PROTEIN DIFFERENCES IN DROSOPHILA. I. DROSOPHILA MELANOGASTER[1]

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ZONE electrophoresis affords a simple, rapid technique for comparing and characterizing proteins. It is especially suitable for investigations of genetically altered proteins, and notable results have come from the use of this method in studying hemoglobins (INGRAM 1961) and haptoglobins (CONNELL, DIXON and SMITHIES 1962). *Drosophila melanogaster* is extraordinary in terms of the extensive array of genetic techniques which have been developed for analyses of its genetic system. However, it has seldom provided material for physiological genetic analysis. This is largely true because few biochemical differences, especially protein differences, are readily demonstrable in the organism. For analyzing controlling mechanisms in development, variegated position effects, or other genetic phenomena characteristic of higher organisms, *Drosophila melanogaster* remains the experimental animal par excellence. The demonstration of protein differences in a system amenable to sophisticated genetic analysis offers unusual opportunity for integrating genetic, biochemical, and developmental approaches at the level of gene action. To this end, methods have been developed for analyzing protein differences in mutant stocks of Drosophila. The techniques employed have also been used with a variety of materials in this laboratory. Over thirty-five species in the genus Drosophila have been analyzed, and the results of this screening will be the subject to a separate paper in this series. Tetrahymena has been analyzed using identical methods, and a large number of migrating protein bands was resolved (DUNHAM 1962). Similar techniques have been successfully employed using embryonic and adult chick brain as the starting material (WYTTENBACH, personal communication). Thus, the methods to be described here are potentially useful with a broad array of materials and for investigating a wide variety of problems. The report includes a description of the methods employed, the results obtained from the initial screening of mutant stocks, and a preliminary analysis of a protein difference detected in strains of the mutant glass of *D. melanogaster*.

MATERIALS AND METHODS

The soluble materials obtained by macerating adult Drosophila in a small amount of buffer include few proteins whose individual concentrations are high

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enough to be seen after zone electrophoresis in either starch gel or acrylamide gel. Therefore, some method of fractionation was necessary in order to increase the concentration of particular proteins before application of screening techniques. Numerous methods for fractionation are available at the present time. Of these, salting out with ammonium sulfate proved most useful for the consistent, efficient and technically simple separation of the complex protein mixture into specifically enriched fractions. The procedure which proved to be most suitable is given below.

Drosophila stocks were grown on yeast-agar medium (CARPENTER 1950) at 25 ± 2°C. Adults were collected within 24 hours of eclosion and stored at −20°C. This frozen material was used for fractionation within a few weeks after collection. For each analysis six grams of flies were suspended in 28 ml 0.1 M Tris-HCl buffer at pH 7.0 and thoroughly ground in a glass grinder. This slurry was centrifuged at an average 54,450 × gravity for 15 minutes. The supernatant was taken to 20 percent saturation with ammonium sulfate (calculated for 0°C). The mixture was allowed to equilibrate for 30 minutes and then centrifuged as above. The resulting supernatant was treated with ammonium sulfate to bring it to 30 percent saturation, allowed to equilibrate for 30 minutes, and then centrifuged. This procedure was followed to obtain precipitates at 40, 50, 60 and 80 percent saturation. The equilibration time and centrifugal force were identical throughout the treatment. Both equilibration and centrifugation were carried out at 0°C, and the pH was maintained at 7.0 during the treatment.

The sediments from each ammonium sulfate precipitation were suspended in 3 ml distilled water and dialyzed for 18 hours against four liters of distilled water. Dialysis was also carried out near 0°C. The dialyzed material was lyophilyzed and then stored as a dry powder at −20°C. These powders are stable for months.

From starting material of 6.0 grams wet weight, an average yield of 300 mg of lyophilyzed material was obtained from the ammonium sulfate precipitations. This material was distributed in the fractions approximately as follows: 50 mg in 20 percent; 100 mg in 30 percent; 75 mg in 40 percent; 50 mg in 50 percent; 20 mg in 60 percent; and 5 mg in 80 percent. The different fractions vary in their relative proportions of protein as determined by the Folin Phenol method (LOWRY, ROSEBROUGH, FARR and RANDALL 1951), but approximately 50 percent of the material is accountable as protein. The material precipitated at 20 and 30 percent saturation contained almost half of the recovered material. However, it included the lowest proportion (18 percent) of the protein. Most of the material from these two fractions remained at the origin when applied to gels, and both fractions produced only a few faint bands. Consequently, no effort was made to use these two fractions for detection of protein differences.

