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Matsui, Tsuyoshi
Horticultural Laboratory, Department of Agriculture, Kyushu University

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On the application of the serological method towards the studies on the phylogenetic differentiation in cultivated plants*

Tsuyoshi MATSUI

GENERAL INTRODUCTION

It is well known that the evolutional process of an organism is accomplished in the main through the mutations of genes. Although an individual mutation is generally a quite small in its extent, during the lapse of many generations the accumulations of these small mutations exposed to the natural selections and the isolation mechanisms become to introduce a new species? Moreover, phylogenetic differentiations of cultivated plants are very complex, mainly because of the enormous complex characteristics resulted from the cultivation in general.

For the appropriate understanding of cultivated entities, such as strains and as forms, it is necessary to be able to distinguish in detail among strains, variety-complexes, and commercial varieties, and then the crop plants would be correctly comprehended after we could make those differentiation procedures quite clear. It has hitherto been improbable to define clearly such actual circumstances by means of any biological methods, such as the classic systematics which based mainly on morphorogy and as the cyto-genetic analyses. So that, there are prevailing urgent needs to introduce the entirely new methods to solve those serious problems concerned.

The precipitin reaction was first reported by Kraus[30] in 1879. After its discovery by Kraus the precipitin reaction was extensively used by Nuttall[37] in his study of animal relationships. These studies of Nuttall gave a fresh impetus of applying this new tool to the contemporary workers in plant systematics. In 1901, Kowarski[27]

* Contribution from the Horticultural Laboratory, Faculty of Agriculture, Kyushu University.

found that the wheat seed-extracts induce in rabbits the production of precipitins which react strongly with wheat extracts, but react weakly or not at all with the seed-extracts of rye, barley, oats, and of peas. Magnus and Friedenthal[32] also reported the results obtained by the precipitin tests with rye and other cereals. In 1908 Magnus,[33] working with some members of grass family, suggested that in the progressive immunization of an animal, the serum first shows reactivity only with closely related antigens, later with more and more distantly related ones, the homologous reaction always remaining strongest. Relander[44] reported his success in differentiation of species and varieties of barley and *Vicia* by means of the precipitin reaction.

Wells[48] used the purified zein and gliadin, and succeeded in demonstrating that the anaphylactic reaction is equally as specific and as severe with vegetable antigens as with animal proteins. Wells and his associates[49,50] followed up their early works with considerable additional experiments by the anaphylactic reaction with purified plant proteins.

In 1910 Chapman" prepared the precipitating sera for the *Acacia* seed-extracts, and discarding all precipitates produced by the reaction with normal serum, showed that *Acacia* has much stronger affinity with *Pisum* than with *Phaseolus, Vicia*, and various non-leguminous vegetables. Wendelstadt and Fellmer[51] applied both the compliment fixation and precipitin tests for the distinction of cereals from legumes, and found that some artefact reaction which are sometimes introduced with normal serum, can be eliminated to some extent by dilution. Galli-Valerio and Bornand[15] immunized rabbits to the sunflower seed-extracts and showed that such antiserum reacts most strongly with the sunflower seed- extracts, less strongly with *Aster, Cynara*, artichocke, and non-*Compositae* in the order named.

In 1914 Zade[54] showed by means of the precipitin tests with numerous species and varieties of *Avena* and of *Triticum* that the serological relationships agree well with the contemporary views of the plant systematists. And moreover, Zade was able to demonstrate that *Trifolium pratense* and *T. repens* are related systematically, but they are serologically quite distinct and their hybrid, *T. hybridum*, reacts so strongly with both as to demonstrate clearly its hybrid nature.

Mez and his collaborators[34] applied the precipitin and "conglutinin" (Mez's reaction) reactions to a great many species of plants. From the enormous amounts of their results a genealogical tree of plant relationships, the so-called serological "Stammbaum," was successfully compiled up and presented in 1926. Gilg and Schürhoff[17] and their students in Berlin began a series of experiments strongly criticizing the Königsberg's

studies supervised by Mez. Mutual controversies developed extensively between Königsberg and Berlin schools were mentioned in detail by Chester[7] in his excellent review.

Kōketsu[28] and Kojima[27] in Japan, showed that the Dicotyledons is separated from the Gymnosperms in its systematic position by means of the precipitin reaction. Within the Gymnosperms their results in general agreed with the views of the contemporary systematists with a few deviations. They eliminated the artefact reactions introduced by the normal serum by the removal of all precipitations formed between an antigen and a normal serum before testing.

In 1923 Rives[45] applied the precipitin technique to a number of varieties of grape and showed that such forms as grafted readily react positively, while poor graft-combinations are distantly related serologically. Green[19] studied to make clear the possible correlation between serological affinity and graft compatibility in *Citrus*, ***Rosaceae,*** and *Solanaceae*, and arrived at the similar conclusions as Rives found.

Arzt[2] applied the precipitin tests to the study of relationships in *Gramineae*. And he demonstrated that barley is nearer to oat than to wheat, and that among the barley forms there are certain minor serological differences, the latter, however, being rather weak and not entirely convincing, in good accordance with the failure experienced by most other workers in separating varieties within a species by the ordinary precipitin test. Kato and Maruyama[26] were also unable to demonstrate intraspecific differences in rice plant by the ordinary method, although the saturation technique in vivo afforded the distinctions among Japanese, Chinese, Korean, and Formosan rices to some extent.

Nelson and Dworak[35] was able to separate wilt-resistant flax varieties from wilt-susceptible one by using the seed globulins in precipitin tests. Baldwin, Fred, and Hastings") found a close parallel between the serological differences of numerous legumes and relationships of legumes as measured by their resistance or susceptibility to various strains of nodule bacteria. Nelson and Birkeland[36] also showed that their serological characterization of wheats tested has some correlations with such genetic characteristics as resistance to stem-rust and yield. Edgecome[11] found that precipitin reactions with wheat globulin bear a strong and consistent relationship to the differential susceptibilities of these wheats to *Puccinia.*

By using the leaf extracts in precipitin tests, Fukushima and Maruyama[12] succeeded in classifying 8 species in ***Brassica*** into four distinct groups; i. e., I group (including *B. japonica*, ***B.*** *rapa*, ***B.*** *pekinensis*, and *B. chinensis*), II group (B. *Napella*), III group (*B.*

juncea), and IV group (*B. oleracea*, and *Raphanus sativus*). And further, they examined F_1 hybrids, *B. chinensis* × *B. Napella*, artificially raised and made clear that the hybrids are situated serologically somewhat intermediate between their parental forms.

As mentioned above, from the beginning of the 1900's, phytoserological reseaches were carried out rather actively in Europe, America, and in Japan, but they soon fell into decay after the 1930's. Such decline is considered to be due to some defects in the classic serological techniques used, and also to the frequent masking of the specific reactions by non-specific ones which are introduced by the normal serum. These two very important obstacles to the progress of phytoserological studies, which induced the controversy between Königsberg and Berlin schools and also the failure experienced by most workers in separating varieties within a species, are discussed later in some detail in this paper.

