PART I

TURNOVER OF THE HEMOLYMPH PROTEINS OF *DROSOPHILA MELANOGASTER*

PART II

A NEW METHOD FOR THE DETECTION OF DEOXYRIBONUCLEASES AND ITS APPLICATION TO STUDIES OF *DROSOPHILA MELANOGASTER*

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My thesis is dedicated to the teacher who most clearly stands out among the many capable instructors that I have had over the past twenty-one years, Mrs. Arlene E. Luce LaBar. This amazing woman has the rare talent for teaching which enables her to stimulate each and every member of her tenth grade geometry classes truly to think. For many of us this was our first encounter with the phenomenon.

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"Love never faileth, but whether there be prophecies, they shall fail; whether there be tongues, they shall cease; whether there be knowledge it shall vanish away. For we know in part, and we prophesy in part."
(I Corinthians 13:8 & 9)

The heart of the present negro revolt in this country is based in part on the desire of every human being for the opportunity to develop his potential to its fullest. Judged by this standard I have every reason to be grateful for my education at Cal Tech. With free access to the best minds and equipment it has been both sobering and stimulating to know that my only limitation has been my own effort and capability. To the innumerable people who have contributed to this environment I am truly thankful.

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ABSTRACT

Part I

A method has been developed for measuring the radioactivity of proteins labeled with C^{14} or H^3 following their separation by disc electrophoresis. The radioactivity is measured directly in acrylamide gel with scintillation techniques after the water in the gel has been replaced with a toluene-based scintillator solution. Seventy-four gel slices can be prepared with a minimum of handling in 9 to 18 hours depending upon the size of the slices.

This technique has been used in combination with densitometry measurements of separately stained gels to study the turnover of the hemolymph proteins of Drosophila melanogaster. By injecting labeled homologous proteins into unlabeled animals, active turnover of most of the hemolymph proteins has been demonstrated in both larvae and pupae. One particular group of proteins begins to turn over rapidly after puparium formation with a half life of 13 hours. Another group of proteins has been shown to be relatively inert in pupae. A postulated mechanism for protein turnover in metamorphosing insects is discussed.

Part II

A method is described for detecting deoxyribonucleases which have been separated in acrylamide gel containing trapped DNA. After electrophoresis the enzymes are incubated at their final resting positions with the substrate found at those positions. The remaining DNA is stained and recorded with a densitometer. The assay is sensitive to less than 0.25 nanograms of crystallized deoxyribonuclease I and is quantitatively reproducible.

An investigation of the deoxyribonucleases of Drosophila melanogaster with this technique has revealed a large number of peaks of enzymatic activity which undergo marked changes during the course of development. By varying the substrate and the magnesium concentration used for incubation, it has been shown that this organism probably produces at least eight different enzymes which can degrade DNA. A preliminary determination of the properties of some of these enzymes has been made.

CONTENTS

I. Introduction

Because the mechanisms of protein and nucleic acid synthesis have important implications for problems in genetics and development, it is understandable that these processes have recently been the object of intensive investigation. This emphasis has, however, resulted in a relative neglect of the methods which an organism uses to degrade and reshuffle macromolecular constituents. These processes represent the other side of the coin from synthesis and are of vital importance for the balance and maintenance of an organism. In the studies reported here the process of protein degradation and the deoxyribonucleases of Drosophila melanogaster have been investigated.

II. Turnover of the Hemolymph Proteins of Drosophila melanogaster

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A. Introduction

1. Historical Background

The current concepts of protein turnover are closely allied to the general theory of protein metabolism which has had such an interesting evolution over the last hundred years. In the middle of the last century Liebig dominated the field of protein metabolism with the idea that proteins are the source of muscular energy whereas carbohydrates are utilized to provide body heat (1). In 1865 Fick and Wislicenus (2) made their historic climb of 6,000 feet on a low protein diet and showed that the small amount of nitrogen excreted in their urine was not sufficient to account for the work of the climb. After the disproof of Liebig's theory by this and other experiments, Voit advanced the hypothesis that the body proteins exist in two clearly separated forms (3). One of these pools was represented by the tissue proteins, and the other pool was conceived as being a variable circulating body of storage protein. At the turn of the century Folin (4) extended the concept that protein metabolism could be considered to be compartmentalized with the introduction of the terms endogenous and exogenous metabolism. Folin believed that the body proteins are stable with the exception of a slow steady replacement (endogenous metabolism) which is necessitated by the aging and wear of the tissue. Exogenous metabolism, on the other hand, was thought to be a variable process which depended on the ingestion of excess nitrogen.

Folin's theory was accepted for over a quarter of a century until it was challenged in 1935 by Borsook and Keighley (5). These authors postulated that a large part of the ingested amino acids are rapidly incorporated into body proteins in a process of "continuing metabolism". This thesis was subjected to direct experimental test when the use of stable isotopes was introduced by Schoehheimer and his group (6). These investigators found that when amino acids labeled with heavy isotopes are fed to animals in nitrogen balance, a high percentage of

the label is subsequently found in the tissue proteins. A number of experiments of this nature confirmed the expectation of Borsook and Keighley that some of the body proteins experience a continual breakdown and resynthesis. In 1955, however, Hogness et al (7) were unable to detect any turnover in the proteins of growing E. coli. With this observation in mind they reviewed the work on mammalian protein metabolism and concluded that:

> There seems to be at present no conclusive evidence that the protein molecules within the cells of mammalian tissues are in a dynamic state. Moreover our experiments have shown that the proteins of growing E. coli are static. Therefore, it seems necessary to conclude that the synthesis and maintenance of proteins within growing cells is not necessarily or inherently associated with a "dynamic state."

This challenge to the "dynamic state" hypothesis has been taken up by several groups over the past ten years. Since many of the difficulties involved in measuring turnover (see reviews, 8-12) have been overcome in studies of microbial systems, we shall turn to these investigations before considering the work which has been performed in the more complex studies with mammals.

2. Protein Turnover in Microorganisms

Most investigators agree that in E. coli there is little or no protein degradation during growth (7, 13-16). This conclusion has been reached by two groups (7, 13) who were unable to detect any significant transfer of radioactivity between β-galactosidase and the remainder of the cellular proteins. An alternate approach was used by Koch and Levy (14) who devised an internal trap to detect protein degradation. After measuring the transfer of label from protein to nucleic acid under carefully defined conditions, they concluded that greater than 95% of the protein in growing E. coli has a half life longer than 30 days. Halvorson (17) points out, however, that none of the approaches so far employed would have been able to detect the presence of proteins with high rates of turnover. The data, nevertheless, permit the conclusion that a large majority of the proteins of E. coli

are extremely stable during growth.

In nongrowing bacteria an entirely different picture has emerged (9,10). Mandelstam has found that the proteins of nongrowing E. coli are degraded at the rate of 5% per hour for several hours (16). This rate was determined by measuring the release of C^{14} -leucine from prelabeled cells. A similar figure was obtained by measuring the incorporation of labeled glycine into the proteins of stationary cultures. Borek et al (18) measured the rate of protein turnover in stationary cultures by determining the rate with which H^2- and O^{18}-labeled waters were incorporated into protein. The results of these experiments are in good agreement with those obtained by Mandelstam. The correspondence between these two types of experiments not only establishes the presence of protein turnover in these cultures, but it also strongly suggests that the products of protein degradation are free amino acids. The evidence for protein turnover in other microorganisms has been reviewed by Halvorson (19).

3. Protein Turnover in Higher Organisms

Since the existence of plasma-protein turnover has been firmly established, we shall consider it before returning to the more controversial question of intracellular-protein turnover. In a comprehensive review of plasma-protein metabolism McFarlane (12) points out that the function of the observed plasma-protein turnover is very poorly understood. Indeed a controversy even exists over the location in the body at which these proteins are degraded. By starvation and injection experiments Whipple (20) has shown that a large percentage of the body proteins are interconvertible with the plasma proteins. However, it is not clear how direct a conversion this is, because at least in the formation of milk proteins it has been shown that amino acids rather than plasma proteins are absorbed from the blood as a source of raw material for protein synthesis (21, 22). Thus very little of a fundamental nature is known about the turnover of these proteins other than the fact that, as a whole, they are replaced at a rate of about 1% per hour.

Hogness et al (7) pointed out in their paper on turnover in growing bacteria that the data which had been obtained from whole animals or tissues did not prove the existence of intracellular-protein turnover. They noted that the available data, which had been accumulated with the aid of isotopic tracers, could also be accounted for by protein secretion, cell turnover, or the direct replacement of amino acids in proteins. With the advent of tissue culture, however, it was possible to eliminate many of the ambiguities which had been encountered in studies with whole animals or tissue slices. Studies with cultured cells have shown that intracellular proteins in animal cells are replaced at an average rate of about 1% per hour (9). The most extensive studies of this problem have been performed by Eagle et al (23), who have measured the rates of protein synthesis and degradation in several strains of animal cells. In contrast to the results found with bacteria, these authors have not detected any difference in the turnover rates of the proteins in growing and non-growing cells. Eagle and his group also performed a critical test for the occurrence of protein secretion or cell turnover. After exposing one unlabeled and one labeled culture to a common medium, they did not find any significant transfer of label between the two cultures. This result demonstrates that protein turnover in these cells is an intracellular process. If turnover had occurred extracellularly, half of the label released from the labeled culture would have been incorporated into the unlabeled culture.

More recently, the problem of tissue protein turnover has been studied by measuring the replacement rate of individual proteins. In rat liver, for example, arginase has been found to have a turnover rate of 0.6% per hour (24), and catalase is replaced at 3% per hour (25). Although there is evidence for protein turnover in plants, the nature of this turnover is by no means clear (26-28).

4. The Mechanism of Protein Turnover

In contrast to the tremendous attention which protein synthesis has received, attempts to determine the mechanism of protein

degradation have been meager. As a result very little is known about this process, even though the available experimental tools should be equally applicable to the investigation of both problems. The cathepsins have long stood out as logical candidates for the intracellular degradation of proteins although no direct evidence for this view has been produced. Data have recently been obtained which suggest that foreign proteins injected into rats are taken up by pinocytosis and incorporated into the lysosomes of liver and kidney cells (29, 30). Since the lysosomes are known to contain cathepsins (31), this finding represents circumstantial evidence that these enzymes are involved in the destruction of foreign proteins. Whether this concept can be extended to the degradation of homologous or intracellular proteins remains to be established.

Simpson (32) had shown earlier, however, that the mechanism of protein degradation may be more complex than simple hydrolytic cleavage. His experiments demonstrated that the in vitro release of amino acids from previously labeled rat liver is energy dependent. Steinberg and Vaughan (33), who subsequently confirmed and extended this result, also discovered that the release of amino acids from the proteins of liver slices is inhibited by certain amino acid analogues. By incubating labeled rat serum albumin with subcellular fractions from rat liver, Penn (34) has found that the mitochondrial fraction in conjunction with ATP and coenzyme A will degrade protein to amino acids and peptide-nucleotide complexes. Compounds of the latter type have been isolated from a variety of organisms (35, 36), and the suggestion has been made that they are involved in protein degradation (37). More recently, workers in two laboratories have concluded that stabilization of enzymes against denaturation may be an important factor in controlling the rate of protein turnover (38, 39). Since this short summary covers much of the information available on the subject, it is clear that a direct experimental attack on the mechanism of protein degradation has just begun.

5. Basis for Present Studies

Morphological studies of insects with holometabolous develop-

ment have shown that most of the tissues of the larvae are replaced during metamorphosis. Since the existence of massive tissue turnover is well documented in Drosophila (40), this organism is a favorable subject for investigating protein turnover. The hemolymph proteins of Drosophila were, therefore, selected for a study whose purpose was to define a system for investigating the mechanism of protein turnover.

Wyatt has reviewed most of the studies which have been carried out on insect hemolymph proteins (41). One of the primary conclusions to arise from this work is that the spectrum of the hemolymph proteins varies in a striking and characteristic manner during development. The most complete developmental study of Drosophila hemolymph proteins has been carried out by Duke and Pantelouris (42) with the aid of starch gel electrophoresis. They claim to have separated a total of 19 separate protein components over the course of development, but no more than eleven components were found at any single stage of development. By altering the procedure slightly these authors have obtained better resolution than was previously reported by Chen (43). In other insects some of the hemolymph proteins possess enzymatic activity (44), and at least one protein has been shown to be absorbed directly into the egg (45). Other authors have suggested that some of the proteins are involved in lipid transport (46), protein storage, clotting, and immune response (41).

Virtually nothing is known about the turnover of any of the insect proteins. Telfer and Williams (47) have reported the incorporation of labeled amino acids into hemolymph proteins during diapause. Although this result suggests that these proteins may be turning over, the experiments are ambiguous, because wounding frequently stimulates the production of hemolymph proteins (48). A Belgian group which attempted to study the mechanism of tissue-protein turnover showed only that free amino acids are incorporated into these proteins in developing and diapausing pupae (49).

B. Procedures and Controls

1. Introduction to the Methods

The approach which was chosen to study the turnover of Drosophila hemolymph proteins involves isolation and injection of radioactively labeled proteins. Most of the techniques necessary for the culture, isolation, and injection of Drosophila had previously been worked out by H. K. Mitchell (50, 51). Additional procedures for labeling, separating, and isolating proteins have been developed or modified to meet the small-scale requirements imposed by this organism. The recently developed technique of disc electrophoresis, which has proven to be extremely powerful for studies on this scale, has been used extensively here.

The utility of disc electrophoresis has been extended in these studies by the development of a technique for counting radioactively labeled proteins directly in the electrophoretic supporting medium. Since this method was developed, several other procedures for measuring radioactivity in acrylamide gels have been reported. Those methods which have been used for counting I^{131} or S^{35} appear not to be applicable to studies with C^{14} or H^3 (52-54). An indirect report (53) indicates that it is possible to hydrolyze acrylamide gel with HCl in glass ampoules. This procedure permits the recovery of H^3 and C^{14} from the gel for counting. In addition, these isotopes can be measured after electrophoresis by first allowing the labeled substances to diffuse out of gel slices (55). The hydrolysis procedure is extremely laborious compared to the method which has been worked out in these studies, and the elution procedure is relatively slow and inaccurate. Therefore, the procedure for counting C^{14} or H^3 directly in gel slices is still the best method for rapidly handling large numbers of samples.

2. Materials and Methods

a. Culture and Isolation of Drosophila. The Oregon R wild type strain, which was used exclusively in these studies, was grown and isolated according to the procedures described by Cole (56) and

Mitchell and Mitchell (51). Synchronous cultures of animals were obtained by collecting eggs in half-pint milk bottles for two hours. Two days after the initial egg collection an additional slurry of yeast was added to each bottle to provide optimum growth conditions. When the age of the animals was measured from the time of egg-laying, the age variation within a culture was about 6 hours. The developmental stages of individual pupae within a batch floated according to Mitchell and Mitchell (51) did not vary by more than an hour. The age of floated animals is recorded as the time after floating plus 124 hours.

Animals from each stage of development were isolated in a different fashion. Flies were obtained by etherization and used without further purification. Bottles containing pupae were filled with water, and the animals were loosened from the glass with a soft brush. After the pupae had been collected on a wire screen, they were washed thoroughly under a stream of distilled water and dried on filter paper. Depending on their size, larvae were collected on either silk or wire screens after having been floated in saline. Any adhering yeast was removed with a strong stream of water, and the remaining food was separated from the animals by differential floating in saline of the appropriate density. The larvae were kept for up to two hours on damp filter paper after a final distilled water wash.

b. Feeding of Radioactive Compounds. The hemolymph proteins were labeled by biological incorporation of C^{14} -labeled amino acid either after the labeled amino acid had been injected directly into the insect hemolymph or after it had been fed to larvae. The two types of amino acid preparations which were used in these studies were obtained from New England Nuclear Corporation. They were: uniformly labeled C^{14} -valine (150-200 millicuries per millimole), and C^{14} -algal protein hydrolysate (0.01 - 0.1 millicuries per milligram).

When free amino acid was fed, an aliquot of the amino acid solution was evaporated in the bottom of a test tube (10 x 2.5 cm).

The residue was then redissolved in enough water to give a tacky consistency to 10 mg of pressed bakers yeast. About 120 larvae, which were around 85 hours old, were added to the yeast and kept in a small pile by maintaining the yeast at the proper consistency. The highest incorporation was obtained by adding 10 mg portions of yeast at one-hour intervals. A feeding of three hours at 25° C was sufficient to permit the incorporation of 2 - 7% of the total amino acid into the recovered hemolymph proteins.

c. Injection of Radioactive Compounds. Although the injection technique, which was worked out by Mitchell (50), has been briefly outlined by Simmons (57), it is described in more detail here, because the individual details of the procedure had to be closely followed in order to obtain adequate survival of the injected animals.

Needle construction. Needles were formed from 1.0 mm glass capillaries which had first been given fine fiber tips by heating and pulling in the vicinity of a hot platinum wire. The tip of the fiber was broken off to give a point whose diameter was approximately the distance between the jaw hooks of the larvae to be injected. Subsequent operations were observed under a dissecting microscope. A very sharp well-beveled needle was formed by placing the point of a water filled capillary on the top of a rapidly rotating brass rod covered with a slurry of glycerine and finely powdered carborundum. The capillary was held at an angle which produced a sharp bevel while the rod rotated toward the point. After the needle was cleaned of excess carborundum by pulling water through it, it was sterilized in the autoclave. Such needles had to be kept wet and used within a week.

Preparation of the injector. All parts of the machine (50) which are in fluid contact with the needle were sterilized either by autoclaving them or soaking them in ethanol. Both ends of a fresh section of Intramedic polyethylene tubing (Pe 20) were expanded in a flame. One end was connected to the machine, and the other end was used to hold a well-fire-polished needle. The entire

system was then filled with sterilized water in a manner that excluded any air bubbles. Just before use, the tip of the needle was filled with a short mineral-oil plug. This served to separate the water from the small volume of sample which was next sucked into the tip.

Animal preparation. The larvae and the pupae which were injected in these experiments were sterilized by immersion in 70% ethanol for 30 seconds prior to being washed in sterilized water. The larvae were then etherized for an additional one minute after the last larvae moved in order to insure that the animals remained completely limp during injection. Both pupae and larvae were stuck on glass microscope slides in even rows by means of cellophane tape coated with adhesive on both sides.

Injection. The needle was inserted at an angle of about 30°, between two segments, and into the posterior end of larvae which had their heads facing the investigator. After a quick insertion the motion of the needle was reversed very slightly before the fluid was injected. The animals were then removed from the slides and washed according to the description of Simmons (57). Animals which were to be incubated at 25° C after injection were gently transferred to a vial containing a few grams of damp yeast.

