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Comperative Studies with *Culex pipiens* Egg Rafts. Immunogenetic, Electrophoretic and Enzymatic Analysis of Unfertilized, Compatible and Incompatible Fertilized Eggs

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Summary. By applying immunologic, electrophoretic and enzymatic methods, extracts of different raft types of *Culex pipiens* were analysed. Rafts of the crosses Pa × Pa and Ha × Ha contained four common antigens, while unfertilized rafts of Pa and Ha (no antisera were prepared against them) and rafts of the crosses Og × Og, Og × Pa, and Pa × Og shared three common antigens with the remaining raft extracts. Disk-electrophoresis of raft extracts in acrylamide gel resulted in different electropherograms. Ten protein bands were common to all these raft types. The unfertilized rafts of Pa and Ha yielded three more protein bands, the crosses Pa × Ha and Ha × Pa one more, the crosses Og × Og and Pa × Og three more, and Og × Pa two more. Many enzymes were demonstrated in the raft extracts after they were separated in acrylamide gel and incubated with the corresponding substrate solutions. All the raft types possessed one enzyme type for glutaminate-, lactate-, glucose-6-phosphate-dehydrogenase and catalase. Malate-dehydrogenase and leucine aminopeptidase occurred in each raft type as two isoenzymes. Alkaline phosphatase was observed as a single enzyme, but was lacking in rafts of the crosses Pa × Pa and Ha × Ha. While rafts of the crosses Og × Og and Og × Pa possessed two acid phosphatases, three could be demonstrated for the remaining raft types. Up to eight esterases appeared; rafts of the crosses Og × Og and Og × Pa possessed seven such activities. The results obtained by the Ouchterlony test, disk-electrophoresis and the histochemical enzyme tests are discussed in context and checked according to the phenomenon of incompatibility.

Introduction

In crosses between populations of *Culex pipiens* of different geographical origin, the following crossing types are found:

1. bilateral compatibility: normal offspring in both directions

2. unilateral incompatibility: only one of the crosses gives offspring, and

3. bilateral incompatibility: both reciprocal crosses are sterile.

The determination of these different mating types was made by Laven (1953, 1957) with the aid of the embryonation rate, the hatching rate and the outcoming offspring. In the case of compatibility there is a hatching percentage of 70—100%; if an incompatible cross was made, 99.9% of the developing embryos are lethal and only 0.1% of the embryos go through the larval and pupal stages and emerge as fertile diploid females. These exceptional females develop from a diploid oocyte or from a diploid nucleus formed by fusion of the pronucleus and the last polar body (Laven, 1956; Jost, 1970).

The first systematic studies of the *Culex-pipiens*-complex were done by Marshall and Staley (1937), Robaud (1941; 1945; 1950), Laven (1951), and Ghelelovitch (1952). Intensive studies were performed by Laven (1957, 1967), who tested 37 different strains from Europe, Africa, Asia and North-America. They were subdivided into 17 different crossing types according to their crossing relationships with other strains.

What is the cytological difference between a compatible and an incompatible cross? In both crossing types, one, two, or sometimes more than two, sperms enter the egg through the micropyple (Jost, 1970). This event stimulates the initiation of meiotic divisions of the oocyte. After activation of the egg, the sperm moves to the centre of the egg. About 30 to 45 min (Idris, 1960) after insemination, the sperm reaches the centre of the egg and, in a compatible fertilized egg, fuses with the female pronucleus, which has meanwhile been formed and has also migrated to the centre. In the incompatible fertilized egg, the sperm is blocked on its way to the centre and no karyogamy takes place. The embryo develops as a haploid and dies at different stages during embryogenesis, while the male pronucleus is resorbed by the egg plasm.

The aim of the present paper, as well as comparative analysis of the protein pattern of different egg types, was to examine the differences for their relevance to the development of incompatibility. The experimental investigations were based on the following working hypothesis. After each insemination there will be an interaction between sperm and egg which results in blocked karyogamy in the incompatible fertilized egg. This interaction must underlie a biochemical mechanism based on enzyme reactions, which catalyse the fusion of the two pronuclei in the

normal case. This must be preceded by "recognition" of the entered sperm. The egg, as well as the sperm, must dispose of at least one factor which takes part in the events resulting in karyogamy. Strains belonging to different crossing types should differ in the composition of their factors.