Recently several investigators reported on the new method applicable to the antigenic analyses of complex biological materials, utilizing the diffusion of antigens and antibodies in the gelified media. Oudin[51,42] developed originally the single gel diffusion method. Ouchterlony[39,40] soon developed the double diffusion method in gel. Moreover, the immunoelectrophoresis was added as a new remarkable technique by Grabar and Williams? These development of the techniques of immunochemical analyses in gelified media advanced the progress of studies on natural protein mixtures. Gell et al.[16] divided many species of mexican potatoes into three main groups by means of the double diffusion technique and the immunoelectrophoresis.

With the development of gel diffusion techniques described above, the author employed these new techniques for his purpose. And soon, he became to modifly the Ouchterlony's method, and showed that his modified method is very suitable for the agro-systematic studies on cultivated plants?

Materials and Methods

Antigens

The material plants investigated in the present work are shown in Table I. Seeds obtained from each of these material plants were pulverized by a mill, and treated with ethyl-ether to remove oils contained. Five grams of defatted seed-powder were extracted with 50 grams of 0.9 per cent sodium chloride solution over-night in refrigerator and then filtered. This saline extract was used as the antigen. Antigens were newly prepared as injections and tests.

Total nitrogen of the antigen was estimated by the micro-Kjeldhal

method. 'When the gel-diffusion analyses were done, the quantity of total protein in each antigen was determined beforehand, and this content was equalized among the antigens to be compared.

Table 1. Plant materials used.

Leguminoseae

Phaseolus vulgaris L. var. " Kintoki "
P. vulgaris L. var. " America "
P. vulgaris L. var. "Beni-shibori"
P. vulgaris L. var. " Master peas"
P. chrysanthos Savi.
Arachis hypogaea Savi. L.
Vicia faba L.

Cucurbitaceae

Cucurbita pepo L. var. " Table queen "
C. pepo L. var. " Large pumpkin "
C. *pepo* L. var. "Oblonga"
C. *moschata* Duch. var. " Shirokawa-sato-kikuza "
C. *maxima* Duch. var. " Delicious"

Antisera

Antiserum was prepared against the saline extract of seeds of bean, *Phaseolus vulgaris* var. " Kintoki." Five healthy 2 Kg. rabbits fed on the standard laboratory rations were used for the preparation of the antisera. The saline extract (the Kintoki-antigen) used for the immunization contained 2 mg. nitrogen per ml. Each animal had been given 2 intravenous injections every week. During the first two weeks 20 ml of the extract was injected in total, during the next three weeks 36 ml, and during the last three weeks 42 ml. Seven days after the last injection the animals were bled. The blood was allowed to clot and the resultant serum centrifuged to clear up. The serum was inactivated and added 1 per cent marzonin solution, making up 0.1 per cent concentration, and stored in refrigerator.

And also, the antiserum against the *Cucurbita pepo* var. "Table queen" antigen was prepared in the same way.

Methods

The author modified Ouchterlony's method. Ouchterlony had prepared his agar plate with three basins as shown in Fig. 1, but the

author prepared his agar plate using four basins and arranging these basins in the form of a rectangle as shown in Fig. 2.

Agar plates were prepared from filtered 1.5 per cent agar containing 1 g of sodium azaide, 0.02 g of methyl orange and 9 g of sodium chloride per liter of medium. The sodium azaide prevented bacterial growth on the plates, while the methyl orange imparted color contrast to the plates for photographic convenience. First, 6 ml of melted agar were evenly distributed over the bottom of a Petri dish of 9 mm in diameter. After the base layer had solidified and four brass moulds for basins had been placed on the agar, 15 ml of melted agar were poured upon to make four basins, each having a capacity of 0.2 to 0.3 ml. Each dish was covered with its lid and stored in refrigerator until used. The testings were carried out by charging each of the lower two basins with 0.2 ml of the same antiserum of desired dilution, and one of the upper two was filled with 0.2 ml of the antigen solution and the other one with 0.2 ml of another antigen to be compared. The antigens compared were adjusted beforehand to their respective concentrations based upon the nitrogen content measured by the micro-Kjeldhal method. All the dilution of reagents were effected using the saline of 0.9 per cent.

All experimental plates were set up in triplicate, incubated at 37°C and each one of the triplicate plates was examined individually and photographed when the precipitate reached at its maximum stage of development.

For the descriptions of these experiments, the upper left basin is designated as No. 1, the upper right one as No. 2, the lower left one as No. 3 and lower right one as No. 4, respectively.

EXPERIMENT I

MODIFING OUCHTERLONY'S METHOD

Introduction

Antigenic materials derived from the biological materials are, as a rule, extremely heterogenous immunologically. Such heterogeneity of the antigen can not be always demonstrable by the ordinary precipitation, agglutination, compliment fixation, and by anaphylaxis procedures. More information can be gained by the use of the quantitative precipitin method developed by Heidelberger,[26] but this method has intrinsic weak points attendant upon the reactions in liquid media. The serological reactions in liquid media have the intrinsic weak points which do not permit the enumeration of precipitating antigen-antibody systems in a sure and direct fashion

and which sometimes introduce the non-specific reaction to the antigen-antibody reactions.

In the technique of single gel diffusion method developed originally by Oudin the antiserum is mixed with agar in the bottom of small tube and the antigen is layered on the top. Then each antigen-antibody system forms a band of precipitate which migrates into the agar. Since the rate of migration is dependent upon the coefficient of diffusion of antigen and upon the relative concentration of antigen and antibody, each band of precipitation can be distinguished by its characteristic rate of migration. This method has a defect that the specific precipitate bands are sometimes covered by the non-specific one and the comparison between antigens is not always easy.

When one wishes to compare two antigens with each other, Ouchterlony's method is most suitably employed. Ouchterlony has developed the double diffusion method in agar. According to his technique, the antigen and the antibody are placed in 3 suitably spaced basins in an agar plate. When the reagents are present in proper concentrations, antigens and their corresponding antibodies diffuse towards each other in a gel and react by forming sharply defined precipitate lines, the morphologic characteristics of which permit detection of minimum number of reacting components and make possible the determination by comparison of identity, partial identity, or non-identity of any two antigens or antibodies (Fig. 1). The interesting technique of immunoelectrophoresis developed by Grabar and Williams[18] may be advantageous to the identification of antigens.