Pupae were much more difficult to inject, and special measures had to be taken to overcome the high fluid pressure in their bodies. It was necessary to use settings of the machine which gave the most forceful fluid injection. In addition, the needles had to be especially sharp and well beveled. In spite of these precautions fluid would sometimes seep just beneath the pupal case. Since this fluid was subsequently recovered with the C^{14} -activity which actually entered the body of the animal, the estimates of the amount of a protein which was degraded in pupae may be low. This complication, in addition to the poor survival obtained with injected pupae, made these experiments less satisfactory than those in which larvae were used.

d. Preparation of Hemolymph. Two methods have been developed for preparing crude hemolymph on a large scale from Drosophila. The purest hemolymph preparations from larvae were obtained by treating animals over 70 hours old in much the same manner that is used to prepare salivary glands. Individual animals were held over the corner of a microscope slide with two pairs of fine tweezers and their heads pulled until they bled. After a pile of such partially decapitated larvae had been collected, it was gently pushed into a 0.2 ml glass centrifuge tube made from 5 mm (o.d.) pyrex tubing. The slide was washed with 25-50λ of electrophoresis buffer, and a glass rod was used to mix the wash with the animals. Prior to centrifugation the outside of the tube was given a thin coat of glycerine to facilitate its insertion into an adapter. The adapter was contained by a 2.0 ml cellulose nitrate tube which is designed for the Spinco Model L ultracentrifuge. Rubber liners for the adapter were drilled with a motor driven cork borer from neoprene rubber stoppers. The adapter was sealed with a standard aluminum cap and spun at 65,000 g (30,000 rpm) for 10 minutes. Pliers, which had their biting surfaces covered with tygon tubing, were used to squeeze the tubes back out of the adapters. The supernatent had to be removed from the tubes immediately after centrifugation in order to prevent remixing. This was accomplished with 50λ calibrated capillaries (Drummond Scientific Co.) whose tips had been pulled out to a very fine point in a micro burner. Withdrawal of the hemolymph was controlled by mouth with the aid of a section of polyethylene tubing. The volume of the recovered fluid was determined by measuring the length of the fluid column in the capillary. All operations were carried out at 4° C.

Preparation of hemolymph from pupae followed a similar pattern except that whole animals were placed directly in the centrifuge tube. The pupae were crushed in the tube with a glass rod, 2 mm in diameter, and the rod and sides of the tube were washed with electrophoresis buffer. Larval hemolymph has also been prepared

in this manner with satisfactory results.

e. Separation of Amino Acids From Hemolymph Proteins. In order to separate the unincorporated amino acids from the hemolymph proteins, the recovered hemolymph was run through a column of Sephadex G75 (Pharmacia, Uppsala). A Pyrex tube, constricted at the bottom and partially plugged with glass wool, was used to contain a column of Sephadex 5 cm long by 0.4 cm in diameter. Sephadex G75, suspended in 30% sucrose, was allowed slowly to settle in the column before it was thoroughly washed with electrophoresis buffer. Up to 50λ of sample were applied to the column at one time and eluted with electrophoresis buffer at 4° C. Under these conditions rabbit hemoglobin was separated from valine or 2,4-dinitrophenol, and greater than 95% of the hemolymph proteins eluted with the hemoglobin in 0.2 ml. Over 98% of the resulting hemolymph "protein" fraction was precipitable with TCA after this treatment. For larger samples a similar column from which the protein was eluted in 0.65 ml was utilized. After four or five uses the Sephadex was discarded.

f. Disc Electrophoresis. The use of disc electrophoresis for separating hemolymph proteins overcame several difficulties which had been encountered earlier in this study with starch gel. In the later experiments densitometry tracings were obtained from circular gels which had been used to separate the hemolymph proteins by standard disc electrophoresis (59). For preparative purposes the original procedure of Ornstein and Davis (58, 59) was scaled up by over a factor of 7. Two changes which were made in the gel solutions included a reduction of the potassium ferricyanide in solution "C" from 15 to 5 mg and a reduction of the final acrylamide concentration from 7.5 to 6.4%.

In the preparative procedure gels were polymerized from 12 ml of solution contained in 12 cm long acid washed Pyrex tubes constructed from 16 mm (o.d.) tubing. Stands, which were made by gluing 3 mm thick rubber sheets to plexiglass, were used to hold the tubes upright. The bottoms of the tubes contacted the plastic through holes which

had been cut in the rubber to provide a water-tight fit around the outside of the tubes. The final gel solution was mixed at room temperature from the lettered stock solutions. A 1.5 ml layer of large-pore gel solution containing about 2λ of TEMED was polymerized on top of the small-pore gel after the tube had been placed in front of a 20 watt daylight fluorescent tube. Prior to electrophoresis the gels were placed at 4° C for one half-hour. About 0.6 to 1.0 mg of protein in up to 1.0 ml of 15% sucrose was layered under the buffer and on top of the large-pore gel. Electrophoresis at 8 ma per gel was discontinued after about three and one half-hours when the tracking dye had traveled exactly 6.0 cm into the small-pore gel. Gels were removed from the tubes as described in Section III, B.

Under the conditions used in these experiments it was not always possible to predict the exact location of a protein band in a given gel. The protein pattern in each gel was, therefore, determined by removing part of the gel for staining. Matching nicks in the stained strip and the remainder of the gel were used as guides to find the corresponding positions in the two pieces. These nicks were derived from a row of slashes which were placed in the original gel after electrophoresis with three #10 scalpel blades. The blades were held together with rubber bands and rigidly separated by 0.4 cm thick strips of Plexiglas in such a manner that only the tips of the blades were exposed. Half of each slash was included in a narrow strip of gel which was cut from the side of the gel cylinder by pinching the gel between two sharp razor blades. With practice a uniform strip amounting to 7-10% of the total gel could be obtained from the entire length of the gel.

Protein positions in the gel were revealed by staining the strip in amido black for 15 minutes at 35° C. The strip was then placed in 7% acetic acid at 100° C for about 30 minutes in order to remove the excess stain. Meanwhile the remainder of the gel was kept at 0° C. Independent tests revealed that after

one hour at this temperature there was no significant broadening of the protein bands. Once the protein bands had been located in the gel, the gel was cut transversely to include each band in a single slice. When the gel was cut at the notches, the weight of the slices varied by 5% (coefficient of variation). In the more recent experiments all cutting blades were periodically wet with a detergent solution in order to prevent the gel from shattering as it was cut. The gel slices obtained by this method were used to recovery the protein or to count its C^{14}.

g. Electrophoresis in Slabs of Acrylamide. At the time this investigation was initiated the available densitometers would accept only flat slabs of gel. In order to obtain quantitative measurements from stained gels it was, therefore, necessary to change the shape of the gel. The apparatus depicted in Fig. 1 permitted this modification with very little change in the disc electrophoresis procedure. A 2 x 15 x 100 mm slab of small-pore gel was polymerized in this apparatus after the bottom of the slit had been sealed by pressing the mold firmly into a pad of plasticine. When the gel had been cooled to 4° C, about 100 μg of protein in 50-100λ of 15% sucrose was layered on top of the 1.5 cm layer of large-pore gel. Buffer was carefully layered over the sample. Electrophoresis was carried out for the first 15 minutes at 4 ma and then for four to five hours at 1 ma.

At the termination of electrophoresis the mold was dismanteled and the slab removed. The proteins were stained either with amido black (59) or light green SF yellowish. The latter stain (dye content 92%, C.I. No. 42095, National Aniline Division, Allied Chemical) was prepared by dissolving 200 mg of dye in 100 ml of 3% sulfosalicylic acid (60). After the gel had been stained overnight, the excess stain was soaked out in 2% acetic acid. The gel was prepared for densitometry with a final soaking in 20% glycerine, 2% acetic

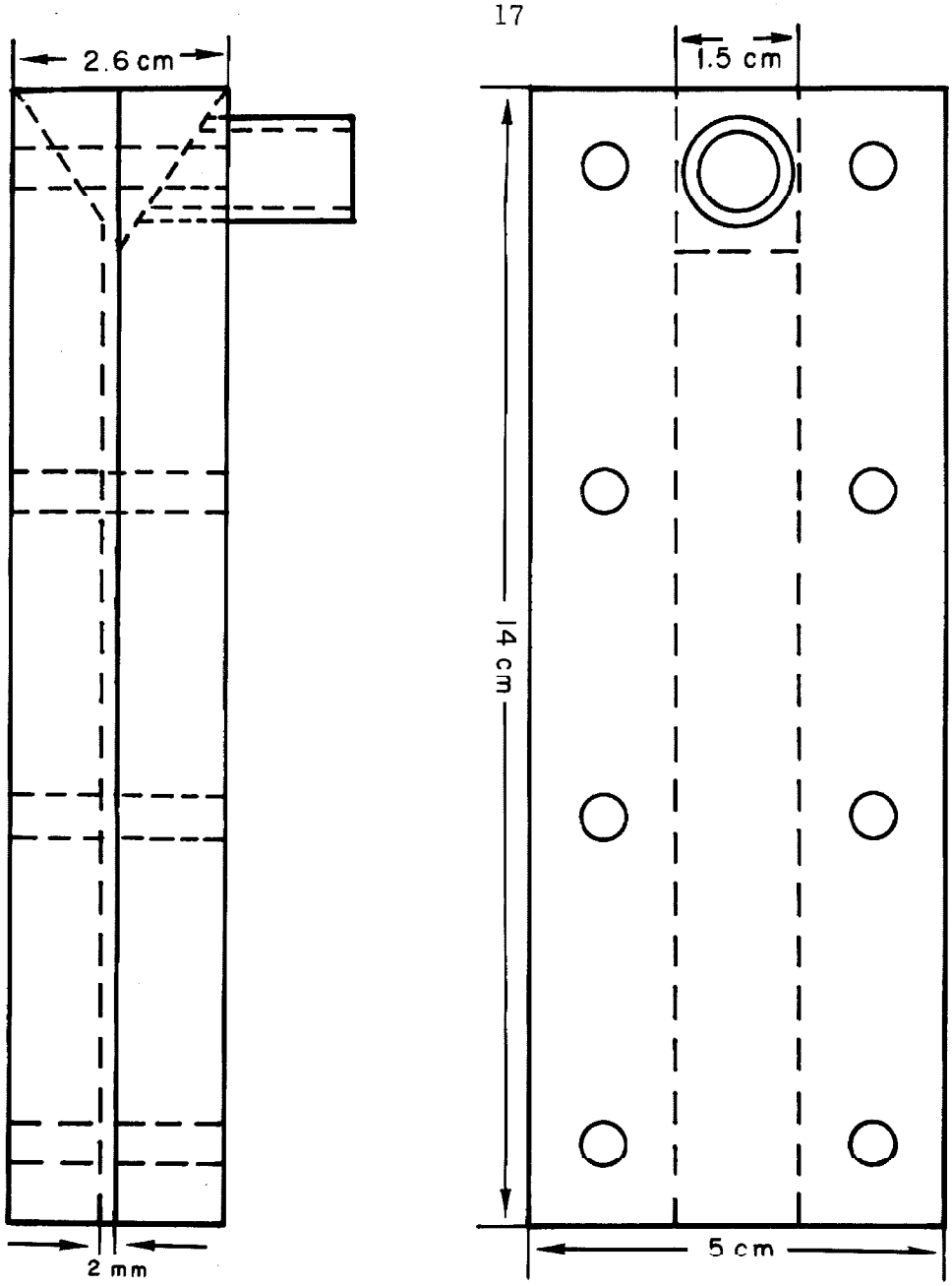

Fig. 1 Plexiglass form for performing electrophoresis in acrylamide gel. The slit which contained the gel was milled in one of two plastic blocks that were held together with washers, bolts, and wing nuts. Prior to assembly the contacting surfaces were coated with a thin layer of silicone grease. The quantity of grease was adequate to exclude air from between the blocks without allowing any grease to squeeze into the slit. Electrophoresis was run vertically with the bottom of the gel in contact with the buffer in the lower reservoir. The tube in the upper reservoir provided direct fluid contact with a larger reservoir which contained the second electrode.

acid solution.

h. Densitometry. For scanning gels in slab form the sample holder of the Joyce Loebl Chromoscan was modified with 2 mm thick rubber sheets. The carriage was placed face down on a table and assembled in the following order: 1. Carriage; 2. Rubber sheet with a 1.3 x 9 cm window cut in it; 3. Rubber sheet with a 1.7 x 10.5 cm hole for holding the gel; 4. Gel, just removed from solution; 5. Damp strip of filter paper; 6. Opaque white solid backing. The gel was scanned with the red filter and the slit provided with the instrument. The scanning operation was performed as quickly as possible in order to reduce the effects of slight changes which the gels underwent in the air. The background for each scan was reset in the area of the gel below the dye marker.

In the later experiments densitometry tracings were obtained from protein patterns in standard-sized gel columns (59) with the aid of the Joyce Loebl Microdensitometer. Scanning was performed by placing a glass stoppered cuvet containing the gel in a horizontal position on the densitometer scanning table. The gel was completely immersed in 7% acetic acid. In order to focus this instrument properly, it was necessary to "lever up" the condensor optics as described in the instructions accompanying the instrument. A Joyce Loebl red filter was used in the light path between the gel and the photocell. Before the beam was centered in the gel, the latter was aligned parallel to the direction of scan by following the outside edge of the gel with the cross-hairs.

i. Recovery of Proteins from Acrylamide. Proteins were removed electrophoretically from gel slices by a procedure similar to that described by Lewis and Clark (61). The same size Pyrex tubing that was used for electrophoresis was used

to construct 8 cm long elution tubes which were tapered at one end to a diameter of 0.5 cm (i.d.). One and one half ml of large-pore gel was polymerized in the constricted end of the tube with overhead illumination. The bottom of the tubes were held against Plexiglas discs which were mounted in rubber serum bottle stoppers. It was found that most of a nonprotein residue could be eliminated from the final protein preparation by prerunning these gel pads at 3 ma per gel for 30 minutes. A 9 cm long dialysis bag, knotted from 18/32 tubing, was then filled with 1.5 ml of electrophoresis buffer and slipped over the constricted end of the tube. Any air in the bag was expelled according to the method of Lewis and Clark (61), and the bag was sealed to the elution tube with a rubber band. The protein in a single gel slice was eluted into the dialysis bag after the slice had been placed on top of the large-pore gel pad. Electrophoresis was conducted at 5 ma per tube for two hours with electrophoresis buffer.

After elution the dialysis bags were slipped off the elution tubes and sealed by tying the tops with #8 cotton thread. Several such samples were dialyzed overnight in three liters of stirred 0.01 M NaCl. Preparatory to injection the volume of each sample was reduced to about 25λ in a collodion bag supported in a suction apparatus obtained from Carl Schleigher and Schuell Co. The sample had to be watched carefully to prevent it from reaching complete dryness. Over 75% of the initial protein was recovered in this final step. All protein recovery operations up to injection were carried out below 4° C.

j. Counting C^{14}-Labeled Proteins in Acrylamide Gel. In order to measure the radioactivity in a gel slice with liquid scintillation techniques, it was necessary first to replace the

water in the gel with toluene and scintillators. This was accomplished by subjecting gel slices obtained from 12 ml gels to a three-step soaking procedure. Gels were first soaked in solvent 1. (See Table 1) for five or more hours and then in solvent 2. for a similar period of time. The combined soaking time for these two steps was 14 hours or greater. The third and final step was a soaking in solvent 3. for three hours or longer. After this step the gels were individually transferred to glass counting vials containing 5 ml of solvent 3.. The gels were allowed to sit for one half-hour at room temperature in the vials before they were cooled for counting. Routine handling of the 0.4 cm thick gel slices was facilitated by the apparatus depicted in Fig. 2. Gel slices were placed in the tubes of this rack while it was supported in an empty refrigerator dish. Since the dish was marked, the gels could be identified by returning the rack to its original position in the dish. When each cylinder of gel was cut into 16-18 slices, one frame could accomodate the 36 slices from two whole columns. Two such racks, or 72 samples, were frequently soaked at one time.

The soaking steps were carried out by transferring a rack of gel slices between dishes containing the proper solutions. These solution changes were completed by washing the gels after each soaking with a small amount of the solvent that was to be used in the following step. All soaking was done at room temperature in tightly sealed polyethylene dishes. Each dish contained one liter of solvent which was stirred with a teflon-coated magnet.