For purposes of comparison, the materials obtained as described above were screened using both starch gel (SMITHIES 1959) and acrylamide gel (RAYMOND and WEINTRAUB 1959) as the supporting medium. Acrylamide gels proved to be much more satisfactory, both with respect to degree of resolution and with respect to number of protein components detected, and they were used exclusively for all further investigations.

Zone electrophoresis was performed vertically in a Model EC 470 cell obtained from E. C. Apparatus Company, Swarthmore, Pennsylvania. The supporting medium was a 5 percent solution of "Cyanogum-41" (a mixture of acrylamide and N,N-methylene bis-acrylamide monomers obtained from American Cyanamid Corporation) in 0.1 M Tris-borate buffer at a pH of 8.9, and containing 1.5 mM EDTA. Polymerization of the monomers was achieved by addition of ammonium persulfate to 0.16 percent and dimethylaminopropionitrile to 0.13 percent. Polymerization was complete at 30 minutes at room temperature. At this time the buffer compartments were filled with buffer having the same composition as that used in preparing the gel. The polymerization catalysts interfered with electrophoretic resolution and were removed by applying 200 ma for a period of two hours. During this period, and during the protein separation, the cells were cooled by circulating water from an ice bath.

Samples consisted of a 5 percent solution of the lyophilyzed material dissolved in the Tris-borate-EDTA buffer. Generally a 35 μl aliquot of such a solution was delivered to each trough at the top of the gel. On occasion, aliquots ranging from 1 to 35 μl were used, depending on the concentration of the proteins in the sample. After the samples had been delivered to the troughs a potential difference of 400 volts was maintained across the gel for 90 minutes. The gels were then removed to a solution of 0.5 percent Acid Black I in 20 percent acetic acid. Staining was complete after 30 minutes. Excess dye was removed by washing the gel in a continuous stream of 20 percent acetic acid overnight. Photographs were taken of the gels for further analysis.

Two standard comparative procedures were used in analyzing the protein fractions. The object of the first was to determine the distribution of individual proteins from a given strain among the different ammonium sulfate fractions. The object of the second was to determine protein similarities and differences between identical fractions from different strains. In order to determine which proteins were common to two or more fractions, individual samples from the 40, 50, 60 and 80 percent fractions of a given series were applied to adjacent troughs of a single gel. A solution of Bovine serum albumen was applied to another trough to serve as a reference. There were many proteins which were restricted to a particular fraction. However, some of the proteins, particularly those in high concentration, could be found in more than one fraction. Usually they predominated in one fraction and traces could be detected in adjacent fractions.

For interstrain comparisons, samples from a given fraction (e.g. the 40 percent fraction) from each of six strains were delivered to adjacent troughs on a single gel. Here, the same fraction from a standard wild type strain (usually Oregon-R) was placed in one trough as a reference. A solution of Bovine serum albumen was placed in another trough as an additional reference.

RESULTS

In the four fractions (40, 50, 60 and 80 percent) studied most intensively, thirty-six distinctly resolved proteins were consistently observed. An additional

twenty to thirty proteins were observed in several independent fractionations. For the most part these were in very low concentration and they will not be considered here. A diagram of the thirty-six major protein components is presented in Figure 1. The mobilities indicated were computed relative to that of Bovine serum albumen. The intensity of staining is indicated by the shading and the width of the band. The photographs in Figure 2 show the characteristic patterns for each of the four fractions studied.

In the course of these experiments Oregon-R has been fractionated independently twenty-two times. Except for minor concentration differences in the various fractions the results were consistent. A total of sixty stocks carrying mutant