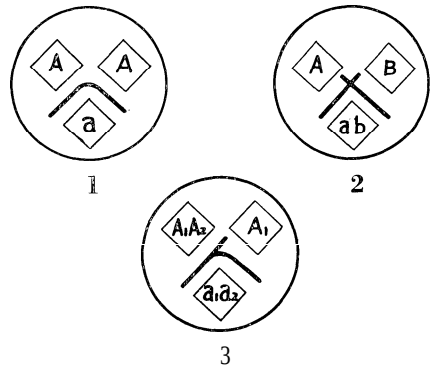

Fig. 1. Diagrammatic representation of the precipitate patterns resulted from the Ouchterlony's method. A, B, A_1, A_2,: antigens, a, b, al, a_2,: antibodies. 1 : a reaction of identity, 2: a reaction of non-identity, 3 : a reaction of partial identity.

As mentioned above, the second serious obstacle in the development of phytoserology is the frequent occurrence of the non specific reaction introduced with the normal serum. Several workers have attempted to eliminate such a non-specific reaction by pre-extraction of antigens with ether or acetone, the alcohol dilution of antigen, the absorption with normal serum and the addition of phosphate buffer. Rohringer and Stahman[46] prepared the r-globulin fractions by

the alcohol fractionation from the anti-tomato leaf protein serum, and employed it as the antiserum and then were able to eliminate the non-specific reaction. These techniques do not always go right in every case.

Materials and Methods

The antigens were the saline extracts of seeds of *Phaseolus vulgaris* and of *Phaseolus chrysanthos.* Antiserum was produced against the *P. vulgaris* antigen in adult rabbits. The details of the modified method were already mentioned in the chapter of general materials and methods.

Results

The four basins were placed in rectangular form in an agar plate (Fig. 2). Each of two comparator basins was filled with different kind of antigens to be compared and each of lower two basins with the same antiserum.

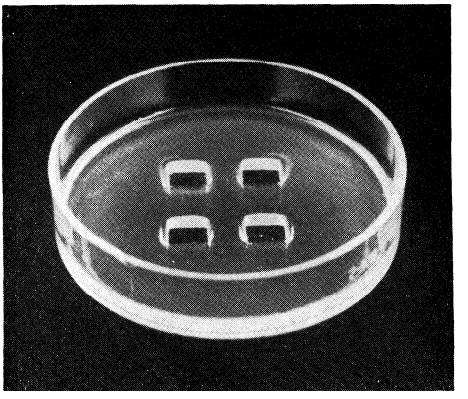

Fig. 2. The agar plate in Petri dish contains 4 basins in the form of a rectangle (according to the author's modified method). Two upper basins are designated as Nos. 1 and 2, and the lower two as Nos. 3 and 4, from left to right, respectively.

In this experiment, the comparator basins, Nos. 1 and 2, were charged with the same antigen of the same concentration and the lower two basins with the homologous antiserum. All reagents were added to 0.2 ml.

As shown in Fig. 3, the same number of the precipitate lines appeared at the same site, i. e., at the same location and the same orientation as well, respectively. Every precipitate line connected straightly as a single line extending from left to right, and all the precipitates on this plate showed the reaction pattern of " perfect identity " which was named by the author.

Several experiments were performed in order to make clear the precipitation patterns which changed into proper correlation with the varied concentrations of the same antigen. Five doubling dilutions of the *P. vulgaris* antigen were prepared, i. e., 1 : 2, 1: 4, 1: 16, 1 : 32, and 1: 64, respectively. One of the comparator basins was charged with the diluted *P. vulgaris* antigen and the other with the undiluted

one. As all the plates examined yielded the essentially similar result, only one example will be represented herewith.

No. 1 basin was filled with the undiluted *P. vulgaris* antigen and No. 2 basin with the 1 : 16 dilution of the same antigen. The lower two basins were filled with the anti- I-'. *vulgaris* rabbit serum. All. reagents were added to 0.2 ml. On the left part of this plate, the precipitate line appeared much more apart from the No. 1 basin than on the right part where the corresponding precipitate line appeared much nearer to the No. 2 basin, and thus these right and left precipitate lines which belong to the same antigen-antibody system appeared at the different locations, but united together at the middle portion, drawing a curved line, because these two lines belong to the same antigen-antibody system. This kind of pattern was termed under the name of the reaction of " imperfect identity " after the Ouchterlony's three different categories of patterns (Fig. 4).

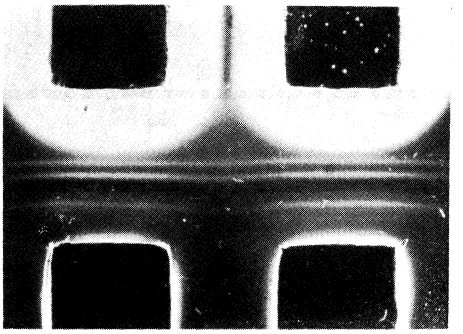

Fig. 3. Reaction of perfect identity. Plate charged with 0.2 ml of the P. *vdgaris* antigen of the same concentration to No. 1 and No. 2 basin, 02 ml of the anti-P. *vulgaris* antiserum of the same concentration to the lower two basins. The precipitate pattern represents "a reaction of perfect identity."

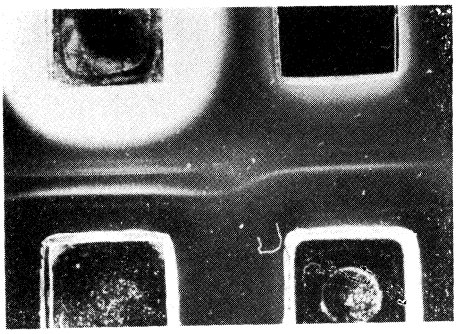

Fig. 4. Reaction of imperfect identity. Plates charged with 0.2 ml of the undiluted ***P.*** *vdgaris* antigen to No. 1 basin, 0.2 ml of the diluted same antigen (1 : 16) to No. 2 basin, the anti-***P.*** *vdgaris* rabbit serum to the lower two basins. The precipitate pattern represents "a reaction of imperfect identity ".

Now the author proceeded to his third experiment in order to effect the elimination of the non-specific reaction which was frequently introduced by the normal serum. No. 1 basin was filled with the *P. vulgaris* antigen and No. 2 basin with the *P. chrysanthos* antigen. The lower two basins were filled with the normal rabbit serum. As shown in Fig. 5, there appeared no precipitations in the intervening zone, revealing that the undesirable non-specific reaction

was able to be excluded. It is, however, not yet certain with the exact reasoning of such favourable excluding procedure under the double diffusion techniques in an agar.

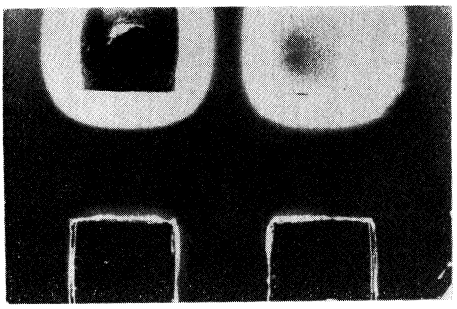

Fig. 5. Elimination of non-specific reaction. Plate charged with the *P. vulgaris* antigen to No. 1 basin, the *P. chrysanthos* antigen to No. 2 basin, the normal rabbit serum to the Power two basins. From the intervening space the non-specific reactions are completely eliminated.