A slightly different procedure was used to determine the amount of radioactivity lost into the solvents. A gel cylinder, uniformly labeled with radioactive protein, was prepared by mixing the protein with the gel solution and polymerizing them together. The protein

TABLE 1

Solutions Used to Prepare Gel Slices for Counting

Solution designation	Composition	Chemical source
1	2.5 g phosphotungstic acid	Merck
	One liter glacial acetic acid (reagent grade)	Baker and Adamson
2.	500 ml glacial acetic acid (reagent grade)	Baker and Adamson
	500 ml ethylene glycol monoethyl ether (cellosolve, purified)	Fisher Scientific
3.	3 g 2,5-diphenyloxazole (PPO)	Nuclear-Chicago
	0.1 g 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP)	Packard Instrument
	One liter toluene (reagent grade)	Baker and Adamson

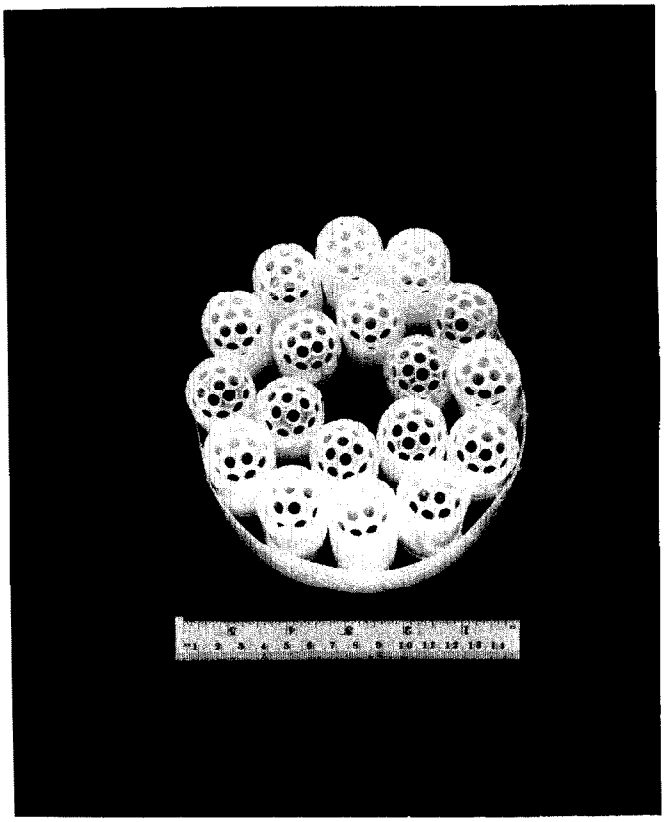

Fig. 2 Apparatus for soaking gel slices. Holes were melted in the bottoms of 50 ml polypropylene centrifuge tubes with a hot brass rod. A ring of polyethylene cut from a refrigerator dish held the apparatus together. The tubes were sewn to it and to each other with Intramedia polyethylene tubing threaded through holes made with a hot wire. The tubes in the outer and inner rows were cut off to lengths of 6.3 and 5.5 cm respectively. In use the apparatus was suspended in a two-quart polyethylene refrigerator dish which held the bottom of the longest tubes 1.5 cm from the bottom of the dish. In this position one liter of solvent was almost sufficient to cover the tops of the tubes. Each tube could be adapted to hold more than one gel by partitioning it with one of the polyethylene strips shown in the upper field.

used in most of these tests was C^{14} -labeled hemoglobin (125,000 dpm/mg). Individual slices cut from such a cylinder were weighed to provide a means of calculating the radioactivity in each slice. Slices were then subjected to various soaking conditions in 125 ml Erlyenmeyer flasks containing 50 ml of solvent. During the soaking the flasks were sealed with Parafilm (Marathon) and shaken at 25° C. In this modified procedure soaking in solvent 3. was performed directly in the 20 ml counting vials. Any radioactivity remaining behind in the solvents was measured with a low background gas flow counter (Nuclear-Chicago). To do this the solvent was first evaporated in a stream of air, and the residue was partially hydrolyzed by boiling it in 6 N HCl for one half-hour. The HCl was then evaporated in the same way, and the hydrolysate was dissolved in 2 ml of 7% ammonium hydroxide. After centrifugation the supernatant was plated and counted. For each set of gel soaking conditions the efficiency with which activity was recovered from the solvent was estimated from parallel controls. The controls were performed by adding an aliquot of radioactive protein to solvents which had been used to soak blank gels. By using this correction the amount of protein lost from the original gel was calculated. This more laborious soaking procedure was used only to develop and test the method.

Gel slices were counted in a Nuclear-Chicago 720 Series liquid scintillation counter by balance point counting. The channels ratio method (62) was used to monitor quenching. Vials were washed and reused after the gels had been discarded and the remaining solvent tested for contamination.

k. Determination of Counting Efficiency. The efficiency with which C^{14} was measured in gels was determined by first counting an aliquot of protein in a gel. A separate aliquot of the same protein was then counted with a planchet counter of known counting efficiency. This latter result was used to calculate the efficiency with which the original gel sample had been counted. The counting efficiency

of the planchet counter was determined with a sample of standard C^{14}-benzoic acid (8260 $\pm$ 5% dpm/mg, New England Nuclear). Three equivalent solutions of benzoic acid were independently titrated to a phenolphthalein end-point with NaOH, and the dilutions were determined by weighing. Three different aliquots of each solution were applied to planchets with calibrated pipettes, spread with acetone, and counted. None of the samples had a density greater than 0.1 mg sodium benzoate per cm^2 of planchet area. The activity of the samples did not change (within 1%) over a period of 10 days. Triplicate efficiencies determined at each sample size were averaged, plotted versus sample weight, and an extrapolation was made to zero sample size. All experimental errors were insignificant compared to the 5% error in the initial sample and the estimated 5% error in the extrapolation. A commercial C^{14} standard was counted at identical machine conditions in order to provide a basis for subsequent monitoring of the counter efficiency.

An analogous procedure was used to determine the efficiency with which tritium is counted in acrylamide gel. For this purpose acid-acetone precipitated globin, which was labeled with 3, 4 H^3-leucine, was incorporated in 7.5% gel as before. An aliquot of the globin was also counted in Bray's solution (63) with an efficiency which was determined by the channels ratio method (62). From the result obtained from Bray's solution the absolute specific activity of the globin was calculated. This value was in turn used to calculate the efficiency with which tritium is counted in the gel.

3. Characteristics of the Methods

a. Injection Controls. The data in Table 2 show that the preparation of larvae for injection did not effect the survival of the isolated animals. The survival of injected larvae varied from 30 to 80%. Fortunately, this mortality did not complicate the results, because the dead animals could be discarded before the next step of the experiment. In the experiments in which pupae were injected, however, it was often difficult to identify the dead animals.

TABLE 2

Effect of Injection Procedure on Survival

Treatment received by the larvae	Total sample	% of total which formed puparia	% of total which reached fly stage
Initial isolation	43	88 ±10	72 ±10
Through ethanol wash step	41	90	81
Through etherization step	44	93	84
Entire procedure without injection	45	89	76
Injected and fed*	21	86	71
Injected and* not fed	27	67	30

* Larvae were injected with 0.1 mg/ml penicillin G and 0.03 mg/ml streptomycin in sterile 0.154 M KCl. A different group of animals was used for the two injection experiments.

b. Quantitative Protein Determination in the Gel. The stained gels shown in Fig. 3 demonstrate that equivalent electrophoretic patterns were obtained in the slab and disc procedures. Both gel forms were used at different points in this investigation to obtain densitometry tracings of the protein patterns produced by electrophoresis. To obtain specific activity measurements, aliquots of a hemolymph preparation were subjected to electrophoresis in both a 12 ml gel and either a small slab or cylinder of gel. Protein quantities were determined from densitometry tracings of the small stained gel and C^{14} was measured in the larger gel. Small gel columns were stained with amido black (59). Light green SF yellowish was used as a protein stain when the slab form of the gel was stained, because excess light green soaked out faster and gave a lower background than did amido black. Light green was shown to be stable (within 5%) in solution for over two months and stable in stained gels for several days.

The densitometry tracings shown in Fig. 4 demonstrate that the Chromoscan densitometer is able to resolve the visible bands in the slab shaped gels presented in Fig. 3. The relative areas under corresponding peaks stained with different stains are identical for the four fastest bands shown in A and C of Fig. 4. On the other hand, light green is seen to stain the low-mobility components more strongly than does amido black. Although densitometry tracings of standard disc-electrophoresis gels are superior in resolution to those obtained from a slab, the machines for scanning columns were only available for the most recent studies. Tracings, which were obtained in these later studies with the Joyce Loebl Microdensitometer, were reproducible within 5%. The Canalco Model E Microdensitometer, which is specifically designed for circular gels, gave an error of over 10% with the same gels. The Joyce Loebl instrument was therefore used to obtain the results reported here. When the amount of hemolymph protein applied to a gel was varied by a factor of two, the areas under the resulting densitometry tracings varied from the

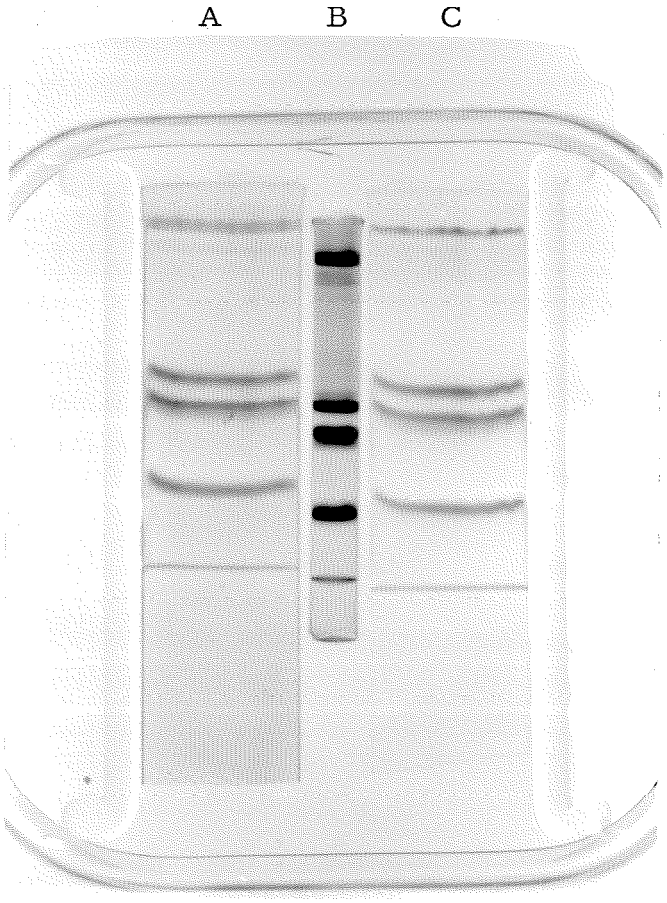

Fig. 3 Electrophoresis patterns of Drosophila hemolymph proteins. Hemolymph was prepared from larvae which were just ready to pupate. An aliquot equivalent to that obtained from six animals was subjected to electrophoresis in each gel. Gels A and C were slabs of acrylamide which were run in the apparatus depicted in Fig. 1. Gel B was a cylinder of gel run by the standard disc procedure at 1 ma and 4° C. Amido black was used to stain Gels A and B, and the excess stain was removed by electrophoresis. Gel C was stained with light green as described in the text.

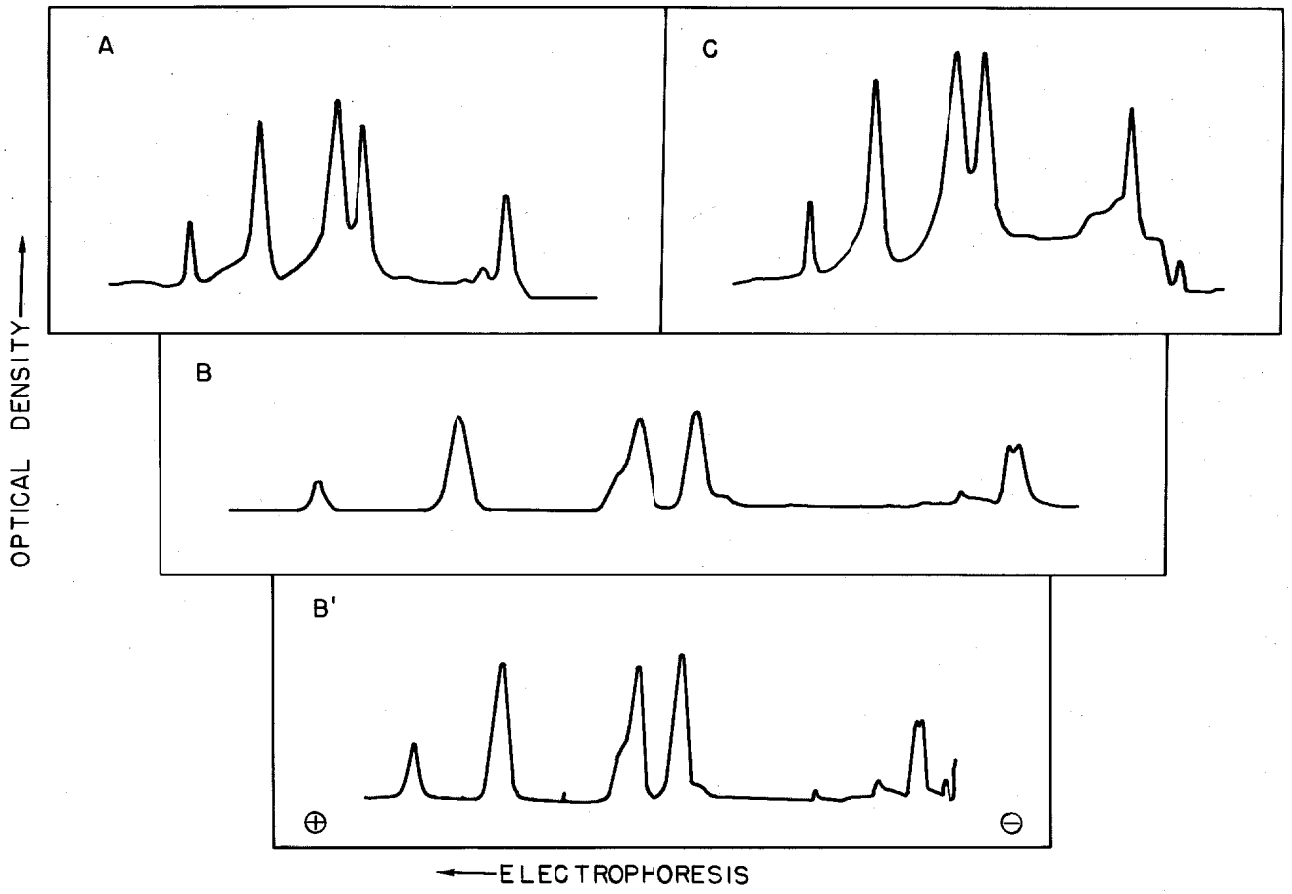

Fig. 4 Quantitative protein analysis. Aliquots of a single hemolymph preparation were subjected to either standard disc or slab electrophoresis. Densitometry tracings A and C were obtained with the Joyce Loebl Chromoscan from gels A and C shown in Fig. 3. The gel used to obtain scans B and B' contained one half the amount of protein contained in gel B of Fig. 3. The two gels were identical in other respects. Scan B was run by the Canal Industrial Corporation on their Model E Microdensitometer. Tracing B' was obtained with a Joyce Loebl Microdensitometer as described in Section II, B. 2.

expected linear relationship by up to 20%. The extent and direction of the variation depended on the protein being studied.

c. Counting C^{14} -Labeled Proteins in Acrylamide Gel. When the difficulty of eluting and handling very small quantities of protein was first encountered in these studies,a method was sought for counting C^{14} -labeled proteins directly in the gel. A test of over a dozen solvents and five scintillator solutions revealed that glacial acetic acid would serve as a satisfactory intermediate for replacing the water in the gel with a toluene-based scintillator solution. Although this procedure gave excellent counting efficiencies, up to ten per cent of the protein was leached out of the gel into the intermediate soaking solutions. Further tests with additional reagents then showed that the addition of phosphotungstic acid to the intermediate soaking solution reduces this loss to less than 3%. Since this reagent reduces the counting efficiency, it was necessary to introduce an additional soaking step which removed the excess PTA without increasing the protein loss.

Two different protein preparations were used to test the method. The most extensive tests were run with hemoglobin as described in Methods (II, B, 2). Although hemoglobin is generally an unsatisfactory protein to count with scintillation techniques because of its color, the availibility of the protein in this laboratory made it the logical choice for large numbers of preliminary tests. It was soon discovered that the high specific activity of the protein, coupled with the fact that most of the heme was leached out of the gel during soaking, permitted the preparation of test gels which possessed little interfering color. Confirmatory experiments were also run with uncolored C^{14} -labeled proteins obtained from Drosophila hemolymph. These latter experiments provided a direct test of the method on the actual proteins under investigation. Since these experiments were performed by conducting proteins into the gel by electrophoresis, they also served to eliminate the possibility in the hemoglobin tests that protein was being artificially trapped in the gel during polymerization. If such trapping had been significant,

the tests of protein leaching into the soaking solutions would have been invalid. The hemolymph tests were run by layering an aliquot of protein solution on 13 mm diameter gels prior to electrophoresis. After the fastest band had traveled 4 mm into the small-pore gel, the upper 6 mm of the gel was cut off and soaked according to the test procedure described in the methods. A similar unreported experiment was used to establish the validity of the hemoglobin results.

The following variables of this method were investigated in detail:

Soaking times. The data in Table 3 indicate that the duration of either of the intermediate soaking steps can be at least as long as 15 hours. The minimum soaking time of five hours which has been set for each of these steps is seen to include a slight safety factor. Inadequate soaking could be recognized immediately by the appearance of a white opaque area in the center of the gel. This effect, which was seen in the last two gels listed in Table 3, seriously reduced the counting efficiency. Dr. M. Fling has found that for gels which are 5 mm in diameter the total acid-soaking time can be reduced to less than 6 hours.

Reagent volumes and concentrations. The effect of varying the concentration of the solutes is shown in Table 4. The PTA concentration in use represents a compromise between quenching at high PTA concentrations and protein loss at lower concentrations. These data, nevertheless, show that the permissible range of solute concentrations is large. Within the examined limits, the purity of the cellosolve and the age of the solutions were not found to be critical.

With regard to the volume of the acid soaking solutions, 50 ml of solution per gram of gel has proven satisfactory. Preliminary evidence suggests that it may be possible to reduce this volume by one half without affecting the counting efficiency. The volume of the final scintillator-soaking step has been varied from 5 to 20 ml

TABLE 3

Variation of Gel Soaking Time

Soaking time in PTA and acetic acid[a.]	Soaking time in cellosolve and acetic acid	Calculated counting efficiency[b.]	Percentage of gel C^{14} lost into soaking solutions[c.]
		Hemoglobin-Containing Gels	
5 ± 0.25 hr.	5 ± 0.25 hr.	74.8 ± 0.5	3.5 ± 1
6	6	74.6	4.2
7	7	74.1	1.0
4	10	74.1	2.0
10	4	73.4	1.4
15	8	74.1	2.2
15	8	74.4	2.3
4	19	74.0	2.9
4	19	74.3	2.1
		Hemolymph-Containing Gels	
8	4.5	80.7	2.1
8	4.5	80.2	--
6	6	78.7	--
4	8	79.8	3.1
9	3	75.6[d.]	5.4
8	4	76.0[d.]	--

a. Hemoglobin-containing gels were uniformly labeled and were soaked in 0.1% PTA. The gels containing hemolymph were soaked in 0.25% PTA after electrophoresis.

b. Quenching for all samples was constant as measured by the channels ratio test.

c. Many tiny gel fragments were left behind in the first soaking solution.

d. These gels had white opaque centers.

TABLE 4

Variation of the Reagent Concentrations*

Phosphotungstic Acid		
Concentration of PTA	Counting efficiency with hemoglobin	Counting efficiency with hemolymph
0.75%		78.2 ±0.5%
0.50	73.5 ±0.5%	79.7
0.25	73.0	80.7
		80.2
0.10	75.0	80.8
		81.1
		81.8
0.05	73.5	80.9
0.01	72.8	

Fluors		
Concentration of PPO	Concentration of dimethyl POPOP	Counting efficiency with hemoglobin
2 g/liter	0.3 g/liter	72.8 ±0.5%
3	0.3	73.6
4	0.3	74.1
3	0.05	73.7
3	0.1	74.0
3	0.2	72.7
3	0.3	73.6
3	0.4	73.5

* Experimental conditions were identical to those described in Table 3.

without any effect on the efficiency of counting. Considerable data have also shown that a third soaking in scintillator solution is unnecessary.