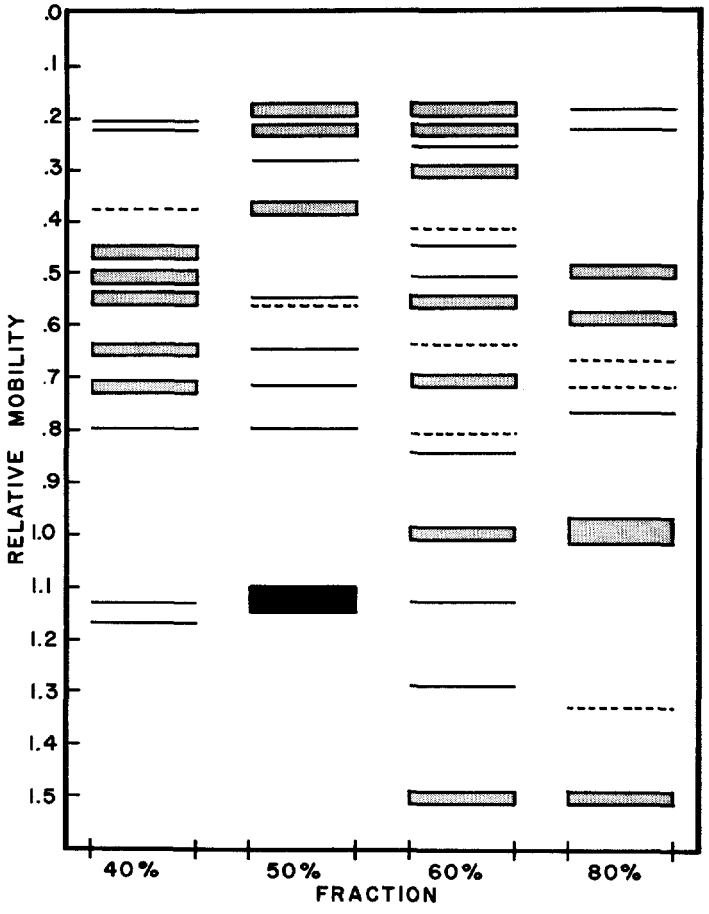
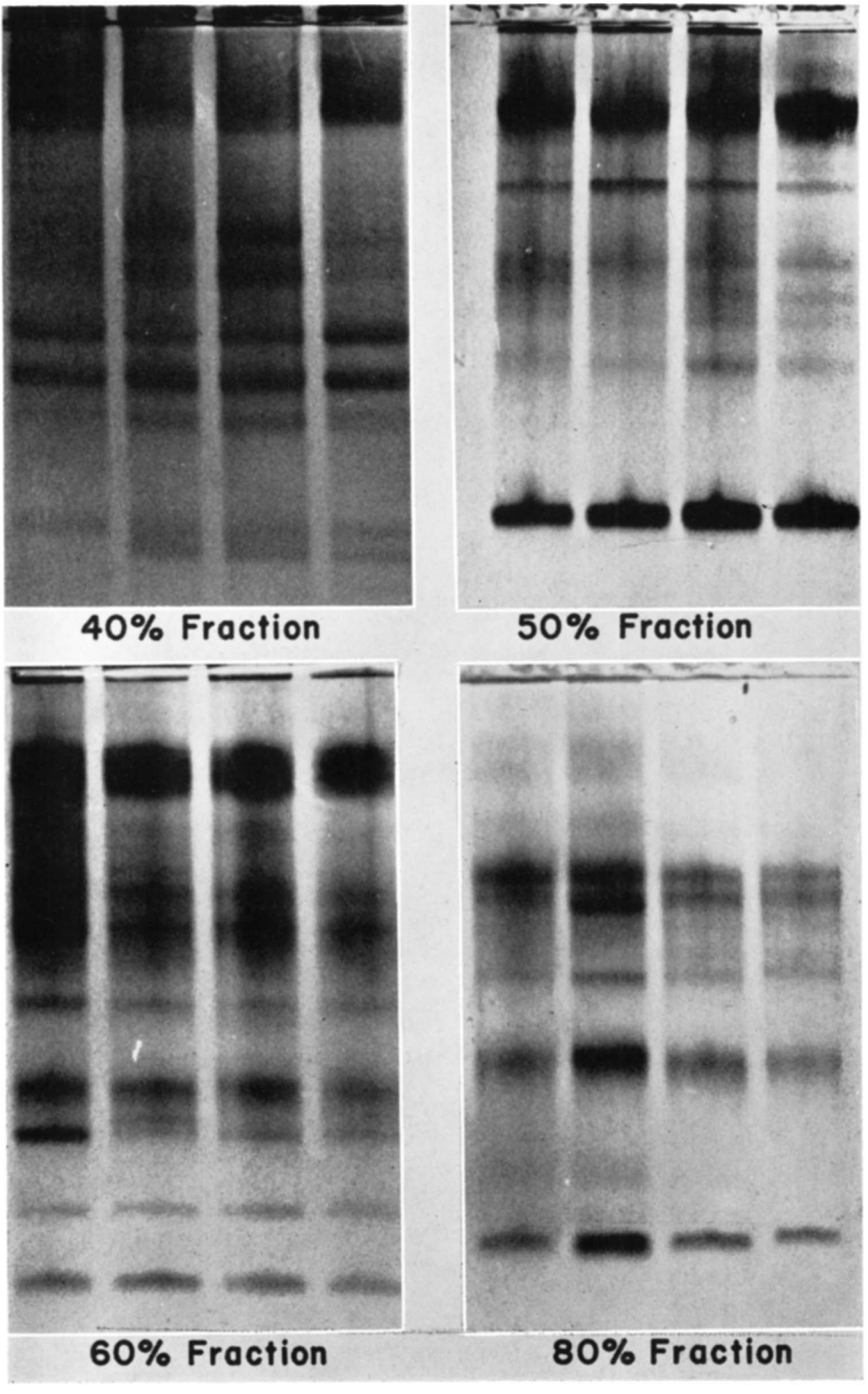


