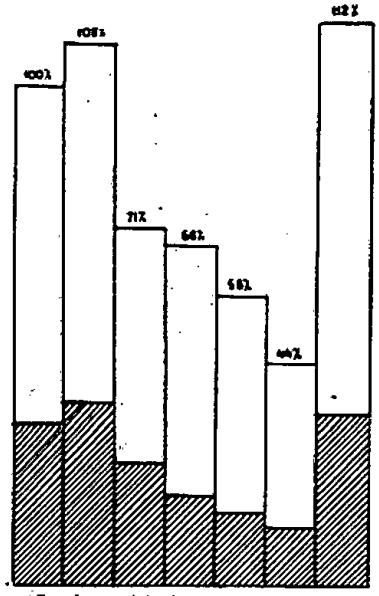
Phages of Rhizobium	Number of phages	Azur I		Toluidine blue		Neutral red		Azur II		Methyl green		Acridine orange		Methylene blue	
		30'	60'	30'	60'	30'	60'	30'	60'	30'	60'	30'	60'	30'	60'
clover	8	4	7	0	7	0	6	1	6	1	8	0	8	0	3
peas	4	3	4	0	2	0	4	0	3	1	3	0	2	0	1
vetch	4	1	4	1	4	0	3	0	3	0	1	0	2	0	0
alfalfa	4	4	4	4	4	3	4	0	4	3	4	2	4	3	4
lupine	5	0	5	2	3	0	4	2	5	1	3	0	2	0	0

Fig 1

Yields of Soybeans and Yellow Lupine inoculated with strains of Rh. lupini or Rh. japonicum cultured beforehand on different media

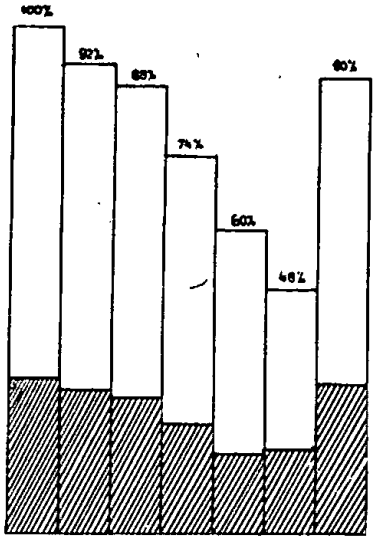


Strain nr. „Cz"

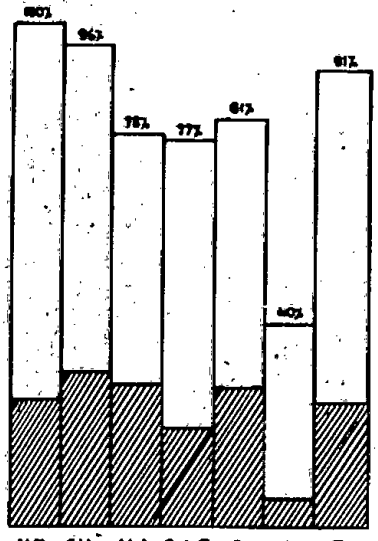


Strain nr. 271

Yellow Lupine



Strain nr. 94 p



Strain nr. 102 C.

Soybeans

Remarks :

Y.E Thorton's medium with yeast extract
CH. — casein hydrolysate
M.A. — mixture of aminoacids
G.AT — of glycine, alanine and tryptophan
G. — glycine
A. — alanine

Content of Nitrogen magnified 10 x

Yield of air dried tops

Content of Nitrogen in d.m. of tops