Materials and Methods

All the investigations were carried out using the three strains "Hamburg" (Ha), "Paris" (Pa), and "Oggelshausen" (Og) of *Culex pipiens*. A description of the origin of these strains is given by Laven (1957). Ha and Pa show bilateral incompatibility. Ha and Og, and Pa and Og, can be crossed only in one direction, ♀ Ha (Pa) × ♂ Og; the reciprocal crosses are incompatible. The mass production of the mosquitoes is described by Schumann (1973).

For the experimental studies, extracts of the normal crosses Pa × Pa, Og × Og, Ha × Ha, Pa × Og, of the incompatible crosses Og × Pa, Pa × Ha, Ha × Pa, and of the unfertilized eggs of Ha and Pa were used. The main problem was synchronization of oviposition. Good results were achieved by the following method. The emerged adults were separated into males and females all at 12 hrs, crossed and kept in cages covered with moist filter paper. After 8–10 days the cages were put in a completely dark room with a dish containing tap water for oviposition. The best time of day was between 5 and 9 p.m. Every 20 min. the dishes were examined and the deposited rafts put on dry-ice and stored at −80 °C in a deep-freeze.

By this method up to 300 rafts could be collected during the first 60 min. After ten days the mortability of the adults increased rapidly, so that a second oviposition after a blood meal yielded only a small number of rafts.

A lot of difficulties arose in getting unfertilized eggs. On one hand the chances of survival of these females in comparison with fertilized ones were essentially decreased, on the other hand only a few of them deposited eggs. A blood meal stimulated oviposition, but the suction rate was low so that immunization with extracts of unfertilized eggs could not be carried out. Table 1 summarizes numbers of collected rafts and their content of soluble protein.

The rafts were ground in Na-phosphate-buffer, pH 7.4, using a Potter-Elvehjen homogenizer at 2000 R.P.M. for 2 min, centrifuged at 20.000 R.P.M., and the clear supernatant was standardized to 1 mg protein per ml solution.

Immunization was carried out with extracts of the crosses Pa × Pa, Ha × Ha, Pa × Ha, and Ha × Pa. Healthy rabbits were given six subcutaneous injections under the neck skin with a total dose of seven mg protein. On the 1st, 8th, 19th, 26th and 35th days, each rabbit received 1 ml antigen with 1 ml Freund's adjuvant-complete (Hyland, Los Angeles, Calif., USA, Nr. 070–150). On the 42nd day a booster injection containing 2 ml antigen was given. One week after the last injection the animals were aseptically exsanguinated from the exterior ear-vein and the serum was stored without preservative in small portions at −80 °C in the deep-freeze.

Table 1. *Preparation of the antigenic material from different Culex pipiens raft types*

Material	Number of rafts	Total amount of soluble protein (mg)
Pa unfert.	275	6.3
Pa × Pa	4173	44.8
Pa × Ha	3634	66.7
Ha unfert.	507	14.0
Ha × Ha	4443	49.6
Ha × Pa	2833	54.6
Og × Og	266	5.0
Og × Pa	596	9.5
Pa × Og	2976	21.0

unfert. = unfertilized rafts

The techniques for controlling antibody production, performing the Ouchterlony diffusion test and the acrylamide gel electrophoresis were identical with those previously reported (Schumann, 1973).

The histochemical demonstration of enzymes was done directly in the acrylamide gel. After the run the gel was incubated with the appropriate substrates. The enzymatic activity was coupled with the formation in the gel of a dye-complex insoluble in the buffer-solution. When testing enzymes working in the acid pH range, the gel had to be equilibrated in the corresponding buffer-solution.

Acid and alkaline phosphatase, esterase, leucine aminopeptidase and catalase were demonstrated according to the methods of Brewbaker (1968), malate-, lactate-, glutaminate-, and glucose-6-phosphate-dehydrogenase according to those of Clausen and Øvlisen (1965).

Results

Determination of the Number of Precipitate Lines with Homologous and Heterologous Tests

The results of the Ouchterlony diffusion test with the egg raft extracts against rabbit antisera are summarized in Table 2. It is convenient to divide the results into two groups. The first contains the four raft types with which immunization took place, and which could be analysed in the homologous and heterologous tests. The second group consists of the remaining five raft types which were not used for immunization.