Discussion

The author have attempted during recent several years to introduce the serological method into agricultural researches, not obtaining any satisfactory result. Numerous way of measuring the intensitv of precipitin reactions, such as the serum dilution titer, the antigen dilution titer, the optimal proportion method, and as the turbiditv measurement. have been tried out, but all the results obtained were nothing but the values represented only on the relative term, and could not stand precise criticism.

The quantitative precipitin method described by Heidelberger and his associates[20, 21] will permit a precise determination of the amount of antibody contained in an antiserum. Amount of the antibody nitrogen precipitated is calculated by subtracting that of antigen nitrogen precipitated from the total amount of nitrogen precipitated. This procedure makes it possible to estimate more precisely the intensity of antigen-antibody reaction than the ordinary precipitin method, but it has also a defect that a serological reaction in liquid media has.

As the value which is estimated by any one of test methods, such as the precipitin test, the agulutination test, the anaphylaxis and as the quantitative precipitin method, is equivalent to the sum of individual values of each of many antigen-antibody systems contained together in the reaction, it is quite difficult to analyze individual antigen-antibody reaction systems contained in the reaction by means of the classic techniques in a liquid media. Therefore, most investigators have hitherto remained in rather complete failure of defining very minute varietal differentiations within a species.

The development of the techniques of immunochemical analysis in gelified media marked an important advance in the progress of the studies on the natural protein mixtures, because it permits the enumeration of antigen-antibody systems.

Thus the author employed the Oudin's method. According to

Oudin's, the serum-agar gel in a small tube was over-layered with the antigen, then the antigen-antibody precipitates and the additional non-specific precipitate were formed at the interface, and moved together down the tube (Fig. 6). Therefore, the number of precipitated bands formed would correspond to the minimum number of the antigen-antibody systems concerned, but the true bands were covered by the non-specific precipitates.

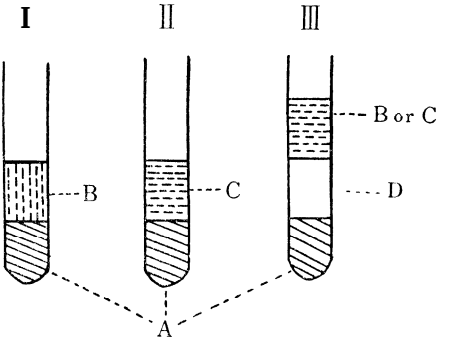

Fig. 6. Diagrammatic representation of the Oudin's method and its modified method. A: antiserum + agar, B : antigen, C : antigen + agar, D: agar.

Soon after, the author had adopted the Ouchterlony's method. In this method, 3 suitably spaced basins placed in an agar plate are filled with antigen or antibodies respectively. Antigens and antibodies, diffusing towards each other in a gel, reacted by forming sharply defined lines of precipitation, the site of which are dependent upon the diffusion rates and the concentrations of the reactants. Two different antigen or antibody systems can be compared directly by means of the phenomena of interactions indicating identity, partial identity or non-identity of individual components, (Fig. 1).

Itoh, Fukushima and Matsui[23] carried out examinations to discriminate among several species and varieties of beans by the Ouchterlony's method and from their results they were able to discriminate among species and also could discern the differences among species from those among varieties, though they could not distinguish among varieties within a species. As reported by Wilson and Pringle,[52,53] the precipitate line usually appeared more or less poorly near the fusion point, but the form of which was the very important criterion for judging the identity, partial identity, or non-identity, especially with the complex biological materials.

As mentioned above, the author tried to make modification of the Ouchterlony's method in order to analyze much more precisely the complex biological materials. The author employed 4 basins in an agar plate instead of 3 basins used in the Ouchterlony's method and also modified the arrangement of the basins. Using the modified method, the author divided one of Ouchterlony's reaction patterns, i. e., "a reaction pattern of identity " into two reaction patterns, i. e., "a reaction pattern of perfect identity " and " a reaction pattern of imperfect

identity."

As shown in Fig. 7, two precipitate lines which resulted from the same antigen-antibody system on both the left and the right parts of the plate became to be tied together at the connecting part for the same reason as precipitate lines in Ouchterlony's method fused. In Fig. 7, two lines which were tied at the region of equal distance from each comparator basin became to make a single straight line. Such a reaction pattern was named by the author as that of "perfect identity," and this may be taken as a particular situation of Ouchterlony's " a reaction of identity." And, moreover, when the concentration of the antiserum in the two comparator basins was constant and each antigen in the upper two basins were different in concentration but of quite the same constitution, the equivalent zone of the heavily concentrated antigen-the constant antibody system was formed much adjacent to the antibody basin than that of of the lightly concentrated antigen-the constant antibody system was to the antibody basin, and the precipitate lines on both parts became to be tied together, drawing a curved line at the connecting part. The author designated such a pattarn under the name of "a reaction of imperfect identity," and introduced this as a new category in addition to 3 kinds of categories already distinguished by Ouchterloney, i. e., identity, partial identity, and non-identity, respectively (Fig. 7).

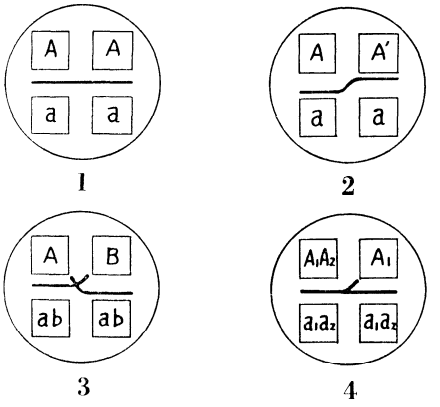

Fig. 7. Diagrammatic representation of the precipitate patterns according to the author's method (modified Ouchterlony's). 1: a reaction of perfect identity, 2: a reaction of imperfect identity, 3: a reaction of non-identity 4: a reaction of partial identity.

Some part of these results is in accordance with the Oudin's statement that when the same two antigens having the same concentration are compared, the precipitate lines develop with the same perpendicular distance from each basin, and when the reactants do not present in the equivalent concentration, the zone of precipitation displaces in a direction away from the source of diffusion of the reactant that is present in excess. Another part of these results is in accordance with the Ouchterlony's statement that the precipitated lines developed by the same antigens fuse. And then, this modified method will make it possible to analyze more precisely antigens or antibodies qualitatively

and quantitatively.

Applying these 4 kinds of categories of identification, Itoh, Fukushima and Matsui[24] succeeded in the distinction of varieties of beans, and Fukushima, Matsui and Miyazaki[13] also made examination to distinguish among varieties of squash, *Cucurbita pepo*. From the results of these experiments mentioned above, it was made clear that the author's modified method of Ouchterlony will duly suffice for the serological studies on the systematics of cultivated plants.