Counting efficiency. The standard deviation of the counting efficiencies calculated for most of the experiments in which C^{14} was used is about $\pm$ 0.5%. That is, the gels in one experiment which were treated within the prescribed range of conditions gave efficiencies with this error. However, the error in the absolute counting efficiency for C^{14} is estimated to be about 15% (12% efficiency). Most of this error is a result of the 10% uncertainty in the efficiency of the planchet counter which was used to determine the gel counting efficiency. From the data in Tables 3 and 4 it may be concluded that the C^{14} in hemolymph protein is counted with an efficiency of 81 $\pm$ 12%. Since the scintillation counter is normally capable of about 76% efficiency (see Fig. 5), the fact that the C^{14} is embedded in gel does not seriously reduce the counting efficiency. The data in Tables 3 and 4 show that the counting efficiency in hemoglobin is somewhat lower. This is probably a result of color quenching by the heme that was not leached out of the gel. The actual efficiencies are slightly higher than the values presented here, because the losses described in the next section have not been taken into account.

The curve in Fig. 5 which was obtained with acrylamide was used to normalize the efficiencies in a given experiment. These data demonstrate that unlike many other systems (62) the ratios obtained with the commercial quenched standards are not accurate for monitoring quenching in gels. By this test no significant quenching has been observed in any of the hemolymph proteins except the fastest moving component.

A sample of tritium-labeled globin was counted in acrylamide gel with an efficiency that was 1.4 times the efficiency with which it was counted in Bray's solution. From this figure and a knowledge of the counting efficiency in Bray's solution it has been estimated that the counting efficiency for tritium in 7.5% acrylamide is about 23%.

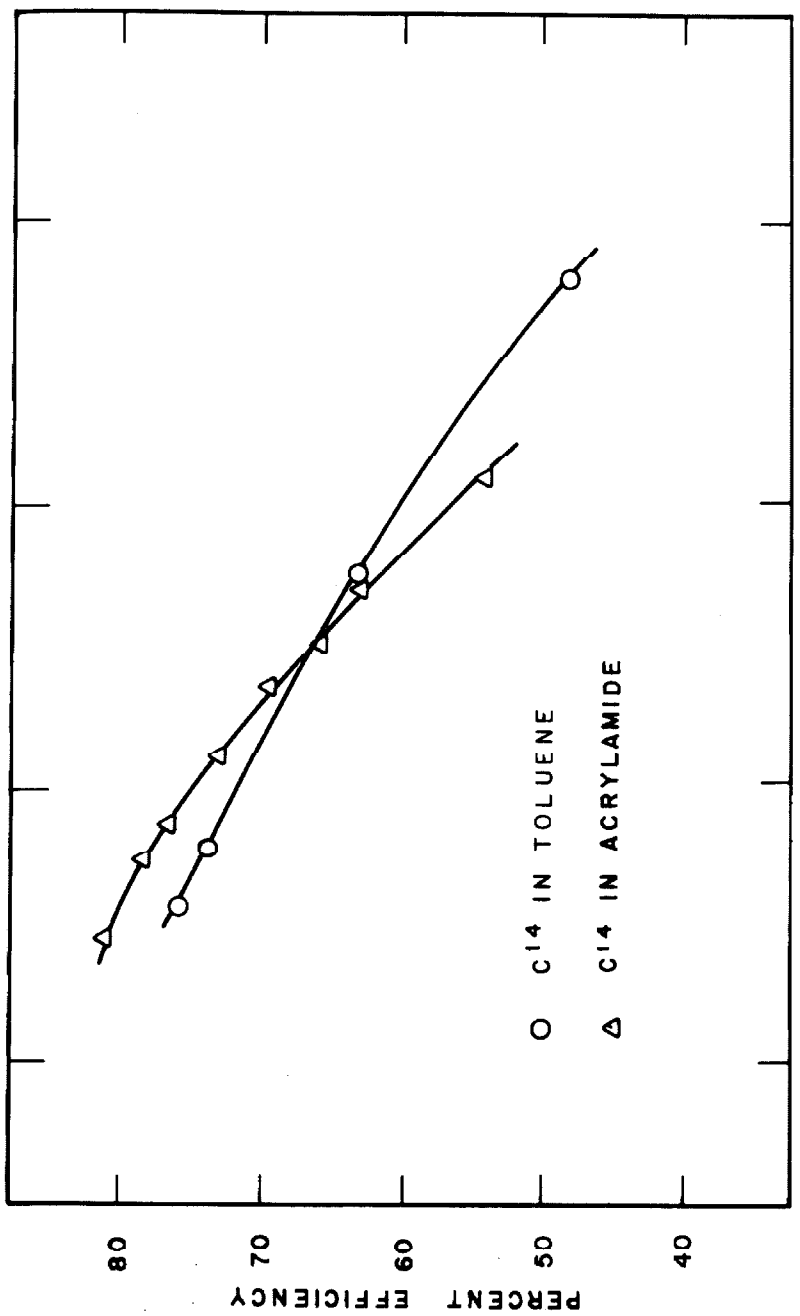

Fig.5 Quench correction by the channels ratio method. A 7.5% gel slice containing C^{14} -labeled hemolymph proteins was prepared for counting and successively quenched by adding acetone to the scintillator solution. When equilibrium had been obtained in each case, the gel was counted to a precision of 0.25% and the channels ratio (62) measured to 0.75%. Activities were converted to efficiencies by assuming that the untreated sample was counted with an efficiency of 81%. The gel and the commercial C^{14} -toluene standards were counted at machine settings which gave maximum efficiencies for the corresponding type of unquenched sample.

Protein loss from the gel. Although protein was never lost into the toluene solution, leaching of protein into the acid solutions was one of the primary concerns in the initial development of this technique. Because the recoveries of leached protein were low, very high initial activities were required in order to detect the small quantities of protein that actually leached out of the gels. As a result the errors in the recovery data are frequently of the order of 30% or higher. This includes a variation of 10% in the recovery and a 20% counting error. Data of the type presented in Table 3 are, nevertheless, accurate enough to permit the conclusion that less than 3% of the total hemoglobin or hemolymph in a 7.5% gel is leached out. This is less than a third of the loss that was found for gels not treated with PTA. The data in Table 5 provide an estimate of the loss from 6.4% gels. Although the loss of hemolymph from this gel was about 5%, the percentage of the larger bands that was lost was very small when the bands were in the center of a slice. Loss from the fastest moving band is not considered typical, because other evidence indicates that it is composed of relatively low molecular weight components. The activity recovered from the soaking solutions included any that was left behind in gel fragments. Since the controls described here were all run before it was found that soaping the cutting blades prevents gel shattering, the actual leaching of label from the gels was probably lower than the data indicate.

Any small molecular weight material which was radioactively labeled had to be separated from the protein prior to electrophoresis. If this were not done, most of it would leach into the common soaking solution and serve as a source of contamination for all the gels being soaked. Thus when C^{14} -valine was introduced into a gel by electrophoresis, all but 6% was lost into the solvent before counting. The soluble activity was found to contaminate other unlabeled slices which were being soaked in the same solvent.

Surface effects. When a C^{14} -toluene standard was counted at the machine settings used to obtain optimum gel counting, only 67% of the obtainable standard counts were registered. Thus when

TABLE 5

Loss of C^{14} -Hemolymph From 6.4% Gels into the Soaking Solutions[a.]

Hemolymph protein pattern	dpm measured in the gel[b.]	dpm lost into the soaking solutions
	1885 ± 25	105 ± 20
	1250	72
	887	60
	572	50
	619	47
	707	77
	6750	58
	6530	74
	391	66
	1180	239
	1070	32
	75	38
	50	13
	44	17
	282	102
	8	22

a. C^{14} -labeled hemolymph was analyzed in the standard manner except that the gels were soaked in individual flasks and the residues in the flasks were counted.

b. dpm was calculated from estimated machine efficiencies of 75% and 19% for the scintillation and planchet counters respectively.

the machine was set to give maximum efficiency for the C^{14} in the gel, any radiation which escaped from the gel into the solvent was also counted at about 51% efficiency. If a significant portion of the β rays were being absorbed in the solution instead of the gel, the result would have been a complex combination of two efficiencies which depended on the geometry of the protein in the slice. Since over 90% of the β rays from C^{14} decay have a penetration distance in toluene less than 0.2 mm (64), this effect need only be considered when the protein band is situated near the surface of the gel slice. The maximum error from this effect would be obtained when a labeled protein band is bisected by the cutting such that each half of the band ends up in two different slices. A crude calculation shows that the overall counting efficiency would not drop by more than 5% for a 1 mm-thick band cut in this manner.

The extent of this effect was tested experimentally by comparing the counting efficiency of a uniformly labeled gel which was counted with and without surrounding solvent. The β rays which escaped from the gel when no solvent was used were presumably stopped in the air without being counted. On the other hand when solvent was used, they were counted with an efficiency of 76%, because the detector was set to give maximum efficiency for C^{14} in toluene rather than in the gel. There was no difference between the results obtained with the two counting methods within a 1% experimental error. This was not a very sensitive test, however, because only 15% of the activity in the gel was within 0.2 mm of the surface from which much less than half of the radiation could theoretically escape. The results of the test do, however, permit the assignment of an upper limit of 3% to the error that would be encountered by bisecting a uniformly labeled 1 mm band. In practice this limit is somewhat higher, because the bands are actually Gaussian in form.

Another indication that any surface error is at least this low is found in the channels ratio data. This ratio is sensitive to the same energy changes that would be expected from this surface effect.

Since the surface effect would vary with the size of a uniformly labeled gel or the position of the label within a gel slice, the ratio should show the effect in these situations. In the experiment presented in Table 5 the ratios were identical for the bands that were centered in a slice and for the band that was bisected. The change in ratio with gel size which is shown in Table 6 is in the opposite direction to that expected from the surface effect. Thus the channels ratio test, which is sensitive to a 2% change in efficiency in 7.5% gels, has not detected this surface effect.

Gel size. The data in Table 6 show that the size of the gel can be varied over a wide range without seriously affecting the counting efficiency. The comparison between gels of 13 and 5 mm diameter was made by running aliquots of a hemolymph sample into gels of both sizes. The large and small gels were cut respectively into regular 4 and 3 mm-thick slices. The total activity in each gel was determined and used for the calculation in Table 6. In both protein preparations the quenching is higher in the small gel slices. In order to compare these data it was necessary to correct them for this additional quenching. Judging from the reproducibility of the channels ratio in larger gels, however, this correction would probably not be necessary if a comparison were being made between small gels of similar size. This quenching may simply be due to the large amount of PTA precipitate which collected on the surface of the gels in these particular experiments. In spite of this correction, the efficiency with which the smallest gels containing hemoglobin were counted is slightly lower than that for the large gels. This probably represents a higher loss into the soaking solutions which was not detected by the insensitive recovery procedure. Within a 6% experimental error the efficiencies with which gels of both sizes were counted were the same when the counter was reset for optimum efficiency with C^{14} -toluene. This suggests that small hemolymph-containing gels are not seriously plagued by the surface effect discussed in the previous section.

TABLE 6

Variation of Gel Size

Weight of gel soaked	Counting efficiency[a.]	Channels ratio
	Slices Homogeneously Labeled with Hemoglobin	
1.336 g	74.3 ± 0.5%	0.29
0.992	75.0	0.29
0.862	74.5	0.29
0.544	73.5	0.29
0.536	74.4	0.29
0.163	71.2	0.33
0.153	72.1	0.37
	Whole Gels after Electrophoresis of Hemolymph[b.]	
About 8 g in 16 slices	72.3 ± 3.5%	0.30
	76.5	0.30
About 1 g in 12 slices	75.8	0.38

a. Corrected for quenching.

b. The machine was reset to give a ratio of 0.30 for these 6.4% acrylamide gels.

Effect of protein concentration. A series of gels containing a constant concentration of C^{14} -hemoglobin and varying amounts of BSA was prepared in order to test the effect of protein concentration on gel counting. The data obtained from duplicate gels are presented in Table 7. Neither these results nor the results of experiments designed to test the effect of added cold hemolymph have shown any indication of protein quenching. Therefore, the counting method is independent of protein concentration within the concentration range used for good electrophoretic separation.

Importance of geometry on counting. Since gel slices do not naturally assume the same position in a vial, it was necessary to determine what effect the position of the gel in the counter had on the counting efficiency. A gel containing labeled hemolymph protein was successively positioned at various locations in a vial whose position with respect to the counter was held constant. In all cases the sectioned surface of the gel rested on the bottom of the vial. The difference between the counting efficiencies obtained at any of these positions was always less than 5%. Since the standard deviation of a large body of efficiency data, like that found in Table 3, is 0.5%, the error due to the position of the gel in the vial cannot be larger than one per cent in these experiments.

The importance of the position of a protein band within a gel was tested with a gel which had a protein band assymetrically positioned in it. C^{14} -hemoglobin was conducted into a gel, and a gel section was cut such that the visible band was very close to one surface. When this gel was counted with first one side and then the other facing up, it was not possible to detect any difference between the two positions within an error of 0.5%. Thus the scintillation counter is relatively insensitive to the possible geometrical variations.

Gel stability. Although a few gel slices have been observed to deteriorate after a few months, by far the majority were stable for over 18 months. Any loss in counting efficiency was immediately detectable by the appearance of a white opaque area in the gel.

TABLE 7

Effect of Protein Concentration on Counting Efficiency

Homogeneously distributed BSA per gel slice	Relative Counting efficiency[a.]	Channels ratio
Slices Homogeneously Labeled with Hemoglobin		
0.05 mg	65.3 ± 0.5%	0.34 ± 0.01
0.10	62.8	--
0.25	63.5	0.35
0.50	63.8	0.35
1.00	63.0	0.35
2.50	63.4	0.35
Whole Gels after Electrophoresis of Hemolymph[b.]		
Protein in nine slices	Total C^{14} activity	Average channels ratio
0.75 mg labeled protein	1540 ± 30 cpm	0.31 ± 0.02
0.75 mg labeled plus 0.42 mg unlabeled protein	1534	0.32

a. Average from two gels subjected to the standard procedure with the exception that the first solvent was 0.25% PTA in acetic acid-cellosolve (3:1) - Absolute efficiency unknown.

b. 0.1% PTA used in first soaking.

d. Reproducibility of the Total Counting Procedure. Tables 8 and 9 provide an indication of the total experimental error in the two major types of counting experiments used in this work. For both of these controls labeled protein was subjected to electrophoresis in duplicate gels in which the C^{14} was counted. The average error between the activities of parallel slices is about 10%. This error could undoubtedly be reduced by introducing mechanical means to cut the 0.4 cm thick slices.

TABLE 8

Reelectrophoresis of Isolated Proteins

Hemolymph protein pattern (approximate)	Percentage of total activity		Percentage difference between parallel slices
	Gel #1	Gel #2	
	1.0 ±0.1	1.2 ±0.1	
	1.1	1.0	
	1.3	1.4	
	1.8	1.6	
	4.1	3.9	5
	67.4	65.5	3
	18.1	20.4	12
	2.0	1.6	
	1.4	1.1	
	0.4	0.5	
	0.5	0.6	
	0.4	0.4	
	0.5	0.6	
	0.2	0.3	
Total C^{14} activity	6,784 cpm	7,111 cpm	

C^{14} -labeled hemolymph was obtained from 87 hour larvae. A mixture of proteins obtained from the electrophoretic areas E and F (Fig. 6) was mixed with unlabeled hemolymph and subjected to electrophoresis. Equal aliquots were run in two separate gels, which were subsequently sliced and counted as described in II, B, 2.

TABLE 9

Electrophoresis of C^{14} -Labeled Hemolymph[a.]

Hemolymph protein pattern (approximate)	Percentage of total C^{14} activity		Percentage difference between parallel slices
	Gel #1	Gel #2	
	14.5 ±0.1	10.3 ±0.1[b.]	--
	11.7	11.0	6
	8.3	8.6	4
	10.7	11.6	8
	6.9	7.2	4
	7.1	7.5	6
	10.9	11.9	9
	6.5	7.1	9
	3.2	2.8	13
	3.7	3.8	3
	10.6	11.2	6
	0.8	1.1	30
	1.2	1.3	8
	0.9	0.9	0
	0.5	0.6	17
	2.3	2.8	19
	0.4	0.2	--
Total C^{14} activity	63,339 cpm	57,839 cpm	

a. Pupae, which had just been floated (124 hr.), were injected with C^{14} -valine in 0.01 M NaCl. After incubation for 18 hr. at 25°C, the animals were used to prepare hemolymph. Equal aliquots were subjected to electrophoresis on two separate gels.

b. This slice was shriveled, and the result is, therefore, invalid.

C. Results

1. Separation of Drosophila Hemolymph Proteins by Disc Electrophoresis

As a foundation for the studies on hemolymph protein turnover disc electrophoresis patterns of the hemolymph proteins were obtained at intervals during the development of the insect. A series of densitometry tracings obtained from five larval and one pupal stages is shown in Fig. 6. For convenience in analyzing the hemolymph patterns, they have been divided into the eight areas indicated above the data obtained from pupae. It should be noted that areas E, F, and H are each known to contain more than one major protein component. The tracings in Fig. 6 demonstrate how the proteins vary with respect to one another, but for technical reasons the total amount of protein shown at different ages cannot be compared.

Tracings representing the hemolymph proteins at various pupal ages were very similar with respect to the relative proportions of the major protein components. To show this quantitatively, the relative amounts of protein in each area were calculated from the areas under the densitometry peaks and plotted in Fig. 7. These data show that the relative concentrations of most of the hemolymph proteins are constant throughout pupal development.