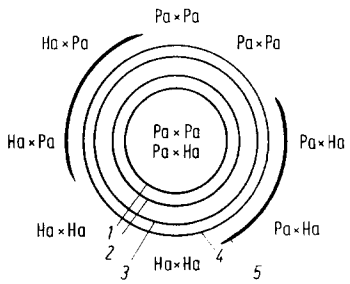
Table 2. *Number of precipitate lines in agar gel resulting from immunodiffusion of Culex pipiens raft extracts against antisera prepared in rabbits*

Ag As	Pa × Pa	Ha × Ha	Pa × Ha	Ha × Pa
Pa unfert.	4	4	5	5
Ha unfert.	4	4	5	5
Pa × Pa	4	4	4	4
Ha × Ha	4	4	4	4
Pa × Ha	4	4	5	5
Ha × Pa	4	4	5	5
Og × Og	3	3	3	3
Og × Pa	3	3	3	3
Pa × Og	3	3	3	3

unfert. = unfertilized rafts
Ag = Antigen; As = Antiserum

Looking at Table 2, it will be noticed at once that there are differences among the raft types. Those belonging to the first group can be subdivided into two further groups according to the number of precipitate bands: one is formed by the two compatible crosses Pa × Pa and Ha × Ha, whose extracts contain four identical antigens; the second subgroup is

composed of the incompatible crosses Pa × Ha and Ha × Pa, which share five common antigens. Comparing both crossing types, it could be proved that the four antigens of the compatible crosses are identical to four antigens of the incompatible crosses. Both the incompatible crosses Pa × Ha and Ha × Pa dispose of an additional antigen. The reaction scheme is as follows:



Precipitate line No. 5 represents the additional antigen. The second group can also be subdivided into two groups. One contains the unfertilized rafts of Pa and Ha. These are able to precipitate five antigen-antibody-complexes with antisera against Pa × Ha and Ha × Pa. This means that a certain protein, which occurs in the unfertilized egg of Pa and Ha, cannot be demonstrated any more. This protein is still present in the incompatible fertilized egg of Pa or Ha, e.g. incompatible rafts show the same behaviour as unfertilized ones. Raft extracts of the other subgroup form only three precipitate lines with all the four antisera. It can be assumed that marked differences exist in all these cases.

Zaman (1964) precipitated four antigens in raft extracts of *Culex pipiens fatigans*; Roberts (1971) succeeded in demonstrating eight different antigens in unfertilized eggs of *Drosophila melanogaster* and ten in fertilized eggs of the same species.

Determination of the Number of Common Antigens in Extracts of Whole Adults and Rafts

Extracts prepared from whole adult males and females (Pa, Ha and Og) and their corresponding antisera (Schumann, 1973) were used to determine common antigens in both adults and eggs. Firstly, the six antisera against adults were analyzed with the nine different homogenates of the seven crosses and the two unfertilized rafts. All the raft extracts share three antigens with the adults. There are no differences among the strains. The reciprocal attempts, namely the diffusion of the four antisera against rafts, revealed only tow common antigens in all 24 possible tests. These differing results express a quantitative effect. The missing third antigen must be present in the adult extracts because it stimulated the formation of antibodies. As it did not succeed in precipitating the corresponding antibodies in the antiserum prepared against the rafts, it must be present in the serum in small quantities and therefore evades demonstration by the Ouchterlony test.

Changes in the protein pattern from the egg to the adult have been reported for four *Anopheles*-species and for *Aedes aegypti* by Smith and Silverman (1966), but no numerical particulars about the number of precipitate lines were given.

Electrophoretic Separation of the Raft Extracts

Fig. 1 shows a diagram of the protein bands which could be stained with amido-black. This scheme was obtained after a run of eight hrs at a gel concentration of 7.5%.

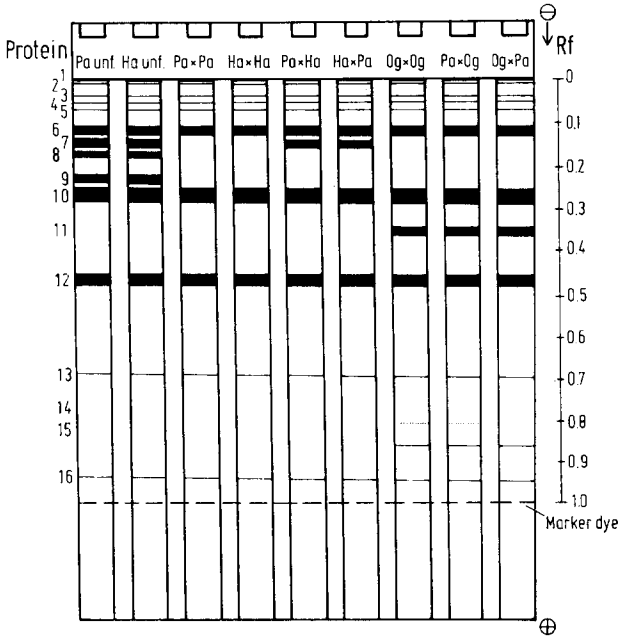


Fig. 1. Diagrammatic representation of the protein fractions of different *Culex pipiens* raft types which could be stained with amido-black in the acrylamide electropherogram