Some crude protein preparations from the plant materials gave not infrequently the non-specific precipitating reactions with the normal rabbit serum, and this fact more or less complicates the immunological tests and makes the results uncertain. So many workers have attempted in vain to eliminate this non-specific reaction. Bean extracts produced very frequently such non-specific reaction with the normal rabbit serum as reported by the several workers. The author, however, found that in his test with Ouchterlony's method, such troublesome non-specific reactions or precipitates did not develop on the plate (Fig. 5). But the author is not certain at present for the reason of such elimination of non-specific reactions though the double-diffusion technique in gel. However, it is evident that this elimination of the non-specific reactions makes it possible to investigate more precisely the plant proteins by the immunochemical methods.

EXPERIMENT II

INTER-SPECIFIC DIFERENCES AND INTRA-SPECIFIC DIFFERENCES

Introduction

As described under the heading of general introduction, many workers have employed the serological techniques in the studies on the plant systematics. The relationship of plants studied by the serological method were manifested by the affinities among them, and the degree of such affinity was based on the differences of intensities of reactions compared. Because of the difficulty of antigen analyses, one could not distinguish between a species and a variety. The description of intensities in precipitin reactions was usually given as the relative term. And further, values estimated by the serological techniques in liquid media were taken as the sum of values of many antigen-antibody systems contained in the reaction. Therefore, most workers have hitherto failed to distinguish among varieties within a species.

However, Gell et al.[13] divided species of mexican potatoes into three main groups judging from the precipitate lines produced by means of a double diffusion technique and an immunoelectrophoresis.

And they have reduced from their results that some proteins are specially confined to a species, or to a closely related group of its species, while some proteins may be found universally throughout the genus of that species.

The author intended to describe the following results obtained with several species and varieties of beans and of *Cucurbita* by means of his modified Ouchterlony's method mentioned above and, in consequence, to present his working hypothesis, concerning the concept of species and of varieties, which was worked out serologically.

Materials and Methods

The plants used for the experiments are composed of several species and varieties of beans and of cucurbits as listed in Table 1. The method employed is the modified Ouchterlony's method improved by the present author, as mentioned above.

Results

(a) Analyses of species and varieties of beans

The control reaction

The basin No. 1 was filled with P. *vulgaris* antigen. The basin No. 2 was filled with the saline which was used as the extractant and as the diluent. Both of the Nos. 3 and 4 basins contained the anti-P. *vdgaris* rabbit serum. All reagents were added to 0.2 ml.

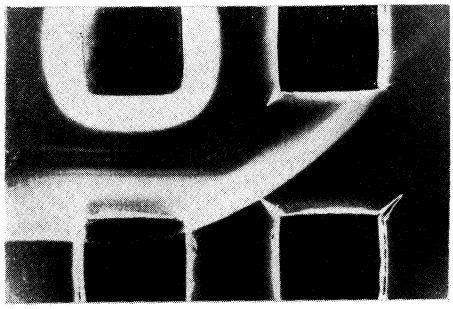

Fig. 8. Control reaction: Plate charged with the *P. vdgaris* antigen to No. 1 basin, the saline to No. 2 basin, the anti- *P. vdgaris* rabbit serum to the lower two basins. The saline and the anti-P. *vdgaris* rabbit serum do not react serologically.

In the intervening space between the *P. vdgaris* antigen basin and the anti-P. *vulgaris* rabbit serum basin, several precipitate lines developed, but between saline basin and the anti-*P. vdgaris* rabbit serum basin, any precipitate line did not develope at all. In other words, the saline and the anti-P. *vulgaris* rabbit serum did not react serologically (Fig. 8).

The two comparator basins, Nos. 1 and 2 were filled with *P. vulgaris* antigen. The basins, Nos. 3 and 4, were filled with the anti- *P. vulgaris* rabbit serum. All reagents were added to 0.2 ml. In the left and right intervening spaces, the equal number of precipitate lines appeared, each being united at the middle spot to make a straight line. The pattern of these precipitate lines will be taken as reaction pattern of "perfect identity" according to the author's designation described above (Fig. 9).

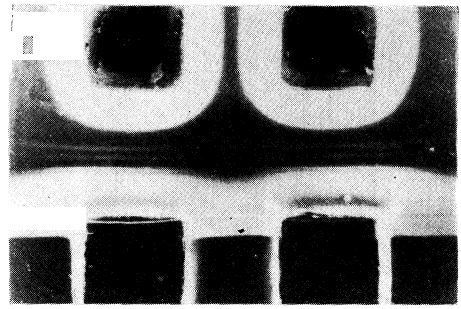

Fig. 9. Reaction pattern between two of the same amounts of the same antigen. Plate charged with the same amounts of the P. *vdgaris* antigen to No. 1 and No. 2 basins, the anti-P. *vulgaris* rabbit serum to the lower two basins. The precipitate pattern represents "a reaction of perfect identity."

Inter-specific differences

The basin No. 1 was filled with the *P. vulgaris* antigen and the basin No. *2* with the *P. chrysanthos* antigen. The Nos. 3 and 4 basins were filled with the anti- *P. vdgaris* rabbit serum. All reagents were added to 0.2 ml. As shown by the shape and the position of the precipitate lines in the intervening space, the *P. vdgaris* antigen and the *P. chrysanthos* antigen held two minor precipitate lines in common, although they were very faint in the reaction system of *P. chrysanthos* antigen -anti-*P. vdgaris* rabbit serum, but apparently the major line was not in common (Fig. 10). Similarly, the *P. vulgaris* antigen and the *Archis hypogaea* antigen did not contain any major line in common (Fig. 11), and also the *P. vulgaris* antigen and the *Vicia faba* antigen did not hold any major line in common (Fig. 12).

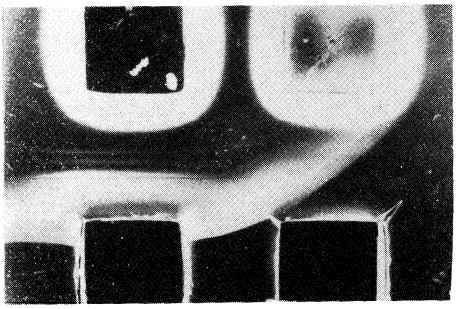

Fig. 10. Reaction pattern between two different species: Plate charged with the P. *vulgaris* antigen to No. 1 basin, the *P. chrysanthos* antigen to No. 2 basin, the anti-P. *vulgaris* rabbit serum to the lower two basins. The P. *vulgarbs* antigen and the ***P.*** *chrysanthos* antigen do not hold the major component in common.

From these results, the author could safely say that the saline

extracts of seeds of the plants belonging to the diff erent species do not, as a rule, hold such major cornponents in common.