A more precise study was made of the absolute amounts of the major hemolymph proteins which are present at various stages of pupal development. In the experiment described in Fig. 8 it is estimated that greater than 95% of the total pupal hemolymph was recovered for analysis. The absolute quantity of protein in this experiment was estimated from a standard curve obtained under the same experimental conditions with known quantities of BSA. The experiment was performed as part of a search for proteins which are maintained in a steady state for at least part of the course of pupal development. Within the experimental error the proteins in area E meet this requirement as a group. The proteins in area F come very close to behaving in the same manner, but those in

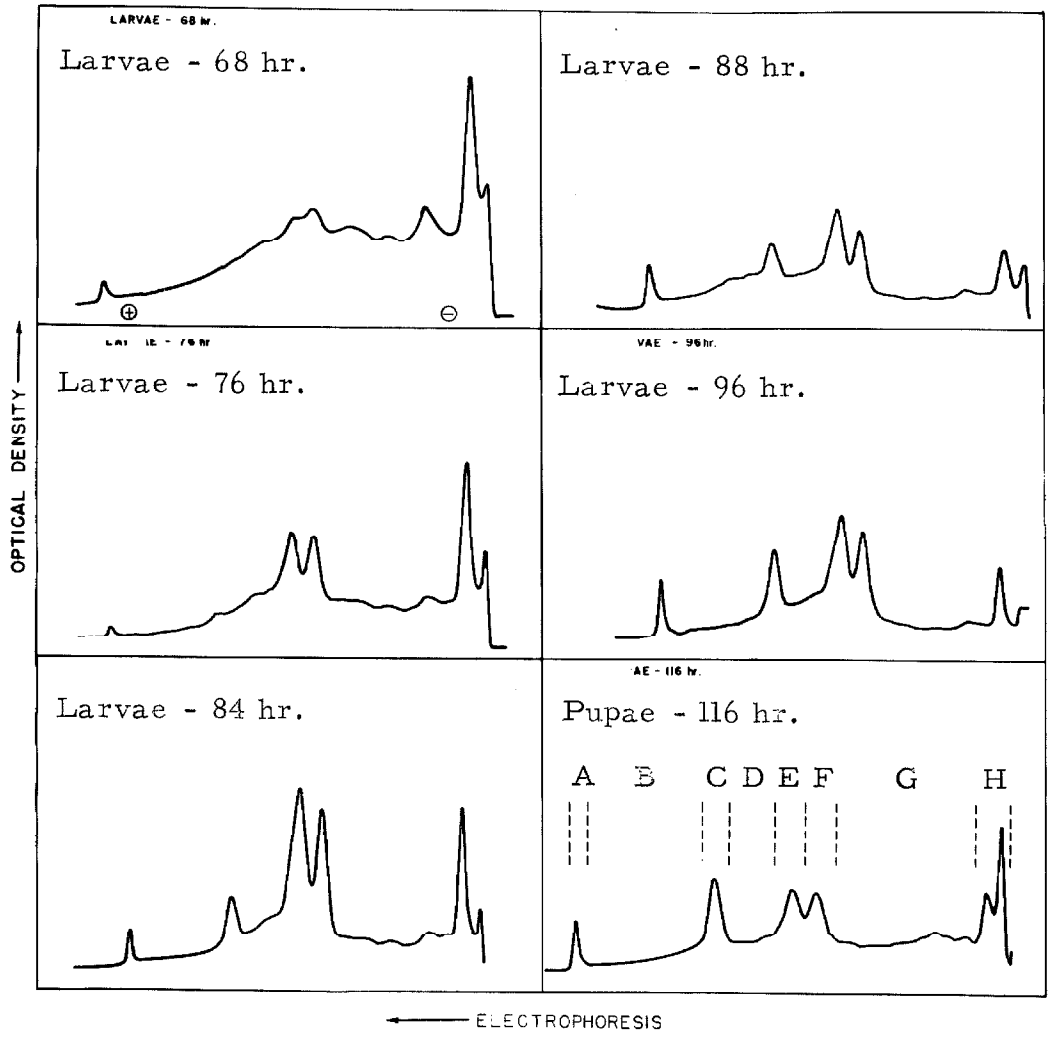

Fig. 6 Hemolymph proteins of Drosophila. Hemolymph from om five larval and one pupal stage was subjected to slab electrophoresis and analyzed with the Chromoscan densitometer. The first and last three groups of animals were taken from separate cultures. The larvae had been fed on pure yeast for differing periods of time.

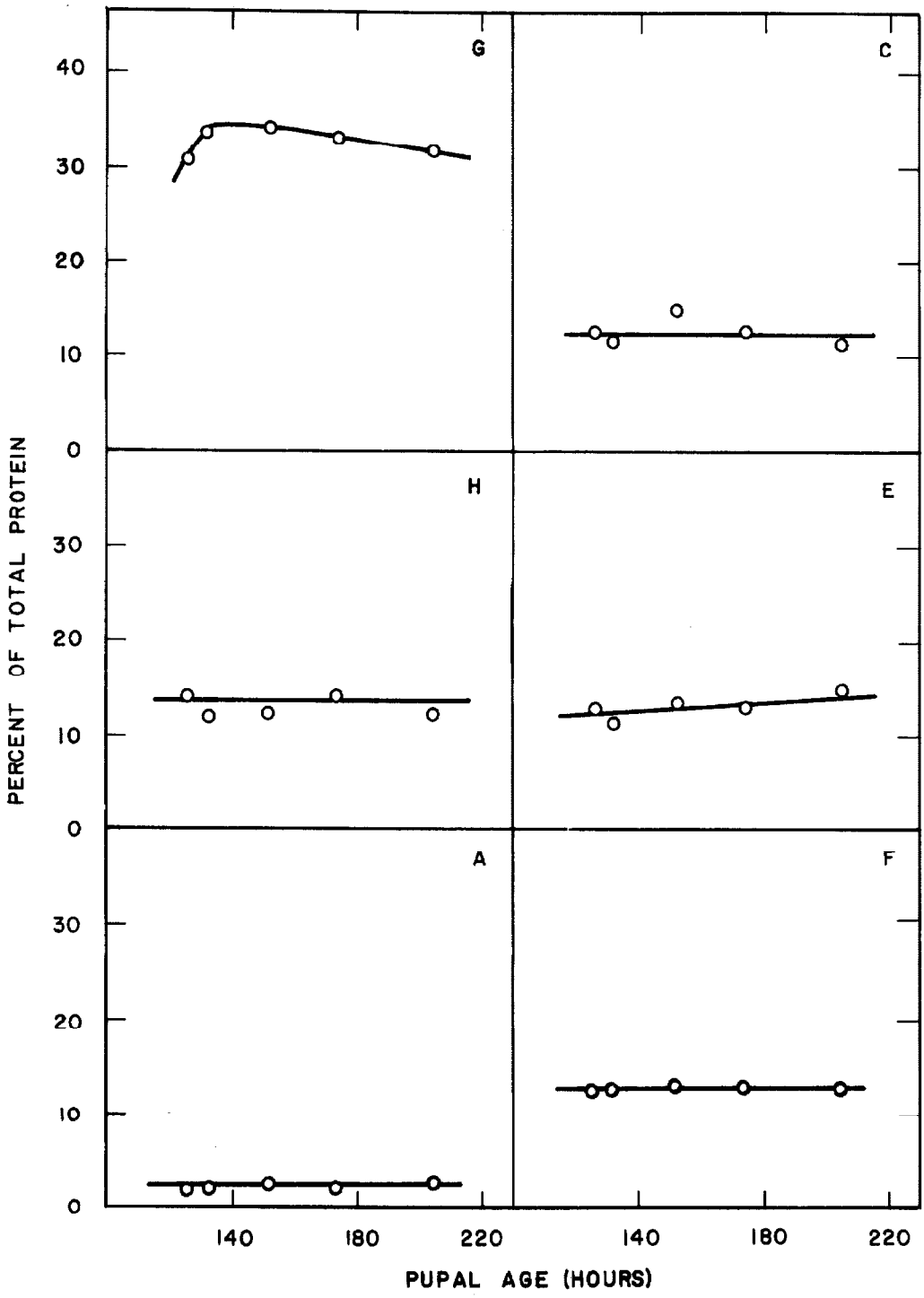

Fig. 7 Relative variations of hemolymph proteins during pupal development in Drosophila. Each curve represents the results obtained from those hemolymph proteins which were found in one of the lettered areas indicated in Fig. 6. The data were measured from densitometry tracings of gel slabs stained with light green SF yellowish. All data were obtained from a single group of animals whose stage of development had been determined by floating them in water.

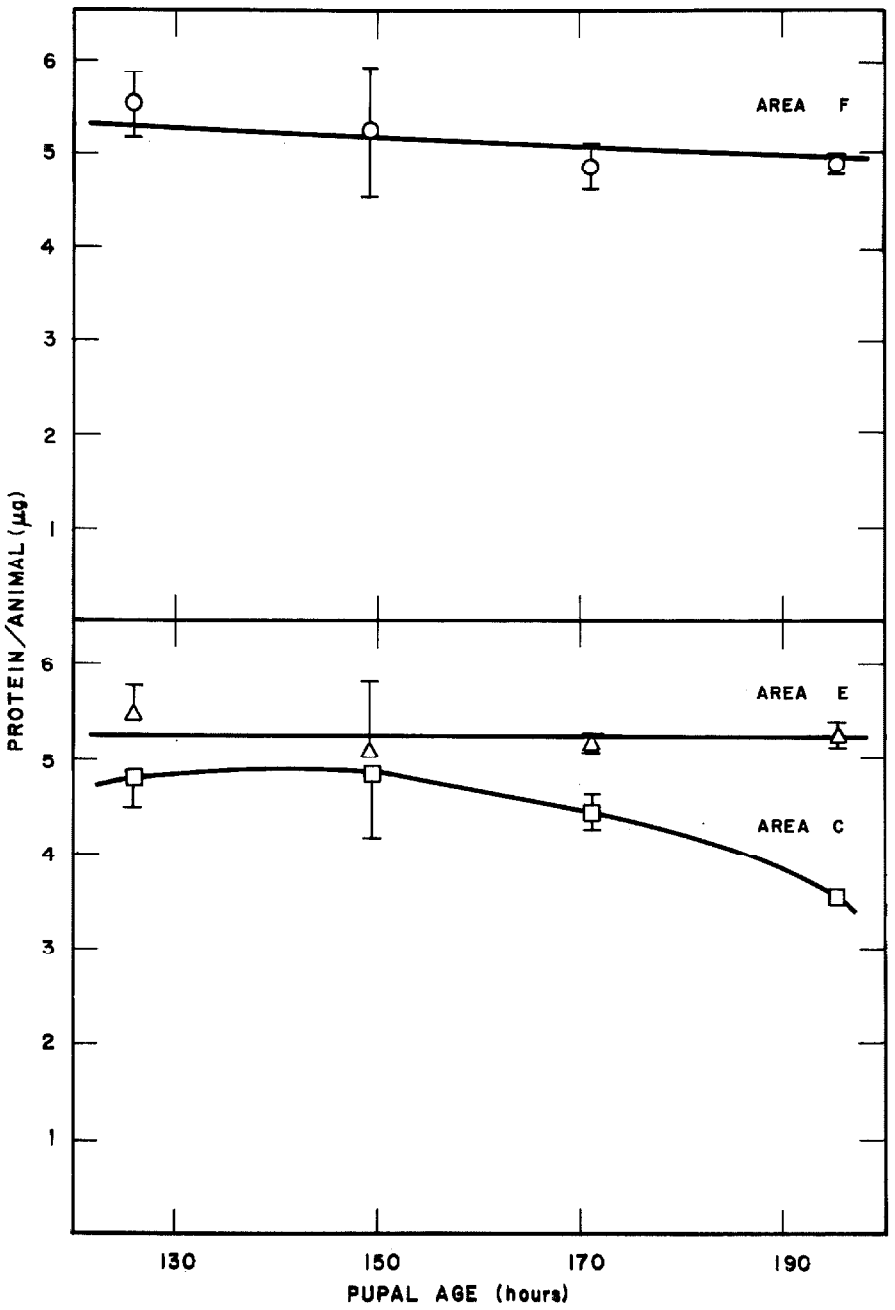

Fig. 8 Total protein in three major groups of hemolymph proteins. Hemolymph was prepared from batches of 24 pupae at various times after they floated on water. An amount of hemolymph equivalent to that obtained from two animals was subjected to standard disc electrophoresis at 1 ma/gel and 4° C. Gels were stained overnight in amido black and destained electrophoretically. Four independent hemolymph preparations were made at each pupal age. The protein quantities in each area of the electrophoretic pattern (Fig. 6) were determined relative to a BSA standard by averaging the areas under the peaks in densitometry tracings obtained from four gels. The error limits represent one standard deviation.

area C do not. The animals at all stages of development studied in this experiment weighed essentially the same with the exception of the 126 hour old animals which were 1.14 times heavier than the older pupae.

2. Electrophoretic Homogeneity of the Proteins

One of the criterion used to establish the existence of protein turnover was the appearance of C^{14} activity in proteins other than the ones injected. This test required that the electrophoretic mobilities of the injected proteins be reproducible. To determine if this requirement was met, a gel containing separated hemolymph proteins was sliced transversely, and each individual section was placed on top of a fresh small-pore gel. Re-electrophoresis of the proteins contained in these slices resulted in sharp bands of reproducible mobility which showed very little streaking. This result demonstrates that when fresh hemolymph is used, the electrophoretic mobilities of the major proteins are probably at least 90% reproducible.

A more sensitive test of the reproducibility of electrophoresis is the isolation and re-electrophoresis of proteins which are labeled with C^{14}. Data from this type of test has already been presented in another context in Table 8. These results show that between 85 and 90% of the combined proteins from areas E and F reappeared at their original position in the gel after a second electrophoresis . The remainder of the activity was found distributed throughout the gel rather than in specific positions as might be expected if aggregation were a problem. A closer examination of the factors which affect the reproducibility of the mobilities of area-E proteins is presented in Table 10. To similate the treatment of the protein during and after injection, samples of protein from area E were incubated at room temperature both with and without unlabeled hemolymph. Since the presence of unlabeled hemolymph did not affect the mobility of unfrozen protein, the proteins in area E probably do not aggregate with other hemolymph proteins. Rather, the result with frozen E protein

TABLE 10

Re-electrophoresis of C^{14} -Labeled Proteins from Area E

Treatment of protein	Percentage of C^{14} recovered at the position of the proteins in the original gel[a].
Isolated protein	94%
Plus added unlabeled hemolymph	93
Frozen, thawed, and rerun	85
Frozen with added unlabeled hemolymph, thawed, and rerun	92

a. The C^{14} activity in the two gel slices closest to the position of the proteins in the original gel was divided by the activity in all sixteen slices of the second gel. All data were taken from one experiment in which the samples were incubated at 25°C for 35 minutes before electrophoresis or freezing.

suggests that the failure of some of the protein to rerun to the same position in the patterns may be due to denaturation. The result obtained with unlabeled protein is compatible with the fact that proteins are often stabilized against denaturation in concentrated solutions (65). It is therefore concluded that aggregation is not a serious problem in these experiments, although the isolation procedure probably denatures a certain percentage of the protein.

The degree of homogeneity of a sample, as measured by the type of test presented in Table 8, varied from one hemolymph preparation to another. In order to correct the results of the injection experiments for this fluctuation it was necessary to monitor each individual protein preparation in this way. In most of the protein preparations that were tested, 85 to 90% of the C^{14} activity appeared at a position in the test gel which corresponded to its position in the original gel. However, values as low as 80% for area E and 70% for area C were encountered.

3. Amino Acid Feeding Experiments

a. Experimental Design. In this series of experiments larvae were pulse fed with radioactive amino acid, and the presence of C^{14} in the hemolymph proteins was studied at intervals thereafter. The data from these experiments served as a preliminary indication of the relative synthesis and degradation of the protein components with respect to one another. In addition it was possible to determine which stage of development was most satisfactory for the preparation of high specific activity proteins.

Since only those values obtained from a single experimental group of animals are compared, the analysis is not complicated by excessive variability in the developmental stages of the animals. The data from two experiments involving larval hemolymph (Figs. 9 and 10) and two involving pupal hemolymph (Fig. 11 and Table 11) are discussed. The error in these experiments is estimated to be about 10% from the results reported in Tables 8 and 9. An additional uncertainty of about 5% must also be added to the one set of specific activity data

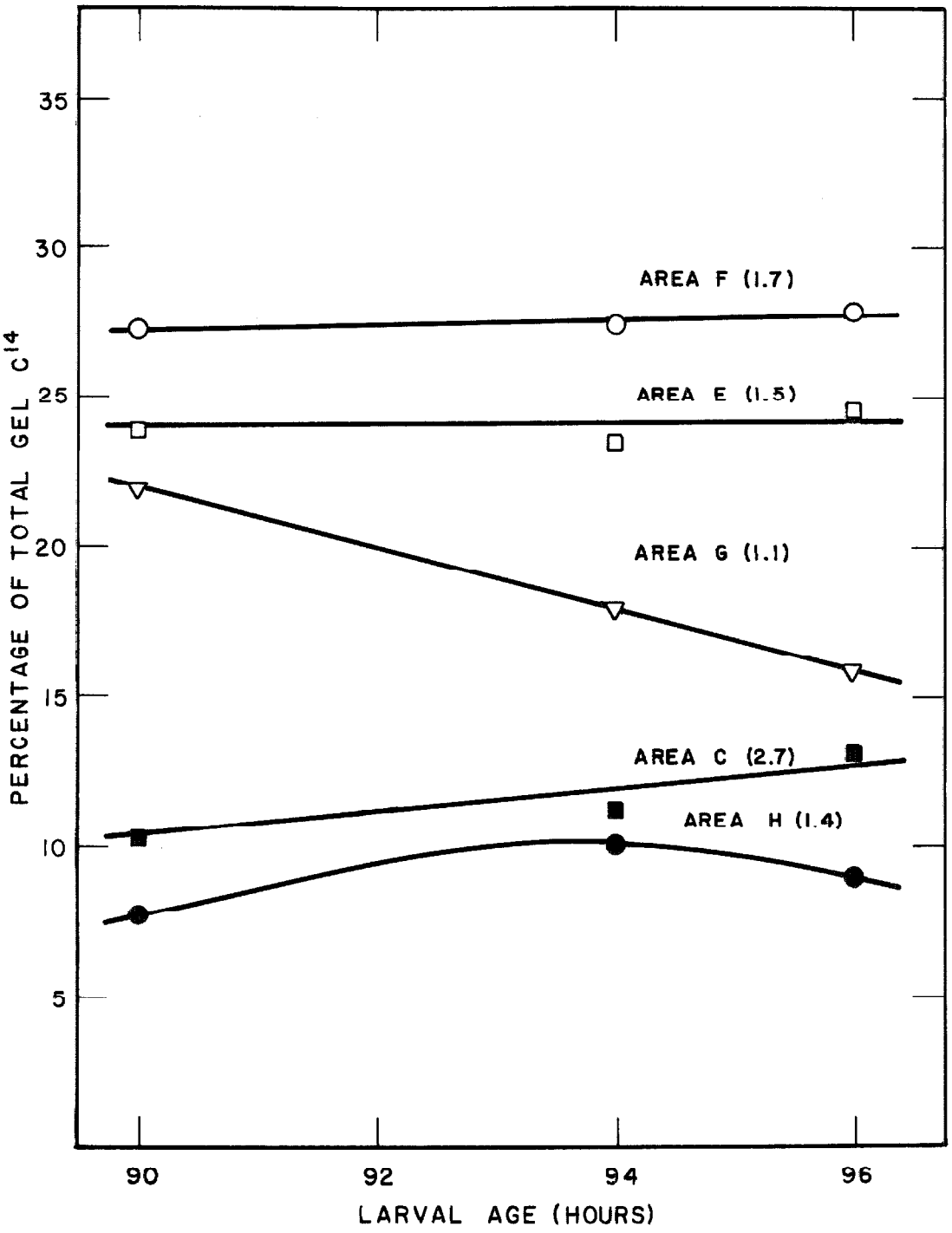

Fig. 9 Amino acid incorporation into hemolymph proteins. Larvae (89 hour) were fed yeast and C^{14} -algal hydrolysate for one hour before being transferred to fresh yeast. Hemolymph was prepared at the designated times and analyzed by disc electrophoresis as described in Methods (II, B). Each curve represents the data obtained from one of the electrophoretic areas designated in Fig. 6. The number in brackets is the factor by which the specific activity increased between 90 and 96 hours.