The diagrammatic representation shows that there are differences in the protein pattern of the raft types. The two unfertilized raft extracts of Pa and Ha share 13 common proteins, the two compatible crosses Pa × Pa and Ha × Ha share ten, and the two incompatible crosses Pa × Ha and Ha × Pa share eleven common proteins. The ten and eleven proteins of the four possible crosses between Pa and Ha are identical with the corresponding proteins from the two unfertilized rafts. The two proteins with the Rf-values 0.18 (protein-8) and 0.23 (protein-9) could be demonstrated only in the unfertilized eggs. On the contrary the protein with the Rf-value, 0.15 (protein-7) is common to the unfertilized rafts and the incompatible ones; it could not be demonstrated in the compatible rafts.

The remaining three raft types differ mainly in additional proteins from the crosses between Pa and Ha. The crosses Og × Og and Pa × Og possess 13

identical protein bands; the cross Og × Pa shows one band less, while the existing twelve bands correspond to those of the previously mentioned crosses. In this case there is also a difference between the crossing types, but it is not as marked as the difference between Pa and Ha.

The decisive difference between both groups of crosses, namely between Pa and Ha on one hand and between Pa and Og on the other hand, concerns protein-7 and protein-11. A striking point is that protein-11 could be demonstrated in crosses of Pa × Og, but not in those of Pa × Pa and Pa × Ha.

Histochemical Demonstration of Enzymes in Raft Extracts Separated in Acrylamide Gel

Fig. 2 shows the zymogram of the phosphatases and esterases. It was possible to demonstrate a maximum of three acid phosphatases, with Rf-values 0.03, 0.32 and 0.37. The phosphatase at 0.32 is not present in homogenates of the crosses Og × Og or Og × Pa, indicating a strain difference. As this enzyme is present in the cross Pa × Og, it can be considered to be specific for eggs. Relative to the activity of the single acid phosphatases the enzyme at Rf 0.27 showed the highest activity; after only

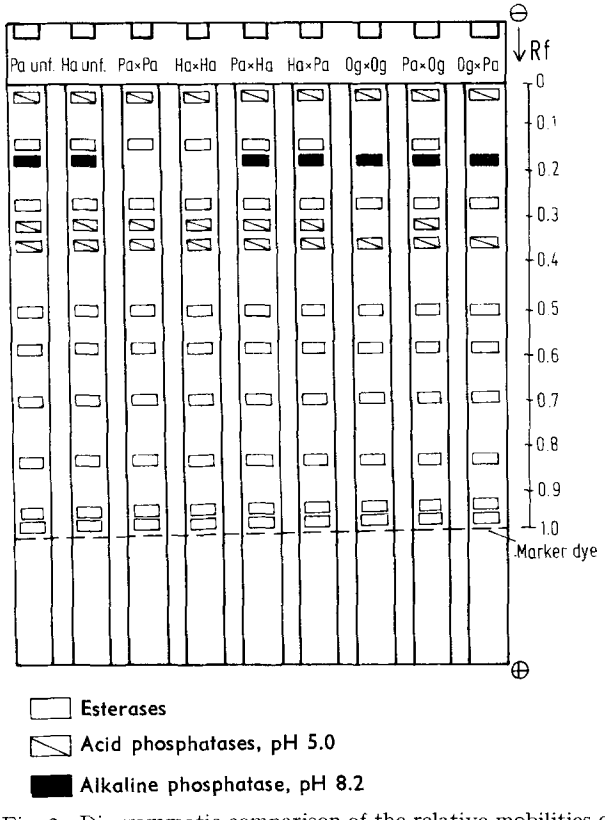


Fig. 2. Diagrammatic comparison of the relative mobilities of the esterase, acid and alkine phosphatase enzymes of different *Culex pipiens* raft types. After disk-electrophoresis of the raft extracts, the histochemical demonstration of the enzymes was directly performed in the acrylamide gel by incubating with the appropriate substrates