Intra-specific differences

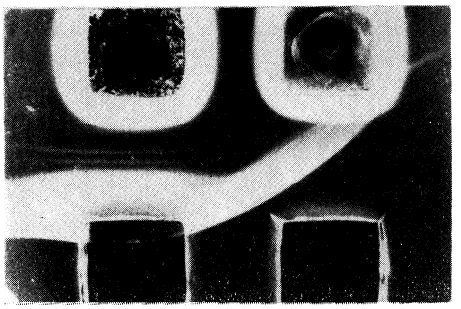

Fig. 11. Reaction pattern between two different species : Plate charged with the *P. vulgaris* antigen to No. 1 basin, the *Arachis hypogaea* antigen to No. 2 basin, the anti- *P. vulgaris* rabbit serum to the lower two basins. The *P. vulgaris* antigen and the *A. hypogaea* antigen do not hold the major component in common.

The basin No. 1 was filled with the *P. vulgaris* var. "Kintoki" antigen and the basin No. 2 was filled with the *P. vulgaris* var. " America " antigen. The basins Nos. 3 and 4 were filled with the anti- *P. vulgaris* var. "Kintoki" rabbit serum. All reagents were added to 0.2 ml. In the intervening space between the "Kintoki" antigen basin and the anti-"Kintoki" rabbit serum basin, the thick major lines, two minor lines and one faint minor line developed. In the intervening space between the "America" antigen basin and the anti-" Kintoki" rabbit serum basin, the thick major lines and three minor lines developed. And moreover, the major lines in the both intervening spaces were connected straightly, showing a typical pattern of a reaction of identity, but with the minor lines, the left three minors were connected with the right ones, making curves, respectively, representing a reaction pattern of a imperfect identity. Especially, this imperfect identity was remarkable for the minor lines located in the nearest position towards the antigen basins (Fig 13).

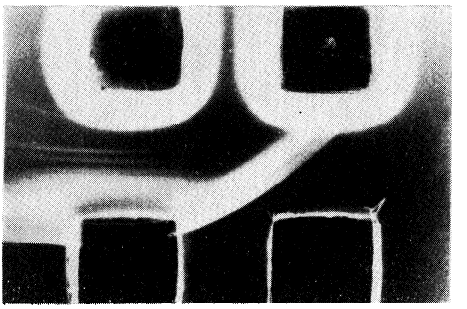

Fig. 12. Reaction pattern between two different species : Plate charged with the *P. vulgaris* antigen to No. 1 basin, the *Vicia fava* antigen to No. 2 basin, the anti- *P. vulgaris* rabbit serum to the lower two basins. The *P. vulgaris* antigen and the *V. fava* antigen do not hold the major component in common.

Therefore, it may be said that both the "Kintoki" and the " America " antigens contain the antigenic substances which de-

velope the major lines, in common and in equal quantity, but hold the antigenic substances which give rise to the minor lines in common but in different quantities. This may be well taken as the characteristic feature of the intra-specific differences.

Further, the comparison of *P. vulgaris* var. "Kintoki" with *P. vulgaris* var. " Benishibori" and also that of P. *vulgaris* var. "Kintoki" with P. *vulgaris* var. " Master-peas" showed the quite similar serological features as described above. These reaction patterns will be easily obtained in Figs. 14 and 15. With "Master-peas" antigen, an additional minor line is clearly developed in Fig. 15.

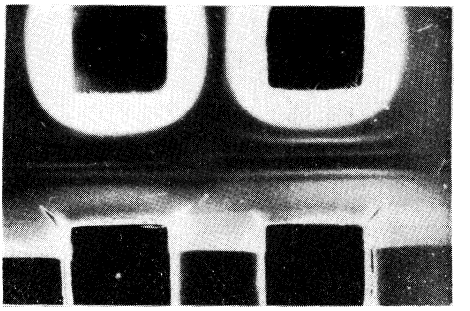

Fig. 13. Reaction pattern between two different varieties : Plate charged with the *P. vulgaris* var. " Kintoki " antigen to No. 1 basin, the *P. vulgaris* var. "America" antigen to No. 2 basin, the anti- *P. vulgaris* var. "Kintoki " rabbit serum to the lower two basins. The *P. vulgaris* var. "Kintoki" antigen and the *P. vulgaris* var. " America " antigen hold the major component in common and some minor components in common, though differing in their quantities.

(b) Analyses of species and varieties of cucurbits.

In order to make certain his methods applied to the experiments with several forms of beans, the author took up several species and varieties of cucurbit vegetables, and examined in the same methods as the former. These experiments gave the quite similar results (Figs. 16–19).

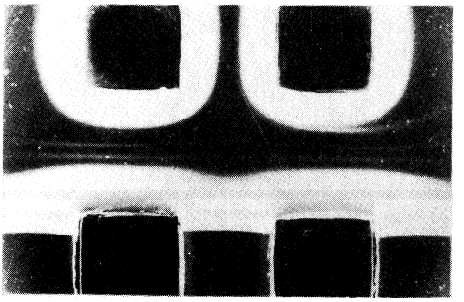

Fig. 14. Reaction pattern between two different varieties : Plate charged with the *P. vulgaris* var. " Kintoki " antigen to No. 1 basin, the *P. vulgaris* var. " Benishibori " antigen to No. 2 basin, the anti- *P. vulgaris* var. " Kintoki" rabbit serum to the lower two basins. The *P. vulgaris* var. "Kintoki " antigen and the *P. vulgaris* var. "Benishibori" antigen hold the major component in common and the some minor components in common, though differing in their quantities.

Discussion

The series of experiments described above indicated that the different species contained a antigenic substance which developed thick major precipitate line (as the major component) not in common, and some antigenic substances which gave rise to minor precipitate lines (as the

minor components) in common, and, in addition, different varieties within a species contained a certain common major component and some minor ones in common but in varied quantities. The author successfully discerned between the intra-specific and the inter-specific differences and also differentiated a species from another, and similarly a variety from another.

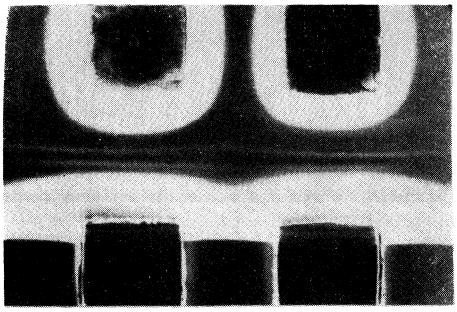

Fig. 15. Reaction pattern between two different varieties : Plate charged with the *P. vulgaris* var. " Kintoki " antigen to No. 1 basin, the *P. vulgaris* var. " Master peas " antigen to No. 2 basin, the anti- *P. vulgaris* var. " Kintoki" rabbit serum to the lower two basins. The " Kintoki" antigen and the " Master peas " antigen hold the major component in common and the some minor components in common, though differing in their quantities.