FIGURE 1.—The major proteins from *Drosophila melanogaster* detected in ammonium sulfate fractions after electrophoresis in acrylamide gel.

FIGURE 2.—Typical gels prepared from precipitates obtained from ammonium sulfate fractionation of *Drosophila melanogaster*.


40% Fraction
50% Fraction
60% Fraction
80% Fraction

markers have also been examined. These stocks contained an aggregate of seventy-five different mutant markers. The majority of these strains could not be differentiated from Oregon-R using the methods described here. However, some differences in the rate of migration of certain proteins were detected. In several stocks the protein bands with relative mobilities of 0.64, 0.72 and 0.80 in the 40 percent fraction migrate at a slightly faster rate than the comparable bands in the standard Oregon-R. The relative shift in position is the same for all three bands, and no case has yet been detected where one of the bands changes mobility independently of the other two. The protein with a relative mobility of 1.17, also found in the 40 percent fraction, has been missing in a few of the stocks examined. Analysis of the 30 and 50 percent fractions from these stocks failed to reveal a protein with this migratory rate. The protein with mobility 0.54 in the 60 percent fraction was observed to migrate somewhat faster in one stock. Other minor differences have been observed also, but neither these nor the ones just mentioned have been analyzed further. Detailed analysis has been restricted to one case. This involves a change in the electrophoretic behavior of what appears to be the major soluble protein constituent revealed by this technique.

A protein with relative mobility 1.13, found principally in the 50 percent fraction (see Figure 1), stains more intensively than any of the other proteins detected. In a stock of glass^3 (3–63.1) a densely staining protein having a mobility of 1.01 was detected. The 1.13 band was absent. Analysis of glass^1 and glass^2 ebony^4 strains revealed that the former contained the 1.13 band, whereas the latter contained a 1.01 band. Heterozygotes of Oregon-R with glass^2 ebony^4 and glass^3, made in all possible reciprocal combinations, revealed two bands having relative mobilities of 1.01 and 1.13. The concentration of each of the two proteins in these heterozygotes was approximately one half that of the single protein present in the presumably homozygous parental stocks (Figure 3).

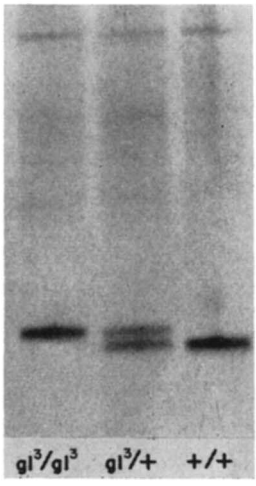


FIGURE 3.—Gel illustrating the protein difference found in the glass^3 stock and in heterozygotes of glass^3 and Oregon-R. 50μg-50 percent Fraction.

A cross was made between the glass^3 stock and a chromosome tester strain, *Cy/Pm;CxD/Sb*, in order to determine whether or not the protein difference was under the control of a locus (or loci) on the second or third chromosome. F_1 females of the constitution *Cy/+; CxD/gl*^{3} were mated to their brothers carrying *Pm/+;Sb/gl*^{3}. From the F_2 generation, two stocks were established; one carrying the markers *Cy/Pm* and homozygous for the third chromosome of the glass^3 stock; the other carrying the markers *CxD/Sb* and homozygous for the second chromosome of the glass^3 stock. The stocks, together with both parental strains, were then analyzed for the mobility of the dense staining protein in the 50 percent fraction. *Cy/Pm;CxD/Sb*, and the *CxD/Sb* derived stock produced a single protein migrating with relative mobility of 1.13, whereas both glass^3 and the derived *Cy/Pm* stock contained a single protein with a relative mobility of 1.01. Therefore it was tentatively concluded that the locus responsible for the protein difference resides on the third chromosome.