15 min incubation the dye-complex became visible. The two other phosphatases appeared after about 90 min. When testing for alkaline phosphatases, one could be detected with a Rf-value 0.16. This enzyme could not be demonstrated in rafts of the crosses Pa × Pa and Ha × Ha. Its activity is also marked and it appeared after 15 min. Electrophoretic variants of an acid phosphatase in the sibling-species of *Drosophila melanogaster* and *Drosophila simulans* were reported by McIntyre (1966). Alkaline phosphatases were demonstrated in *Drosophila melanogaster* (Schneiderman et al., 1966; Sena and McIntyre, 1966) and in two sibling-species of *Anopheles* (Bianchi, 1968).

Up to eight esterases were found; their Rf-values were 0.14, 0.27, 0.50, 0.70, 0.84, 0.96, and 0.99. Even in this case, the absence of the esterase of Rf 0.14 in the rafts of Og × Og and Og × Pa can be interpreted as a strain difference. The greatest activity was shown by the enzyme of Rf value 0.27, followed by those of Rf's 0.50, 0.14, 0.96, 0.99, 0.70 and 0.84.

There have been many reports about the demonstration of esterases. In *Drosophila melanogaster* six different esterases have been detected (Beckman and Johnson, 1964a); in *Drosophila subobscura* there were five, with polymorphism found for the fifth (Gonzales-Duarte and Prevosti, 1970). Cholinesterases have been demonstrated in head extracts of *Musca domestica* (Eldefrawi et al., 1970) and in larvae of *Culiseta inornata* (Wilder, 1970), phospholipases A and B in larvae of *Culex pipiens fatigens* (Rao and Subrahmanyan, 1969a; 1969b; 1970) and esterases in *Anopheles atroparvus* (Bianchi, 1970).

Fig. 3 shows the zymogram of the enzymes glutaminate-, lactate-, malate-, glucose-6-phosphate-dehydrogenase, leucine aminopeptidase und catalase. No differences could be detected for these enzymes among the different raft types. Only one enzyme activity was observed for glutaminate-, lactate-, and glucose-6-phosphate-dehydrogenases. Their Rf-values amounted to 0.01, 0.03, and 0.28. Two enzyme activities were shown for malate-dehydrogenase with Rf-values of 0.16 and 0.49.

Lactate-dehydrogenase has been demonstrated in the tissues of many insects (Zebe and McShan, 1957), in eleven species of *Chironomus* (Wülker et al., 1969), and was characterized in *Aedes aegypti* by determination of the Michaelis constant (Combre et al., 1971). Glucose-6-phosphate-dehydrogenase has been detected in 19 different strains of *Drosophila melanogaster*, occurring in two electrophoretic variations (Young et al., 1964); two variants from the same species were partly purified and biochemically characterized (Steele et al., 1968); and finally, it has been found in embryos and the early stages of *Drosophila* (Wright and Shaw, 1970). Malate-dehydrogenase was observed in *Drosophila melanogaster* (O'Brien, 1969) and in many other insects and its

pH-optimum was designated as pH 8.5—9.0 (Faulkner, 1956).

10 min. after incubation with H_2O_2 the catalase activity became visible, and increased to a maximum at about 20 min. after incubation. The Rf-value for catalase was 0.10.

A catalase present in *Chironomus thummi* was described by Precht and Carlsen (1953).

Testing for leucine aminopeptidase lead to the detection of two enzyme activities with Rf-values 0.22 and 0.77. The dye-complex was formed 5 min. after addition of the substrate. The enzyme at Rf 0.22 showed greater activity than did that at Rf 0.77. Two enzymes of leucine aminopeptidase have also been found in *Drosophila melanogaster* (Beckman and Johnson, 1964b).