All investigators who employed the serological techniques under liquid media could not descriminate between the intra-specific and the inter-specific differences, because the resultant values estimated by the classic serological tests were the sum total values of individual ones of many antigen-antibody systems concerned, and yet the values of intensities of precipitin reactions was usually given as the relative terms.

Gell et al.[16] applied the agar-diffusion methods towards the taxonomy of many species of Mexcan and South American potatoes and succeeded in dividing these species into three main groups. And also they reported that some protein components may be confined only to species, or to a closely related group of species, and that some may be found universally throughout the genus, using the potato juice as an antigen.

The author showed, using the saline extract of seeds of beans and of *Cucurbita*, that different species contain the major component not in common, and

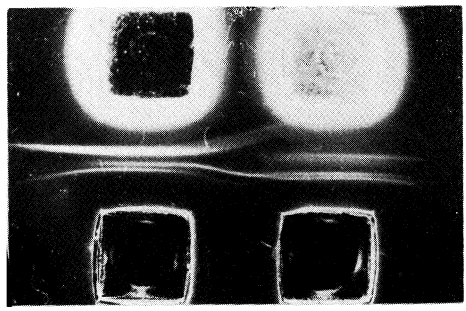

Fig. 16. Reaction pattern between two different species : Plate charged with the *Cucurbita pepo* antigen to No. 1 basin, the *C. moschata* antigen to No. 2 basin, the anti- *C. pepo* rabbit serum to the lower two basins. The C. *pepo* antigen and the C. *moschata* antigen do not hold the major component in common.

varieties within a species contain the major component in common and some minor ones in common but in varied quantities. In consequence, the author discerned the discrimination between the intra-specific and the inter-specific differences and also discriminating a species from another and similarly a variety from another.

The author used the saline extract of seeds, while Gell et al.[16] used the native potato juice as an antigen, so that the proteins reported by Gell et al. and by the author respectively, can not be checked with each other. However, it is interesting to note that in both the potato juice and the seed extract it seems to exist a regular protein constitution respectively.

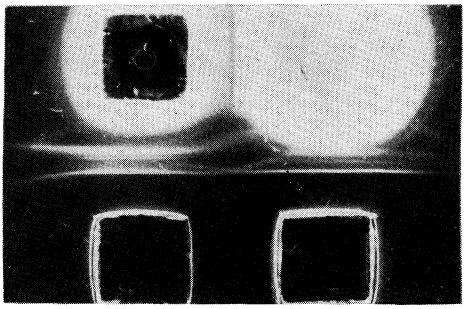

Fig. 17. Reaction pattern between two different species : Plate charged with the *Cucurbita pepo* antigen to No. 1 basin, the C. ***maxima*** antigen to No. 2 basin, the anti-C. *pepo* rabbit serum to the lower two basins. The C. *pepo* antigen and the C. *maxima* antigen do not hold the major component in common.

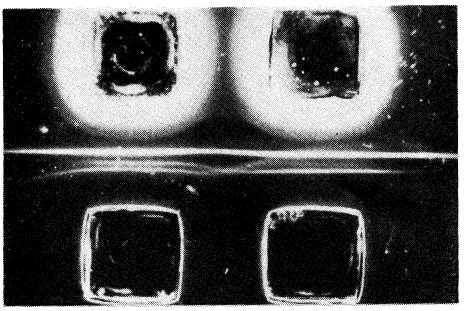

Fig. 18. Reaction pattern between two different varieties: Plate charged with the C. *pepo* var. "Table queen" antigen to No. 1 basin, the C. *pepo* var. " Large pumpkin " antigen to No. 2 basin, the anti-"Table queen" rabbit serum to the lower two basins. The C. *pepo* var. " Table queen " antigen and the C. *pepo* var. " Large pumpkin " antigen hold the major component in common, and the some minor components in common but in different quantities.

All the results presented above are those obtained through investigations used the saline extracts of seeds as the antigens. Further studies to make clear the relationships between such saline extracts and all the protein components contained in seeds will be necessary in this line of studies. However, it is nowadays a rather difficult task to extract all the protein components contained in seeds.

Although there are a considerable number of literatures on sero-systematics of plants, no experiments have yet been carried out which make it possible to distinguish between the inter-specific and the intra-specific differences by means of serological techniques. Therefore,

the author will dare to present here the working hypothesis that (1) each species contains the species-specific major component, (2) varieties within a species contain the species-specific major component in common and also the minor components in common, though in different amounts, or not in common in some cases.

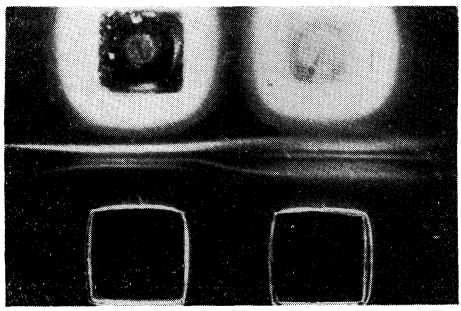

Fig. 19. Reaction pattern between two different varieties : Plate charged with the C. *pepo* var. " Table queen " antigen to No. 1 basin, the C. *pepo* var. "Oblonga" antigen to No. 2 basin, the anti-" Table queen " rabbit serum to the lower two basins. The C. *pepo* var. " Table queen " antigen and the C. *pepo* var. "Oblonga" antigen hold the major component in common, and the some minor components in common but in different quantities.

General Consideration

Since the work of Kowarski in 1901, phytoserological researches had been carried out intensively by many workers, but have fallen into decay after the 1930's. Such decline in phytoserological studies is considered to be due to the serious defects accompanied by the classic method used. As mentioned above, the result of a certain reaction tested can be obtained as a total sum of many antigen-antibody systems concerned to the reaction, and such total intensity can only be represented in relative amount, and further, the reaction itself is disturbed not infrequently by the non-specific reaction which results between the antigen and the normal rabbit serum. Thus the classic serological method seems to be only possible to represent the degrees of reiative affinity among antigens or antibodies.

In recent years, several investigators have been extending the serological techniques into gelified media instead of the classical liquid media. Oudin[41, 42] originally developed the single gel-diffusion method, and later Ouchterlony[39, 40] reported the double diffusion method in gel. In consequence, now we have arrived upon circumstances where we are quite possible to make the antigenic analyses of complex biological materials using these new techniques in gelified media.

When the crude protein preparations from plant tissues were used as the antigen, in the Oudin's method the precipitation due to the non-specific reaction became frequently to appear at the interface between the antigen and the antiserum, and it usually complicated the explanation of the results. So that, to compare two antigens or two antibodies

with each others the Ouchterlony's method may be considered to be more suitable. The Ouchterlony's method, however, accompanied the defects that the precipitate line was poorly developed near the fusion point, form of which was very important in the judgement of identity, partial identity or non-identity, and that the comparisons of the distances from the comparator basins or from the central basins to the precipitated line were not always easy, especially with complex biological materials.