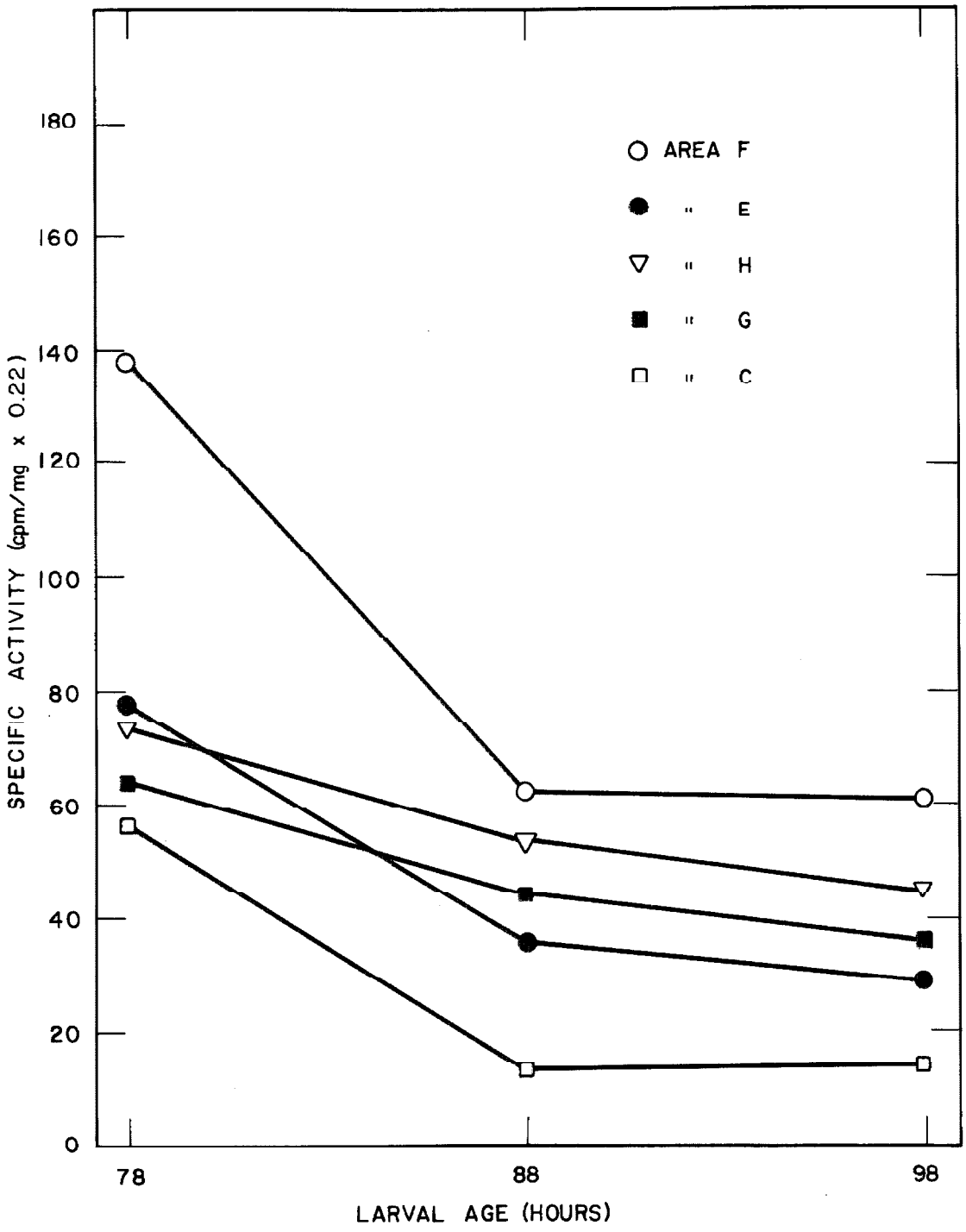

Fig. 10 Pulse-chase labeling of hemolymph proteins. Larvae (70 hour) were fed yeast and C^{14} -algal hydrolyzate for 3 hours. They were then transferred to fresh yeast and fed at 25° C until samples were removed for hemolymph preparation. The average specific activities of the proteins in each area were determined from slab and disc electrophoresis patterns as described in Methods (II, B). Each curve represents the data obtained from one of the electrophoretic areas designated in Fig. 6.

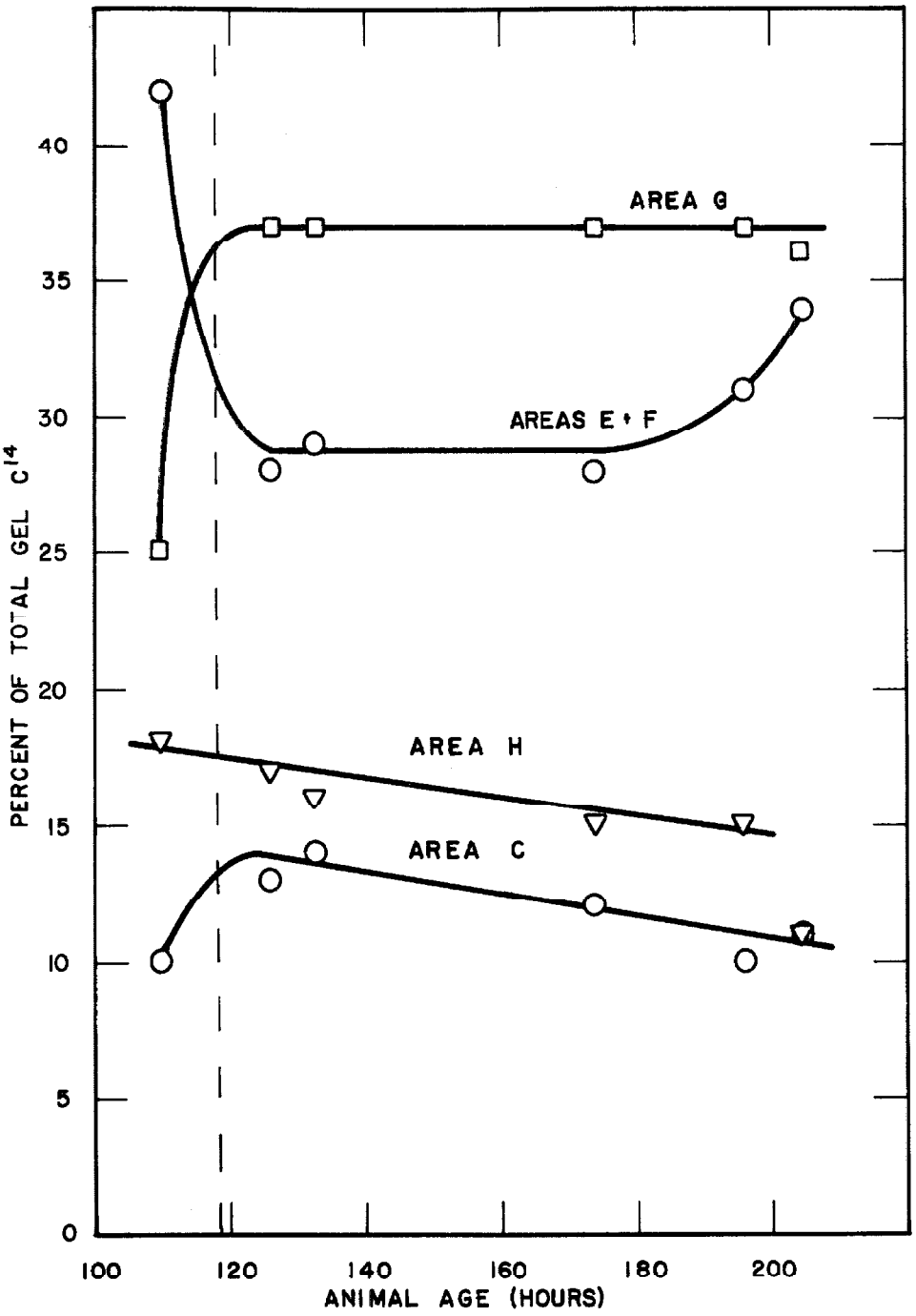

Fig. 11 Pulse-chase labeling of hemolymph proteins. Larvae were given a three hour feeding of C^{14} -algal hydrolysate. Twenty-four hours later the developmental stage of the pupae was measured by floating them on water. Hemolymph from different age pupae was subjected to disc electrophoresis and analyzed for C^{14} as described in Methods (II, B). Hemolymph was also prepared from the remaining larvae which had not formed a puparium twenty-four hours after feeding. Each curve contains the data obtained from one of the electrophoretic areas designated in Fig. 6. The dotted line represents the time of puparium formation.

TABLE 11

Variation in the Pattern of Labeled Hemolymph Proteins at Puparium Formation

Area of the electrophoretic pattern	Percentage of the total gel activity		Relative specific activity	
	96 hr. larvae	115 hr. pupae	96 hr. larvae	115 hr. pupae
H	13.4	17.7	100	116
G	16.2	45.6	73	125
F	29.4	5.2	136	29
E	19.1	11.4	62	63
D	5.6	3.8		
C	13.9	13.5	77	78
B	0.6	1.0		
A	1.7	1.5	4	8

Larvae (80 hr.) were fed C^{14} -algal protein hydrolysate and yeast for three hours. They were then transferred to fresh yeast and fed at 25°C until samples were removed for hemolymph preparation. The hemolymph proteins were separated by disc electrophoresis and analyzed for C^{14} as described in Methods (II,B).

contained in Fig. 10.

b. Distribution of C^{14} in Larval Hemolymph Proteins. Two types of experiments were performed in an attempt to determine if some of the larval hemolymph proteins turn over much faster than others. The data presented in Fig. 9 provide a rough estimate of the relative rates of synthesis of the major hemolymph proteins, and the data in Fig. 10 indicate the relative rates of degradation. A detailed analysis of these data is complicated by many factors including the fact that the relative proportions of the proteins themselves are changing during larval development. Nevertheless, it is clear in both cases that the major groups of proteins behave rather similarly with respect to the rates of synthesis and degradation. Thus these preliminary data suggest that if they turn over at all, the major larval hemolymph proteins turn over at approximately the same rate.

c. Distribution of C^{14} in Pupal Hemolymph Proteins. The type of investigation just described was also carried out on pupal hemolymph which had previously been labeled by feeding C^{14} -amino acids to larvae. The data presented in Table 11 exemplify the types of changes observed in the C^{14} pattern of the hemolymph proteins around the time of puparium formation. The most striking changes are exhibited by the proteins occurring in areas F and G of the electrophoretic pattern. Not only does the relative amount of radioactivity in area F decrease drastically at this time, but the specific activity of the proteins in this area also decline. These results together with those in Fig. 6 strongly suggest that most of the proteins which appear in this part of the pattern are being rapidly synthesized and degraded. The increase in both the specific activity and the relative amount of C^{14} which is observed in area G after puparium formation probably represents an increased recovery of tissue proteins. Since the method of preparing hemolymph from pupae is cruder than the method for preparing larval blood, the preparation from pupae is expected to contain more tissue proteins.

The changes observed in the other areas are not as dramatic; suggesting that protein turnover in these areas may not be as high as it is for the proteins in area F.

The type of experiment just described has also been used to obtain data throughout the pupal stage of development. These data, which are presented in Fig. 11, suggest that many of the proteins in pupal hemolymph undergo some sort of turnover. This conclusion is drawn from a comparison of these data with the data in Fig. 7. Since the curves for a single group of proteins in these two experiments are not parallel, metabolic turnover is suggested.

d. Summary of the Feeding Experiments. As a test of protein turnover, these experiments were complicated by the fact that the whole animal was labeled with C^{14}. In this situation it is difficult to detect protein turnover, because radioactivity which is lost from a given protein pool may be replaced from a labeled amino acid pool. In most of these preliminary experiments the free amino acid pool was being replaced during growth and very probably had a lower specific activity than the pool of amino acids in the proteins. Thus this test for turnover would have been expected to reveal any cases of exceptionally high turnover. With this assumption the following conclusions can be made. 1. During late larval development none of the major groups of hemolymph proteins turn over at a much higher rate than the others. 2. Active synthesis and degradation probably occur among the proteins of area F after puparium formation. 3. Some protein turnover probably occurs during pupal development.

4. Measurement of Radioactivity Transferred From Injected Labeled Proteins

A more rigorous test for the existence of protein turnover was performed by injecting animals with homologous C^{14} -labeled hemolymph proteins. Labeled hemolymph was obtained from larvae which had been fed C^{14} -labeled amino acids. After this hemolymph had been subjected to preparative disc electrophoresis, the proteins were isolated from individual areas of the electrophoretic pattern and injected into unlabeled animals. In the experiments described in this

section the hemolymph of the injected animals was subsequently analyzed at intervals for C^{14} after disc electrophoresis. It was reasoned that in a rapidly growing (larvae) or closed (pupae) system the degradation products of one protein should appear in other proteins being synthesized. This approach required that hemolymph proteins were being synthesized during the period of development that was being tested. Numerous experiments have shown that this requirement is met during the later phases of larval development. The results presented in Table 9 show that some of the recovered proteins are synthesized in pupae as well. Thus it should be possible in both of these stages of development to detect protein turnover by measuring the redistribution of the C^{14} in the electrophoretic pattern after the injection of C^{14} -labeled proteins.

The accumulated data from a number of these injection experiments are presented in Table 12. The figures in the right hand column of this table represent the percentage of the total C^{14} in the gel which was found in proteins other than the ones that were injected. Each of these values has been corrected as described in the methods for any heterogeneity which existed in the original injected sample. These results show that there is a clear difference between the rates of degradation of the proteins in the three areas studied. This is a greater difference than would have been predicted from the results of the feeding experiments. The contrast between the two sets of results can probably be explained by the greater sensitivity of the injection experiments as a test for turnover. These data may not be compared quantitatively since both C^{14} -valine and C^{14} -algal hydrolyzate were used at different times. By rejecting the last value in Table 12, it is possible to rationalize the behavior of the proteins in area F with the dramatic changes which were demonstrated for these proteins in the feeding experiments. With the elimination of this contradiction, the data are consistent with the conclusion that the proteins in area F begin to turn over rapidly sometime after puparium formation. Preliminary experiments performed with

TABLE 12

Redistribution of the C^{14} of Injected C^{14} Labeled Hemolymph Protein

Area of the electrophoretic pattern from which protein was obtained	Age of injected animals	Duration of incubation following injection[a].	Percentage of C^{14} recovered at other than the original position of the protein (corrected)[b].
C.	87 hr. Larvae	4.5 hr.	< 5
	76 hr. "	21	33
	116 hr. "	(22)	33
	90 hr. "	(47)	30
	118 hr. Pupae	(20)	32
	142 hr. "	(19)	52
E.	73 hr. Larvae	24	9
	110 hr. "	(26)	17
	90 hr. "	(47)	24
	115 hr. Pupae	(24)	39
	118 hr. "	(42)	56
F.	87 hr. Larvae	4.5 hr.	< 5
	114 hr. "	8	3
	73 hr. "	24	2
	114 hr. "	(26)	3
	115 hr. Pupae	(24)	40
	118 hr. "	(42)	-3

a. Hemolymph reisolated from pupae is indicated by brackets.

b. The percentage of the total C^{14} activity in the two gel slices surrounding the original protein position was measured. This value was then divided by the corresponding value obtained from an uninjected protein sample. The quotient was subtracted from 100.

some of the other proteins in the electrophoretic pattern have provided evidence suggesting that these proteins may also turn over at a significant rate.

The effect of flooding the hemolymph with unlabeled amino acid was tested in this sytem by injecting the proteins in a saturated amino acid solution. In the experiment described in Table 13 the injected proteins, which had been labeled with C^{14}-valine, were dissolved in buffer saturated with valine. From the data of Chen (66) it is estimated that the injection increased the valine concentration of the hemolymph by a factor of 35. These data show that in each case, in which it was testable, the free valine inhibited transfer of the C^{14} -valine from the injected proteins to the other proteins of the hemolymph. The last value in Table 13 is the same point which was discounted in Table 12. The results of this experiment suggest that as precursors for general protein synthesis the degradation products obtained in the process of protein turnover are in competition with free valine or one of its derivatives.

Table 14 contains the results of a parallel experiment in which non-homologous C^{14} -labeled hemoglobin was injected into Drosophila. In this experiment the animals remained bright red well past the time at which the last data were taken. This fact together with the data itself show that Drosophila is not able to degrade non-homologous proteins at nearly the rate with which it can degrade its own hemolymph proteins.