Discussion

By applying the Ouchterlony diffusion test, extracts of compatible and incompatible fertilized rafts were analysed. Immediately after oviposition fertilized eggs possess at least four or five different antigens. It is necessary to add the expression "at least" because the Ouchterlony test would probably not allow fine differentiation between antigens which diffuse at similar rates. Using the same technique, four distinct precipitate lines have already been observed in fertilized eggs of *Culex pipiens fatigans* (Zaman and Chellappah, 1964), while Roberts (1971), using immunoelectrophoresis, demonstrated ten different antigens in fertilized eggs of *Drosophila melanogaster*. Therefore it can be assumed that, in this case also, the application of more sensitive methods should result in the detection of further antigens. The most striking point during the investigations with raft extracts was the discovery of an antigenic difference in the four possible crosses between Pa and Ha. The compatible crosses Pa × Pa and Ha × Ha showed four different antigens, the two incompatible ones Pa × Ha and Ha × Pa five antigens. Taking the results of the two unfertilized raft types Pa and Ha into consideration, it can be stated that they react in different ways. They will form four bands with antisera prepared against the compatible crosses and five bands with the two other antisera; furthermore, Pa and Ha possess a protein in the unfertilized egg which can not be demonstrated in the compatible fertilized egg, while it is still present in the incompatible fertilized eggs.

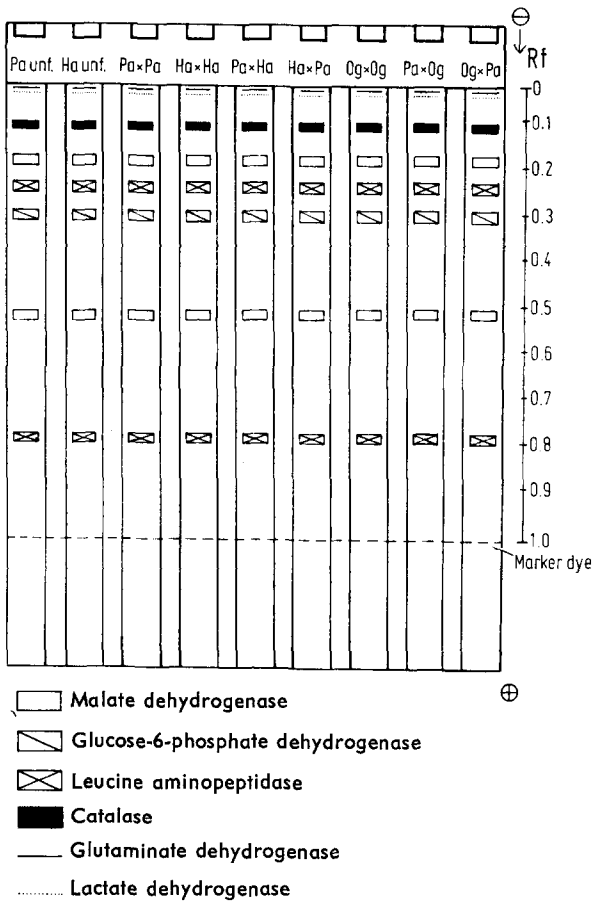


Fig. 3. Diagrammatic comparison of the relative mobilities of the malate-, glucose-6-phosphate-, glutaminate-, lactate dehydrogenase, leucin aminopeptidase and catalase enzymes of different *Culex pipiens* raft types. After disk-electrophoresis of the raft extracts, the histochemical demonstration of the enzymes was directly performed in the acrylamide gel by incubating with the appropriate substrates

Differences among the raft types could also be demonstrated by disk-electrophoresis in acrylamide gel. After staining the protein bands with amidoblack, one protein band (protein-7) appeared in the unfertilized and the incompatible fertilized rafts of Pa and Ha while it was absent from the compatible fertilized rafts. It is assumed that this protein-7 and the fifth precipitate line in the Ouchterlony test are identical. Is there a connection between these different protein patterns and the phenomenon of incompatibility? According to the working hypothesis strains of different crossing types differ in chemical composition of the factors which are responsible for the development of compatibility and incompatibility. When comparing the protein patterns of compatible fertilized rafts, no differences can be observed between Pa and Ha; the same is true for unfertilized rafts of these two strains. The two incompatible fertilized rafts also exhibit the same protein pattern. A difference is detected when the two raft types are compared. As this difference does not appear between two strains, but between two crossing types, it is possible that it can be correlated with the phenomenon of incompatibility.

The electropherograms of the three raft extracts of the crosses Og × Og, Og × Pa and Pa × Og are nearly identical, except for a weak protein band (protein-14) which is lacking in the cross Og × Pa. Nevertheless, this small difference can not be correlated to that in the crosses between Pa and Ha. The

incompatible fertilized rafts (Og × Pa) do not exhibit an additional protein.