As mentioned above, the author modified the Ouchterlony's method in order to analyze more precisely the complex biological materials and became to be able to divide the Ouchterlony's " a reaction of identity " into two categories ; i. e., " a reaction of perfect identity" and "a reaction of imperfect identity " using 4 basins in an agar plate and arranging them in each corner of a rectangle. Using his modified method, the author successfully discerned between the inter-specific and the intra-specific differences, and also differentiated a species from others and a variety from other ones. So that, it may be safely said that the modified method will contribute definitely towards the future serological studies with plant materials.

Although a considerable effort has hitherto been expended to frame a good definition of a species, it is still disputable to formulate a clear definition of a species in many fields of biology. As mentioned above, the author discerned between the inter-specific and the intra-specific differences employing the modification of Ouchterlony's method. The series of experiments here reported apparently indicate that the different kinds of species do not contain the major component in common, while different kinds of varieties within a species contain the major component and some minor components in common, though in different quantities. Generally speaking, there exist a considerable number of literatures of phyto-serosystematics, but no work has yet been done in order to elucidate the inter-specific difference and the intra-specific difierence serologically, mainly because of some shortages accompanied by the classic serological techniques as mentioned above. Although it is a rather difficult task to formuiate a clearcut definition of a species, the author will dare to present from the serological viewpoint his working hypothesis as follows: A species will usually contain the major protein component characteristic of itself and the minor protein components common to several species, and all the varieties within a species also contain the major protein component which is characteristic of its species in common and in addition the minor protein components which are common to several varieties but in variable quantities with the respective varieties. Such definition of species still remains to be inspected further and also to be substantiated

through advanced further studies along many fields of biological sciences.

It is well known that a seed contains several protein components, each of which differ in its quantity. Osborne"") classified the wheat proteins into four main groups, i. e., glutelin, gliadin, globulin and leucosin. He could also isolate three protein fractions from pea seeds, i. e., vicilin, legumelin and legumelin. From barley seeds Quensel[43] distinguished four components of globulin, i. e., a, β, γ, and δ, according to his designation. Danielson') reported on the sedimentation diagram showing the composition of globulin solutions which were prepared by the extraction of seeds of different species in Gramineae, and also he studied on 34 different species of *Legminosae* and by sedimentation analyses showed that latter seeds contain two main globulins. Further details of the studies on the seed proteins will be obtained refering to the reviews, compiled by Osborne,"") Danielson,') and by Brohult and Sandegren[5]. Although there exist a considerable number of literatures with seed proteins, only a little work has been done exclusively from the taxonomic viewpoint.

It is well known that the evolutional processes of an organism are accomplished in the main through the mutations of genes. Although the individual mutation is generally a quite small in its extent, during the lapse of many generations the accumulations of these small mutations exposed to the natural selection and the isolation mechanism, become to introduce a new incipient species or a true species. Studies on the biochemical effects of mutations have endowed the strong support to the notion that individual genes are directly connected with the biosynthesis of individual proteins, especially as reported by Ingram?' Comparisons of the structures of homologous proteins, i. e., proteins with the same kind of biological activity or function, obtained from different species will serve as the strong basis for establishing phylogenetic relationships. The proteins and the polypeptides for which comparisons of their covalent structures have been made until now are relatively few in number, and moreover, the species which had been examined are confined to those which are situated more or less distantly in their phylogenetical relationships. For example, Sanger[47] and his colleagues have determined the amino acid sequences for insulins derived from five different species, i. e.. beef, pig, sheep, horse, and whale. Differences were confined only to the amino acids within the disulfide "loop" of the A chain. A cytoclome C furnished one of examples of species variations in the protein structure, although studies on the variation in sequence have been carried out for only a relatively small portion of the total chain. The species had been examined were beef, horse, pig, salmon, chicken, silkworm, yeast, and *Shodo-*

spirillum rubrum. The amino acid sequences of N- and C- terminal of the proteins from various species had been examined with several proteins. A more extensive discussion of a biochemical approach to the study of evolution will be found in the excellent book by Anfinsen:[1] The molecular basis of evolution.

Species used for the comparison of variations in protein structure which will duly reflect the evolutional relationships were exclusively confined to ones related very distantly. Nowadays, it is rather difficult to detect the minute variations in protein structures which were raised among the closely related species, by means of any ordinary biochemical techniques. From such circumstances the author considers it most suitable to employ the serological species specificity for the ago-systematic inquiries of cultivated plants. It is well known that immune serum acts most intensely with the kind of antigen used for the immunization, and in addition, with the antigen from the related plants, the intensities of the reactions being in proportion to the degree of phylogenetic relationships.[4,25,31]

This series of experiments clearly showed that one can successfully discern the discrimination between the intra-specific and the inter-specific differences, and can also differentiate a species from another and a variety from another by means of the author's modified method. Thus, it may be safely said that the serological method will provide us a new powerful tool to solve the problems of agro-systematics in cultivated plants. And further, it is quite concievable that with the development of serology and protein chemistry, the serological method will contribute effectively towards the solution of the various species problems in cultivated plants and towards the studies on the general agro-systematics. The author's work therefore will become to arouse a large number of fresh experiment to solve the various important species problems at the molecular level.

The author's further experiments of cultivated plants which have direct concern with the phylogenetic differentiation are now under execution.

CONCLUDING REMARKS

From this series of experiments, the concluding remarks are briefly summarized as follow : (1) The history of the application of serology to plant systematics is described. The reason of decay of phytoserological studies during 1930's and the some shortages accompanying the serological techniques under liquid media are discussed in some detail. (2) The author modified the Ouchterlony's method in order to analyze more precisely the complex biological materials, especially the plant

materials, employing 4 basins in an agar plate and arranging these basins in the form of a rectangle, and divided the Ouchterlony's pattern of "a reaction of identity" into 2 categories ; "a reaction of perfect identity," and "a reaction of imperfect identity." (3) On the pattern of " a reaction of imperfect identity," when the concentration of antiserum in the adjacent 2 lower basins are the same and the concentrations of antigens filled in the upper two comparator basins are different between them, the precipitate line produced by the heavily concentrated antigen-antibody system appeared much nearer to the antibody basin than the precipitate line of the less concentrated antigen-the constant antibody system was to the antibody basin. (4) In the Ouchterlony's method, the non-specific reaction which would be brought about with the normal serum did not develope in the plate. (5) The author made clear the distinction between the inter-specific and the intra-specific differences, and also differentiated a species from another, and a variety from another by means of his modified method using the saline extracts of seeds of beans and of cucurbits. (6) The author dares to propose his working hypothesis that a species always contains the major protein component characteristic of itself and the minor protein components common to several related species, and the varieties within a species also contain the major protein component, which is characteristic of its species, in common and in addition, several minor protein components common to its related varieties and that each of these minor components is contained in different quantitiy with each variety. (7) This series of works, therefore, will call for many future experiments to solve various complex species problems at the molecular level.

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