5. Determination of Protein Turnover Rates in Pupae

The data presented in Fig. 8 demonstrate that the total quantity of protein which appears in the electrophoretic areas E and F is very nearly constant for each animal throughout pupal development. Under such steady state conditions, in which the rates of protein synthesis and degradation balance, it is often possible to measure the rate of protein turnover by determining the specific activity of labeled proteins at various times after their injection. This method of analysis, which has been discussed by Traver (8), has been used to measure the half lives of the proteins which appear in these two areas. As

TABLE 13

Effect of Unlabeled Free Valine on the Redistribution of C^{14} Activity After Injection

Electrophoretic area from which protein was obtained	Age of injected animals	Duration of incubation following injection	Percentage of C^{14} recovered at other than the original position of the protein (corrected) [a]	
			With valine [b]	Without valine
C	90 hr. Larvae	47 hours	14%	30%
E	90 hr. Larvae	47	14	24
E	118 hr. Pupae	42	39	56
F	118 hr. Pupae	42	-4	-3

a. The percentage of the total C^{14} activity in the two gel slices surrounding the original protein position was measured. This value was then divided by the corresponding value obtained from an uninjected protein sample. The quotient was subtracted from 100.

b. Protein was dissolved in a saturated valine solution before injection.

TABLE 14

Redistribution of the C^{14} of Injected C^{14} -Labeled Hemoglobin

Age of injected animals	Duration of incubation following injection	Percentage of C^{14} recovered at other than the original position of the protein (corrected)*
77 hr. Larvae	20 hrs.	2
91 hr. "	1	3
91 hr. "	19	6
91 hr. "	(43)	9
118 hr. Pupae	(43)	7

* The percentage of the total C^{14} activity in the three gel slices surrounding the original protein position was measured. This value was then divided by the corresponding value obtained from an uninjected protein sample. The quotient was substracted from 100.

determined from the data in Fig. 12 the proteins in area F have an average half life of 13 hours while the proteins in area E have a combined half life which is too large to be measured. The dotted line in Fig. 12 has the same slope as a curve which Abdou and Traver (67) obtained from similar experiments in which they studied the plasma proteins of rats. In their experiment the average "metabolic" half life of the plasma proteins was estimated to be 74 hours. Thus the two groups of hemolymph proteins that have been analyzed have turnover rates which are higher and lower than the average turnover rate of rat plasma. The amount of protein formed per unit time, or replacement rate, can be calculated for the proteins in area F from the data in Figs. 8 and 12. This figure is 0.56 μg of protein per hour per animal.

A comparison of these data with the data reported in previous sections must take into consideration the fact that the data for the first point in this experiment were taken 43 hours after injection. Thus the injected proteins were incubated longer in this experiment than in previous experiments. This difference can explain the failure to detect turnover of the proteins in area E in this experiment whereas the experiments reported in Table 12 indicated a significant turnover for these proteins. The most probable explanation for this contrast in the data is that area E contains proteins with both high and low rates of turnover. If this is the case, only the radioactivity in the inert proteins of area E would have been found in area E after 43 hours. On the other hand, in the experiments in which radioactivity was measured in proteins appearing outside area E (Table 12), the degradation of the rapidly metabolizing component would have been readily detected. The rapid turnover which was found in this experiment for the proteins in area F is consistent with the earlier conclusions that these proteins start to turn over sometime after puparium formation.

The experimental approach used here to determine turnover rates includes the assumption that the steady state depicted in Fig. 8 is also maintained in animals which have been injected. Additional data

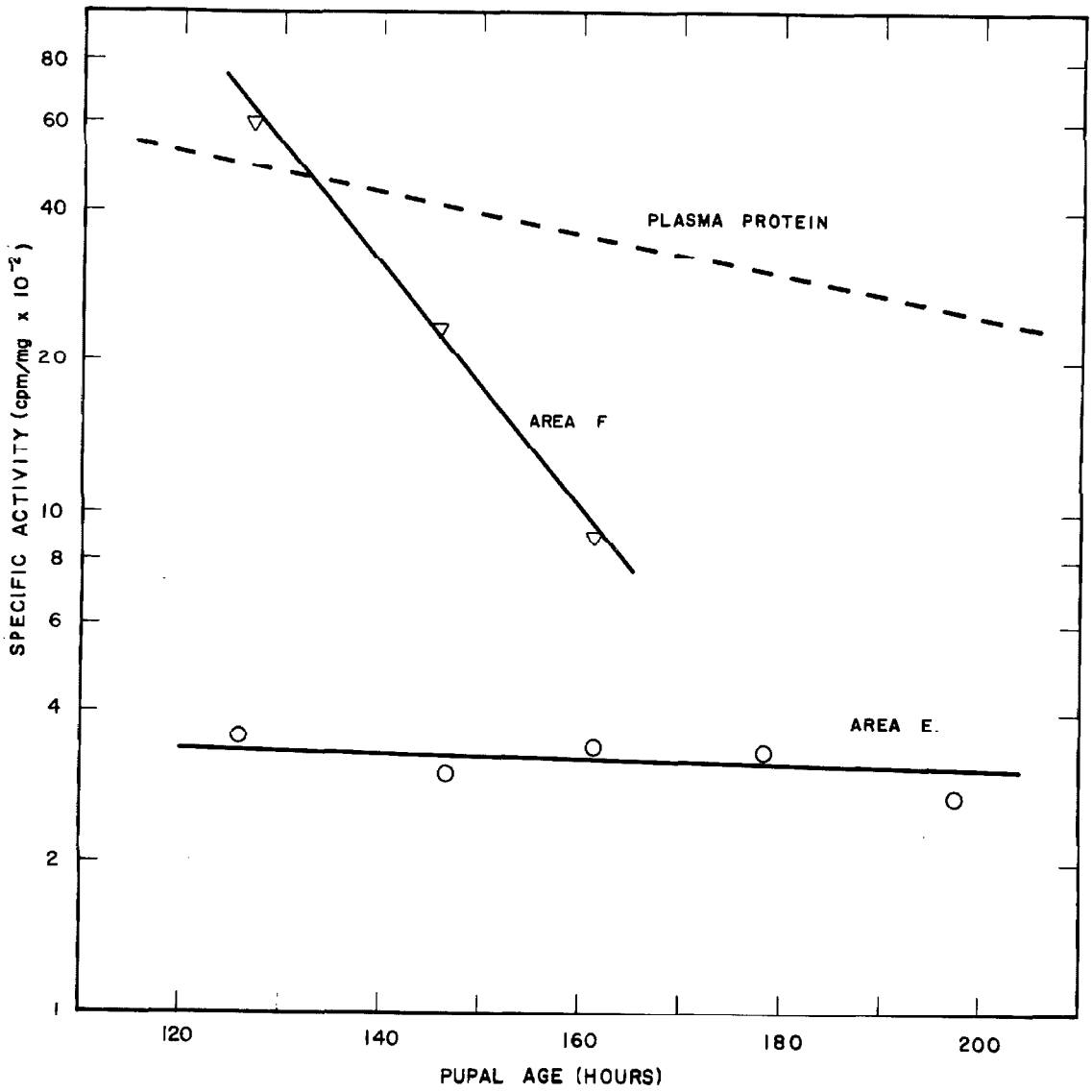

Fig. 12 Determination of hemolymph protein turnover by the Hevesey method (8). Two groups of larvae, 89 hours old, were injected with C^{14} -labeled proteins isolated from areas E and F of the electrophoretic pattern. At various times after puparium formation the specific activity of the protein in areas E and F was determined in the appropriate sample as described in Methods (II, B). Aliquots of each hemolymph sample were subjected to standard disc electrophoresis in triplicate. Protein determinations were made from these gels as described in Fig. 8. The dotted curve has a slope equal to that obtained by Abdou and Traver (67) who performed similar experiments on the plasma proteins of rats.

obtained from the experiment described in Fig. 12 indicate that this assumption was valid with one exception. The amount of protein per animal in band F began to decrease sometime after the third sample was taken. The data taken after this time were, therefore, not valid for analyzing turnover rates and have not been included in Fig. 12. It was also noticed that the proportion of the protein in areas E and F to the total hemolymph protein was lower in injected animals than in those that had not been injected.

D. Discussion

1. Experimental Results

a. Electrophoretic Separation of Hemolymph Proteins. Although the hemolymph proteins of Drosophila had previously been investigated with starch gel electrophoresis (42, 43), it was necessary to repeat this work in acrylamide, because protein mobilities are not directly comparable in the two types of gel. With the experimental conditions used in this investigation, only 5 major bands of hemolymph protein were clearly resolved by disc electrophoresis. Under slightly modified conditions it has been shown that at least three of these bands are composed of two or more protein components. Although this resolution is inferior to that obtained in starch gel, for the purposes of these experiments it was unnecessary to attempt any further separation. When starch gel electrophoresis was employed in the early stages of this investigation, 6-12 protein bands were separated from pupal hemolymph and 5-9 from larval hemolymph. Since these results are comparable to those obtained by the earlier investigators (42, 43), the procedure used here to prepare hemolymph probably yields as satisfactory a preparation as do the more refined methods.

The densitometry tracings shown in Fig. 6 confirm the qualitative observations of the earlier workers that the relative proportions of the proteins change dramatically during larval development. Studies with radioactive tracers have shown that over 98% of the hemolymph proteins enter the gel. This observation is in agreement with the measurements which Chen (68) has made on the isoelectric point of these proteins. The protein nature of the stained components is substantiated in these studies by the observation that the major components stain equally well with very different protein stains. Several lines of evidence indicate that the band with the fastest mobility is partially an artifact and partially made up of low molecular weight components. In contrast, at least some of the proteins appearing in areas E and F of the electrophoretic pattern have

been shown by Mitchell (50) to have a molecular weight greater than 200,000. The consistency of the protein pattern throughout pupal development is somewhat surprising in view of the fact that hemolymph proteins have been shown to be precursors of developing tissues in other insects (45). The existence of this phenomenon has, however, permitted the determination of accurate turnover rates for two groups of hemolymph proteins.

b. Turnover of Drosophila Hemolymph Proteins. Two separate criteria have been employed in this study to establish the existence of hemolymph protein turnover. The data in Table 12 show that the labeled amino acids of one group of injected proteins are progressively redistributed to the other proteins of the hemolymph. This result proves, with very few reservations, that the initially labeled proteins are degraded to provide substrate for the synthesis of other proteins. In addition, protein turnover was detected in the experiment reported in Fig. 12 by monitoring the specific activity of the injected proteins. This method for measuring turnover has been carefully analyzed in mammalian systems and has been found to be generally satisfactory (8). Although the method is not quantitatively exact when there is significant reincorporation of the degradation products into the original protein, this would appear not to be the case here because of the wide redistribution of radioactivity which has been found in the experiments reported in Table 12. The two sets of data therefore show that Drosophila hemolymph proteins are degraded during the course of both larval and pupal development. The failure of Drosophila to degrade hemoglobin indicates that the observed turnover is a specific phenomenon.

The data presented in Table 12 and Fig. 12 reveal that the rate of protein turnover varies from one group of proteins to another. An analysis of all the data together has further indicated that proteins which appear within one electrophoretic area also behave differently from one another in this respect. Significant changes in metabolism are to be expected following puparium formation, because at this time Drosophila transforms from a mobile growing form to a stationary

form in which differentiation predominates. Such a change has been found in the metabolism of the proteins in area F as the data in Table 11 show. Although a second set of data (Table 12) is not entirely consistent on this point, these injection experiments have sometimes been difficult to reproduce. The sudden shift to a high turnover rate which is experienced by at least one of the proteins in area F after puparium formation makes these proteins the most likely candidates for future studies of the mechanism of protein turnover. Some of the other proteins may be equally as useful since they also undergo significant turnover (Table 12).

During the course of this investigation increasingly sensitive tests for protein turnover have been employed. It is now apparent that the early larval feeding experiments (Figs. 9 and 10) were insensitive as they did not detect all the differences in the behavior of the proteins which were found in the later experiments. In spite of its insensitivity, however, this method revealed the same sharp increase in the turnover of the proteins in area F (Table 11) that the more sensitive injection experiments showed. This correspondence in the data indicates that the process of injection did not seriously alter the pattern of protein turnover. By other criteria, however, it has been shown that injection affects the metabolism of the hemolymph proteins. Because of this complication these experiments reveal the metabolic capability of Drosophila, but they may not faithfully represent the normal protein metabolism of the organism.

The finding of different turnover rates for the various hemolymph proteins has a parallel in the results that have been obtained from intensive studies of mammalian systems (8). It is not possible to make significant comparisons between turnover rates in the two phyla, however, because the mammalian turnover rates are very strongly dependent on the size of the animal being studied. The results of experiments in which excess amino acid inhibited the transfer of label between hemolymph proteins are in strong contrast to the results of mammalian (69) and tissue culture experiments (70)

in which amino acid flooding had no effect on such transfer. The result obtained with hemolymph does not prove, however, that the proteins are degraded to free amino acid. The same result would be obtained if the free amino acid and a degradation product of the proteins compete with each other at some common step in protein synthesis. Additional tests are also needed to establish that the excess amino acid did not itself somehow alter the observed rate of protein turnover. Since hydrolysis of C^{14} -valine-labeled hemolymph proteins yields over 80% of the label in valine, the results of the flooding experiments are at least consistent with an interpretation of competition between a free valine pool and valine released from the protein.

2. General Discussion. Since it has not been possible to collect quantitative data on the turnover of all of the hemolymph proteins, the full significance of this phenomenon in metamorphosis is not known. It seems reasonable to assume, however, that the average rate of turnover of all the proteins is somewhere between the two extreme rates that have been measured for the proteins in areas E and F. This then suggests that the turnover of the hemolymph proteins has an important metabolic function, although it may not be the main channel through which the massive protein turnover of the metamorphosing insect passes.

Whitten (71) has recently found that shortly after the larval tissues of Drosophila have begun to degenerate, the resulting fragments are rapidly ingested by a special type of haemocyte. Once engorged, these cells become attached in a regular pattern to developing adult tissues. In the process of attachment the haemocytes radiate cytoplasmic strands which appear to connect the cytoplasm of the haemocytes to that of the developing cells. She concludes from these observations that the haemocyte may be acting as a nurse cell which feeds the developing tissue directly through the cytoplasmic filaments. If this is true, these cells, rather than the hemolymph or its proteins, are the primary means of transport between degenerating larval tissues and developing pupal organs.

Why should this organism employ such an elaborate mechanism for transporting degraded tissue components? It would appear to be more efficient simply to release the products of tissue degeneration into the circulation from where they could be picked up by developing tissues. On the other hand, this complex mechanism of transport could be part of a process for conserving some of the energy which was originally expended for macromolecular synthesis.

Just as glycogen and ribonucleic acid can be degraded by other than hydrolytic reactions (72, 73), an analogous reaction for conserving the energy of the peptide bond might be operative in this organism. Walter has reviewed a large body of circumstantial evidence which is consistent with the existence of such a reaction. He concludes that:

> I have shown how a variety of experimental results dealing with aspects of protein catabolism can all be interpreted uniformly by postulating amino-acid derivatives as intermediates in the re-utilization of amino-acids derived from one protein for the synthesis of another. . . . It is possible that this amino-acid derivative is similar to, or identical with, one of the intermediates known to participate in the anabolic process (74).

To speculate even further, it is possible that the intermediates conceived by Walter are formed by the following scheme, in which "AA" stands for amino acid:

$$\text{I} \qquad AA' - AA'' - (AA)_n + X \longleftrightarrow AA' + X\text{-}AA'' - (AA)_n$$

$$\text{II} \qquad X\text{-}AA'' - (AA)_n \longleftrightarrow X\text{-}AA'' + (AA)_n$$

$$\text{III} \qquad (AA)_{n+2} + X \longleftrightarrow X\text{-}AA'' + AA' + (AA)_n$$

In this hypothetical reaction two amino acids are cleaved from a large peptide in separate steps. One of the amino acids is, however, retained in an "activated" complex. If the receiving group X were an sRNA molecule or some compound interchangeable with sRNA, some of the free energy used to synthesize the original two peptide bonds would be conserved for subsequent protein synthesis.

In reaction III two peptide bonds have simply been replaced by one "high energy" bond. In order to establish the thermodynamic equilibrium of the reaction it is, therefore, necessary to know the standard free energy of formation of these two types of bonds. From work with small model peptides it has been estimated that the standard free energy of formation of an "internal peptide" bond of a protein is about 0.4 kcal/mole (75). The free energy necessary for the formation of a tetrapeptide from dipeptides is about 2.0 kcal/mole, and that for the formation of a dipeptide from free amino acids is about 4.0 kcal/mole (76). It is possible to estimate the average standard free energy of formation of a peptide bond in a protein from the above figures by imagining the hydrolysis of the protein to proceed through tetrapeptides and dipeptides to amino acids. This calculation yields a value of 2.6 kcal/mole. Therefore, the two peptide bonds which have been split in reaction III have a total standard free energy of formation of about 5.2 kcal/mole.

The free energy exchange in the formation of the bond between sRNA and an amino acid with the participation of ATP can be estimated to be about -700 cal/mole from the published equilibrium constants for this reaction (77, 78). Since the hydrolysis of ATP to AMP and pyrophosphate proceeds with a free energy change of -8.6 kcal/mole (79), ΔF° for the formation of the sRNA amino-acid bond can be estimated to be 7.9 kcal/mole. Thus the free energy change in reaction III is 7.9 - 5.2 kcal/mole or 2.7 kcal/mole. Since even the most careful estimates of free energy changes are thought to be accurate to only 1-2 kcal/mole (79), this calculation suggests that a mechanism for condensing the energy of two peptide bonds could provide an intermediate with sufficient energy to be effective in protein synthesis.

A further restriction on the postulated mechanism is that reaction I itself cannot be an insurmountable energy barrier. Studies of amino acyl sRNA and peptidyl sRNA have shown that the latter compound is 6-10 times more resistant to hydrolysis than the former (80, 81). This observation suggests that the free energy of formation of the peptidyl sRNA bond may be low enough to favor reaction I. In

analogy with the results obtained from studies of model peptides, reaction I would be expected have a more negative ΔF° than reaction II, because a free amino acid is released in reaction I whereas in reaction II the carboxyl group of the released amino acid is bound. In similar reactions, where only peptide bonds are involved, the reaction in which the free amino acid is released has the more negative ΔF° (76). Thus two lines of reasoning both suggest that more than half of the energy for driving reaction III is derived from reaction I. Therefore, there is probably not enough energy available in reaction II to support a second transfer reaction to sRNA or a compound exchangeable with sRNA.

The results obtained by Borek et al (18) with 0^{18} indicate that this mechanism is not operative in microorganisms. On the other hand, the data from higher organisms which have been reviewed by Walter (74) can be rationalized to fit the mechanism. The possibility that such a reaction exists suggests that experiments should be performed in higher organisms with 0^{18} similar to those performed in microorganisms. The use of this isotope, as opposed to C^{14} and N^{15} which have so far been used, makes it possible to measure directly the extent to which protein turnover occurs through free amino acid intermediates.