For further support of this conclusion the glass^3 stock was mated reciprocally with a homozygous Muller-5 stock. From these matings two stocks were derived which contained, in the first case, only Muller-5 X chromosomes and the Y chromosome from the glass^3 stock; and in the second case, X chromosomes solely from the glass^3 stock and the Y chromosome of the Muller-5 stock. These two derived stocks contained both the 1.01 and the 1.13 protein in approximately equal amounts while the Muller-5 parental stock contained only the 1.13 and the glass^3 stock contained only the 1.01 one. These results are consistent with the data placing the locus controlling the protein difference on the third chromosome.

In order to establish the relationship between the locus of the phenotypic marker glass^3 and the locus of the protein marker, glass^3 females were crossed to Oregon-R males. F_1 female progeny were backcrossed to glass^3 males. The daughters of the two classes, homozygous glass^3 or heterozygous glass^3, were mated singly to glass^3 males. The population derived from each of these matings was analyzed to determine the electrophoretic behavior of the proteins in the 50 percent fraction. If, in any of the F_1 females, there had been recombination between the glass^3 locus and the locus controlling the protein, then some progenies phenotypically glass^3 would contain both forms of the protein. On the other hand, some heterozygous glass^3 progenies would show only the one form of the protein. Forty-three such populations were analyzed. Two of twenty-three populations which were phenotypically glass^3 were heterozygous for the protein marker. Eight of twenty populations segregating for glass^3 contained only the 1.01 protein. Thus, it was concluded that the locus controlling the protein is separable by crossing-over from the glass^3 locus. The new locus will be designated as the Protein$_1$ locus (Pt_1) and the two alleles will be called $Pt_1^{1.13}$ and $Pt_1^{1.01}$. The superscript refers to the relative mobility of the protein under conditions described in this paper.

DISCUSSION

A genetically controlled altered form of a protein has been demonstrated in *Drosophila melanogaster*. The locus of the genetic material responsible for the

structure, or part of the structure, of the protein is on the third chromosome. The simple pattern of parental forms of the protein in heterozygotes suggests that no stable intermediate or hybrid form of the molecule is formed. This indicates that the synthesis or assembly of the polypeptide chain does not involve the interaction of allelic loci and does not involve aggregation of polypeptide units controlled by allelic loci. However, it does not rule out the possibility of aggregation of polypeptides produced by identical loci. Nor is there any evidence at present concerning the number of different independently controlled polypeptides in the protein.

The only modified forms of the protein detected thus far occur in stocks carrying a glass allele. But since the identity of the alleles has not been investigated, it appears premature to discuss any relationship between them or their probable origin.

It is unnecessary to belabor the point that only changes in protein constituents that result in a change in the net charge of the molecule will be detected by electrophoresis, or that it is possible to detect only rather large changes in size of the molecule by the "sieving action" of the gel. It should be remembered that it has been possible to detect only a minority of the proteins present in the organism and that those detected may represent a special class of protein in view of their high concentration. For these reasons the small number of protein differences detected greatly underestimates the genetic variability within the strains. The organisms that were included in this study were in all cases laboratory stocks. No isogenic lines have been included in this study; therefore, altered forms of proteins present in low frequencies in the populations analyzed would not be detected. This may be a factor in the small number of protein differences found in this survey.

At present the position of the Pt_1 locus is being established and preliminary analyses of purified preparations of the protein are being investigated. Eighteen to twenty-two peptide fragments have been obtained after trypsin and chymotrypsin treatment. These studies may provide an opportunity to investigate a number of gene-protein relationships in a multicellular organism for which there is an extensive array of techniques available for the analysis of its genetic and developmental systems.

SUMMARY

A technique has been described that reveals thirty-six proteins after zone electrophoresis of extracts of *Drosophila melanogaster*. Sixty strains have been examined for protein differences. A protein difference involving a major protein constituent of Drosophila has been found. The locus controlling the protein was found to be on the third chromosome. Heterozygotes for the protein mutant have both parental proteins and no intermediates. A discussion of some of the factors influencing the amount of genetic variability found is given.

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