When comparing the electrophoretic pattern of the crosses between Pa and Og with those between Pa and Ha, an essential difference can be noticed. Protein-7, which appears in the two unfertilized raft types and in the rafts of the crosses Pa × Ha and Ha × Pa, is absent in the crosses between Pa and Og. In contrast, the latter possess protein-11 which is missing in all the other raft types. In any case there is a difference between the crosses with Pa and Ha and those with Pa and Og.

An immunological analysis of the raft extracts from Og × Og, Pa × Og and Og × Pa with the four antisera prepared against rafts showed the existence of three precipitating antigens. All the rafts under investigation share at least three common antigens.

Finally, an attempt was made to correlate specific enzymes to the protein bands in the electropherogram and, at the same time, to look for differences in the enzyme pattern. Glutaminate-, lactate-, glucose-6-phosphate-dehydrogenase and catalase occur as single enzymes in each raft type, and possess the same Rf-value. Malate-dehydrogenase and leucine aminopeptidase exist as two enzyme activities and exhibit the same Rf-values in all three raft types. Up to 8 esterases could be demonstrated per raft type; only the rafts Og × Og and Og × Pa lack one of these enzymes, the same one in both cases. This difference is interpreted as strain-specific. Similar properties occur in the acid phosphatase, where three enzyme activities could be observed, except in Og × Og and Og × Pa. This difference can also be regarded as strain-specific. The most interesting result was produced when testing for alkaline phosphatase. Except in the crosses Pa × Pa and Ha × Ha an alkaline phosphatase with a common Rf-value was demonstrated in all the raft types. This Rf-value corresponds to that of protein-7 of the raft electropherograms. It can be concluded that the greatest part of the protein-7 band stainable with amido-black represents an alkaline phosphatase.

The finding of two or more enzyme activities when testing for one special enzyme can be interpreted in different ways: (1) they represent true isoenzymes; (2) they are monomers of a multimeric enzyme divided by electrophoresis into the still active subunits; (3) they represent different enzymes with an affinity for the same substrate. A decision for one of the three possibilities can not be made without further elaboration of the reaction kinetics of the observed enzyme activities.

How can these results be interpreted in context? Is there a connection between the detected differences and the phenomenon of incompatibility? If the results of the Ouchterlony tests, of the disk-electrophoresis and of enzymatic studies are compared, the following statement is possible: both unfertilized raft types Pa and Ha possess a certain protein (protein-7) which exhibits the same behaviour during electrophoresis. Its identity is not yet proved; this could be achieved only by immunologic methods. This protein-7 can no longer be demonstrated in the crosses Pa × Pa and Ha × Ha, but it is still present in the crosses Pa × Ha and Ha × Pa. As it proved to be identical in both raft types, it can be assumed that it is also identical in the two unfertilized raft types. Assuming that protein-7 is an alkaline phosphatase and that it is identical to the fifth precipitate line in the Ouchterlony test, it can be concluded that this alkaline phosphatase in the unfertilized egg of Pa and Ha is present in an active form. After having entered the egg of the same strain, the sperm catalyzes a still unknown reaction, and then is immediately destroyed. It is not inactivated as it is absent from the electropherogram. No protein band can be found at the corresponding place in the crosses Pa × Pa and Ha × Ha. In contrast, the two crossing types Pa × Ha and Ha × Pa exhibit alkaline phosphatase activity.

The difference between the crossing types can only be caused by interaction between sperm and egg. The egg, as well as the sperm, must contribute at least one factor responsible for the fusion of the two pronuclei.

The results can be summarized as follows: Differences could be detected using two different methods among different raft types from crosses with these strains. Considering the strains Pa and Ha combined by bilateral incompatibility, it is possible to interpret the quantitative differences as an expression of incompatibility, while such a difference could not be found between Pa and Og combined by unilateral imcompatibility. The evidence for the correlation of a discovered difference to incompatibility will be proved only when the factor implicated in the difference can be isolated and, by injection into the corresponding strain, its crossing type can be changed.

The analysis of compatible and incompatible fertilized rafts presents a possible way to clearing up this phenomenon. The studies must continue in two directions: firstly, further raft types from crosses between strains belonging to different groups have to be produced; secondly, the extracts of these rafts must be fractionated by ion exchange- and gel-chromatography. The corresponding fractions should then be analysed comparatively to elucidate differences among the various raft types to the last detail. An extract characterization of the enzymes and their catalysed reactions would also be required. In this way, the biochemical mechanism of incompatibility would be elucidated.

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