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III. A New Method for the Detection of Deoxyribonucleases and its Application to Studies of Drosophila melanogaster

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A. Introduction

1. History and Nomenclature

Since 1903 it has been known that organisms can produce substances which will degrade deoxyribonucleic acid (1, 2, 3). These and later studies, however, were limited by poor assay methods and by a lack of adequate substrate with which to perform the assays. In fact, the rapid increase in our knowledge of nucleases and nucleic acids has been partially due to the close interdependence between the two areas of study. This relationship was clearly demonstrated by the discovery of Avery, McCleod, and McCarty (4) that deoxyribonucleic acid is the substance responsible for transformation. Central to these classic experiments was the use of crude preparations of deoxyribonuclease to substantiate that the active principle was indeed DNA. This demonstration of the tremendous biological importance of DNA simultaneously stimulated an increased interest in both DNA and the enzymes which attack it. Shortly thereafter McCarty (5, 6) and others (7, 8) independently purified a DNase from beef pancreas, thereby initiating the present era of DNase studies.

Although the enzyme obtained from pancreas is still the best purified and understood of the DNases (9), a great variety of DNases with different specificities and from other sources are continually being isolated. Up to the present most of these studies have been directed toward finding enzymes which can be used as tools for the study of DNA itself. Comparatively little attention has been focused on the problems of the function and distribution of the enzymes in the organisms from which they are isolated. As the initial descriptive stage of these studies progresses, however, more attention is being turned in this direction (10). The purpose of the work described here has been to provide a method and an approach to facilitate studies of the function and distribution of DNases.

According to Schmidt and Laskowski (11) enzymes which degrade DNA belong to the nucleophosphodiesterase class according to the

following scheme:

Nucleophosphodiesterases	(nucleases)
Deoxyribonucleases	(DNases)
Ribonucleases	(RNases)
Exonucleases	(phosphodiesterases)

A common or alternative name is given on the right. This system is historically derived and admittedly inadequate. Nevertheless, it is the most commonly used classification, because the present rapid pace of enzyme discoveries prevents the final selection of a consistent classification system (11). For the purposes of this discussion it will be convenient to refer to any enzyme degrading DNA as a DNase. This definition includes enzymes which are sometimes classified as exonucleases, endonucleases, or phosphodiesterases.

2. Diversity of Action and Distribution

An examination of the recent reviews (9-15) reveals the impressive variety of DNases which have been isolated to date. Enzymes have been identified which display individual pH optimums between pH 4.5 and pH 10 (11, 16). Some enzymes are most active on native DNA while others are maximally active on denatured DNA. The modes of action vary from exo- to endo-nucleolytic attack. In most cases enzymes with properties intermediate between these extremes have also been described. The ionic requirements for maximum activity are usually complex and require careful control. In addition, a wide variety of inorganic and specific organic inhibitors have been found.

This tremendous diversity of enzymatic activity immediately raises the question of the distribution of these enzymes within species, individual organisms, tissues, and cells. Such information should provide some clues to the functional significance of these enzymes. Kurnick (13) has amply documented the fact that DNases have been found in all animals, plants, and bacteria in which they have been sought. Likewise activity has been demonstrated in many secretions including serum, bacterial filtrates, pancreatic juice, bile, and snake venoms. DNase activity has also

been demonstrated in all the investigated cell types with the possible exception of the human leucocyte and its precursors (13). Negative results, however, are subject to serious question both because of the widespread distribution of inhibitors and because of the great diversity of conditions required by enzymes of this class for maximum activity (11). As an example, Feinstein and Hagen (17) found no DNase activity in mouse kidney or intestine extracts unless they added albumin. Apparently this protein protects the DNase against denaturation while the inhibitors are being destroyed. In conclusion, the problem of distribution is no longer a question of whether DNase activity is present in an organism but rather how many and what type of activities are present. This latter problem is just beginning to receive serious consideration.

Bacteria have been the subject of the most systematic attempts to define the total DNase spectrum in a single cell or organism. At the present time four distinctive enzymatic activities have been identified both in group A Streptococci (18-22) and in Escherichia coli (14, 23-28). The four DNases of each of these organisms have been at least partially purified, and each enzyme has been shown to differ from the other three DNases of the organism by enzymatic or immunological tests. A recent report indicates that the pleuropneumonia-like organisms also produce at least one DNase (29). Within the past three years evidence has been rapidly accumulating which shows that even the viruses can be responsible for the production of a new DNase in infected cells. This has been demonstrated for the coli phages (16, 30-36) and similar evidence is accumulating for some of the animal viruses as well (37-39). Thus the simplest forms of life with which we are familiar all appear to produce one or more DNases.

After the first investigations in mammals it was thought that the enzyme with a neutral pH optimum (DNase I) was restricted to the pancreas and that the enzyme which is active at acid pH (DNase II) was more regularly distributed throughout the other tissues (40).

At that time, however, the many difficulties of assaying this broad class of enzymes in crude homogenates were not known. Although most of the comparative studies which have been carried out since then have also been performed with crude homogenates, many of these problems have been overcome. As a result, it is now fairly certain that most mammalian tissues contain enzymes which fall into both the DNase I and the DNase II classes (41-43). In these cases DNase II activity is usually predominant.

In order to determine the number and nature of the activities which were being assayed together at a given pH, the next stage of this study has centered around the isolation of pure enzymes. This work is being carried out on several different species of mammals in which enzymes of similar function might be expected to differ slightly in their properties. Therefore, only broad classes of DNases will be considered here in an effort to make a minimum estimate of the number of different DNase functions in mammals. The discovery of DNase II in spleen (44) was the first evidence that mammalian tissues produce a DNase different from that of DNase I. In most cases there is still no clear evidence to indicate whether or not similar activities observed in other tissues are in fact due to the same enzyme (9). Experiments which have indicated that crystalline DNase I itself is composed of two separate DNase activities (45, 46) suggest that more than one enzyme of this general class may be present. Razzell (10) has reviewed the procedures for isolating two distinctive phosphodiesterases from mammals. Both of these exonucleases have been identified in all tissues in which they have been sought. A new class of enzymes has recently been found in lamb brain by Healy et al (47) and in rat liver by Burdon et al (48). After partial purification both activities appear to be similar to each other and yet different from that of any previously known enzyme. This type of enzyme, therefore, represents the fifth class of DNA-degrading enzymes that has

been found in mammalian tissues. As more effort is expended in this direction, it seems likely that still others will be found. Much less information is available from other phyla, although Georgatsos et al (49) has recently reported a preliminary survey in which two to four DNases were tentatively identified in each of several marine organisms.

3. Function

Studies in biochemical genetics have shown that the most minute alterations in the DNA of a cell can have profound consequences for the descendants of that cell (50). DNases might, therefore, be expected to have functions which are basic to the genetic manifestations of an organism. No such correlation has yet been made, and in fact, almost nothing is known about the function of most of the DNases which have been isolated. The presence of DNases in bile and pancreatic juice (13) clearly implicates a digestive function for these enzymes. In addition, DNase II has been found to be localized in the lysosomes of most animal cells (51). Although the complete roll of the lysosomes in the cell has not been rigorously established, it almost certainly includes a digestive function (52).

The great diversity among the DNases which have been isolated from bacteria implies that these enzymes may have important functions in cellular DNA metabolism other than that of digestion. Lehman (14) has suggested several possibilities for DNase function in bacteria and has indicated possible control mechanisms for protecting the genome of the bacterium from destructive action by these enzymes. One of his most interesting points is that DNases may be involved in genetic recombination. Much of the direct evidence for this view comes from the work of Meselson et al (53-56) who have shown that genetic recombination in the bacteriophage λ occurs by a mechanism of breaking and joining of DNA strands. In a recent conclusion from this work Meselson states:

> Whatever the details of the mechanism of recombination, it appears that at least four different reactions are involved: breaking DNA strands, excising portions of strands, resynthesizing them, and joining them to other strands in phosphodiester linkage. Almost certainly these processes are catalyzed by specific enzymes. As yet no such enzymes have been positively identified, but this is likely to be one of the main directions along which further understanding of recombination is to be gained (54).

Of these four reactions the first two clearly suggest the existence of enzymes which could be detected as DNases. A similar mechanism and set of enzymes have also been implicated in the biological repair of bacteria DNA which has been damaged by ultraviolet radiation (57-59). In addition, genetic recombination and transformation in bacteria have been shown to occur by the physical incorporation of one chromosome into another (60-62). These processes represent the most recent and exciting possibilities for identifying DNase functions.

A knowledge of the number and fluctuation of enzyme activities within an organism provides the basic groundwork for investigations of such problems. This information may also provide a foundation for any developmental and genetic studies involving these enzymes. The investigations described here provide a much more rapid analytical method than has previously been available for attacking these problems. In addition, some evidence has been obtained which indicates that higher organisms may produce a greater variety of DNases than has so far been identified.

B. A New Method for DNase Detection*

1. Introduction to the Method

Most of the available DNase assays are very time consuming when they are used to scan the nuclease activities of large numbers of organism, mutants, developmental stages, et cetera. For such studies to be done correctly the enzymes must usually be separated from each other by laborious column chromatographic procedures (31-33). To facilitate these studies a method has been developed for detecting DNases after their separation by disc electrophoresis.

Disc electrophoresis and its predecessor starch gel electrophoresis have both been extremely valuable tools for the separation and identification of enzymes and their multiple molecular forms (63, 64). Although a large number of enzyme systems have been studied with these techniques, the range of the investigations has been limited by the fact that most methods for localizing the enzymes in the gel require low molecular weight substrates. One exception to this limitation is a recent adaptation of the agar gel plate technique of Jeffries. The procedure of Jeffries et al (65) consists of plating a high dilution of microorganisms on an agar medium containing DNA. As the individual colonies secrete DNase, the DNA in the surrounding gel is degraded. Those colonies producing DNase are detected by precipitating the remaining DNA with hydrochloric acid and observing the clear areas of degraded DNA. In analogous experiments DNase was detected after electrophoresis in agar gel by placing the gel in contact with a second gel containing DNA and incubating them together (66-68). Daoust has applied this same concept to histochemical detection of DNases in tissue sections (69).

The method described here is more sensitive and gives better resolution than the earlier indirect detection methods. The procedure differs from previous methods in that the substrate is immobilized in

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* Parts of this section have been taken from a manuscript submitted for publication. The style and tense, therefore, conform to the requirements imposed on the manuscript.

the gel during polymerization and is present in the gel during all subsequent steps. The method, therefore, requires that care be taken to prevent enzymatic activity during the initial electrophoretic separation. With this accomplished, the enzyme is incubated at its final resting position in the gel with the substrate found at that position. The remaining or modified substrate is then revealed by staining, and the enzyme positions are identified by noting the discontinuities in the substrate background.

This same concept has recently been used to study amylases, another class of enzymes which attacks a high molecular weight substrate (70, 71). In the first report all of the electrophoretic supporting medium is substrate, while in the second starch is immobilized in acrylamide as is done here with DNA. Such applications indicate that this approach can extend the application of gel electrophoresis to still other important enzyme classes.

2. Materials

Unless otherwise indicated,the materials and methods used here are those originally described by Ornstein and Davis (72, 73). These authors have also recently published a slightly modified procedure (74, 75).

a. Apparatus and Instruments.

Electrophoresis columns. Pyrex tubing, i. d. 5 mm cut in 8. 5 cm lengths, and lightly fire polished.

Elution columns. Soft glass, i. d. 6 mm in 7. 0 cm lengths, lightly fire polished, and constricted at one end. When these cylinders are constructed by removing both ends from Bacteriological Disposable Capillary Pipettes (Aloe Scientific), they fit the same buffer reservoirs used for electrophoresis.

Buffer reservoirs. Upper reservoirs are constructed with eight electrical grommets per polyethylene refrigerator dish. To eliminate leakage problems the holes for the grommets must be drilled very slowly with a sharp metal drill while the polyethylene is supported against a wooden block. Both reservoirs are fitted with

platinum electrodes (73) which are wound around glass supporting rods.

Polymerization racks. To facilitate handling large numbers of gels at one time, gray base caps for holding the cylinders during polymerization (Canalco) are glued onto a board with rubber cement.

Power supply. Duostat, Model RD-2 (Beckman Instruments).

Camera. Polaroid MP-3 Industrial View Land Camera (Polaroid Corporation) fitted for transmitted fluorescent illumination.

Densitometer. Automatic Recording Microdensitometer Mark III Model (Joyce, Loebl & Co.)

Cuvet for densitometry. Glass stoppered quartz cuvet 1 x 1 x 5.8 cm (Pyrocell Manufacturing Co.)

Compensating polar planimeter. Model 4236M, (Keuffel & Esser Co.)

b. Reagents.

Stock solutions (see Table 1). The DNA solutions are prepared by dissolving highly polymerized calf thymus DNA (sodium salt, type I, Sigma Chemical Co.) in the appropriate solvent at 4° C over 0.5 ml of $CHCl_3$. The solutions are stirred once each day with a glass rod to break up the clumps. All solutions except (I) are stable at 4° C for at least one month.

Electrophoresis stock buffer. Used at 0.1 strength. Tris 6.0 g, glycine 28.8 g, H_2O to 1 liter.

Elution buffers. Although the buffers are used at 4° C, the pH measurements are made at room temperature.

Upper reservoir (anodic) buffer. Electrophoresis stock buffer diluted 5-fold, 0.001 M EDTA, titrated to pH 9.4 with 1.0 N NaOH.

Lower reservoir (cathodic) buffer. Electrophoresis stock buffer diluted 10-fold, 0.001 M EDTA, titrated to pH 8.5 with 0.1 N NaOH.

Gel soaking buffer. Electrophoresis stock buffer diluted 20-fold, 0.001 M EDTA, titrated to pH 9.2 with 1.0 N NaOH.

TABLE 1

Stock Solutions - Modified from Davis (73)

(A)			(F)		
1 N HCl		48 ml	Solution (A)		25 ml
Tris		36.6 g	H_2O		75 ml
TEMED		0.23 ml			
H_2O	to	100 ml (pH 8.9)			
(B)			(G)		
1 N HCl		48 ml	DNA		50 ml
Tris		5.98 g	Solution (F)		25 ml
H_2O	to	100 ml	$CHCl_3$		0.5 ml
(C)			(H)		
Acrylamide		30.0 g	DNA		50 mg
BIS		0.8 g	H_2O		25 ml
H_2O	to	100 ml	$CHCl_3$		0.5 ml
(D)			(I)		
Acrylamide		10.0 g	Ammonium Persulfate		0.28 g
BIS		2.5 g	H_2O		100 ml
H_2O	to	100 ml	(Discard after one week)		
(E)			(J)		
Riboflavin		4.0 mg	Sucrose		30 g
H_2O	to	100 ml	H_2O		70 ml
			BSA	at	2 mg/ml (optional)

Native-DNA stain (76). Dissolve 2.5 g of methyl green (77% dye content, C.I. No. 42590, National Aniline Div., Allied Chemical Corp.) in 1 liter of 0.2 M acetate buffer at pH 4.0. The solution is extracted vigorously with 250 ml portions of $CHCl_3$ until all the contaminating crystal violet has been removed from the aqueous phase. When stored at 4° C in the dark, the solution is stable for many months and after repeated use. It is discarded when the pH falls below 4.5.

Denatured-DNA stain, modified from Kurnick (77). A 2% solution of pyronin B (40% dye content, C.I. No. 45010, National Aniline Division, Allied Chemical Corp.) is prepared in water and extracted five times with 200 ml portions of $CHCl_3$. Any emulsions are allowed to separate between extractions. The aqueous phase is stored at 4° C in the dark and diluted 10-fold with 0.2 M acetate buffer (pH 4.5) before use. The diluted stain is discarded after one use.

DNase I. To test the method, crystallized and lyophilized DNase I from bovine pancreas (Lot No. 103B-0180, one Kurnick methyl green unit per mg, Sigma Chemical Co.) was dissolved in buffer (1.) at a concentration of 0.1 mg/ml. This solution was then diluted into buffer (2.) just before use to give a final concentration of 10^{-7} to 10^{-8} g/ml.

Buffer (1.) 0.1% (W/V) BSA, 0.25 M ammonium sulfate, in 0.05 M Tris, pH 7.0.

Buffer (2.) 15% (W/V) sucrose, 0.05% (W/V) BSA, 0.25 M ammonium sulfate, in electrophoresis buffer.

DNase II. Amorphous powder from bovine spleen, 500 units/mg (Lot No. 73B-1960, Sigma Chemical Co.)

Drosophila melanogaster homogenates. The animals were cultured and collected as described in the materials section of Part II. In the DNase studies, however, pupal ages are recorded from the time the pupae floated on water (78), and fly ages are recorded from the time of emergence. Homogenization was carried out in a motor

driven cone glass grinder which was supported in an ice bath. Grinding was continued for two minutes with a 75 mg/ml suspension of animals in electrophoresis buffer. After the suspension had been ground, it was centrifuged in 2 ml spinco tubes at 60,000 g and 4° C for 15 minutes. The supernatant was mixed 1:1 with stock solution (J) and 100 λ of the mixture were subjected to electrophoresis.

3. Methods

a. General. Fig. 1 is a flow diagram representing the basic sequence of operations employed for detecting enzymes with DNase activity after their separation in polyacrylamide gel containing DNA. After DNA has been trapped in the acrylamide gel matrix, the gel is utilized for separation of the enzymes by the standard disc electrophoresis procedures (73). The gel containing the separated enzymes is then incubated under conditions appropriate for DNase activity. Incubation is followed by electrophoretic removal of the proteins and the DNA fragments which have been freed from the matrix by enzymatic action. Both the incubation and elution stages are preceded by a presoaking step to establish the appropriate buffering conditions in the gel. Finally, the residual DNA is stained, and the resulting nuclease pattern is recorded by photography or densitometry.

b. Preparation of the Gel. Gels containing either native DNA or denatured DNA are prepared in a similar manner with one exception. Just prior to mixing the stock solutions for polymerization, the DNA containing solutions must be pretreated and in different fashions for the two substrates. Native substrate is pretreated by degassing solution (G) over a period of about 20 minutes as it warms to room temperature under a moderately strong vacuum provided by a water pump. This operation is necessary to prevent bubble formation in the final gel. Denatured substrate is prepared by heating solution (H) in a boiling water bath for 15 minutes. Heating is followed by quick cooling in an ice bath.

Room temperature solutions are used to form the mixtures prescribed in Table 2. Mixing is done in 40 ml conical centrifuge tubes to facilitate the removal of air bubbles by centrifugation

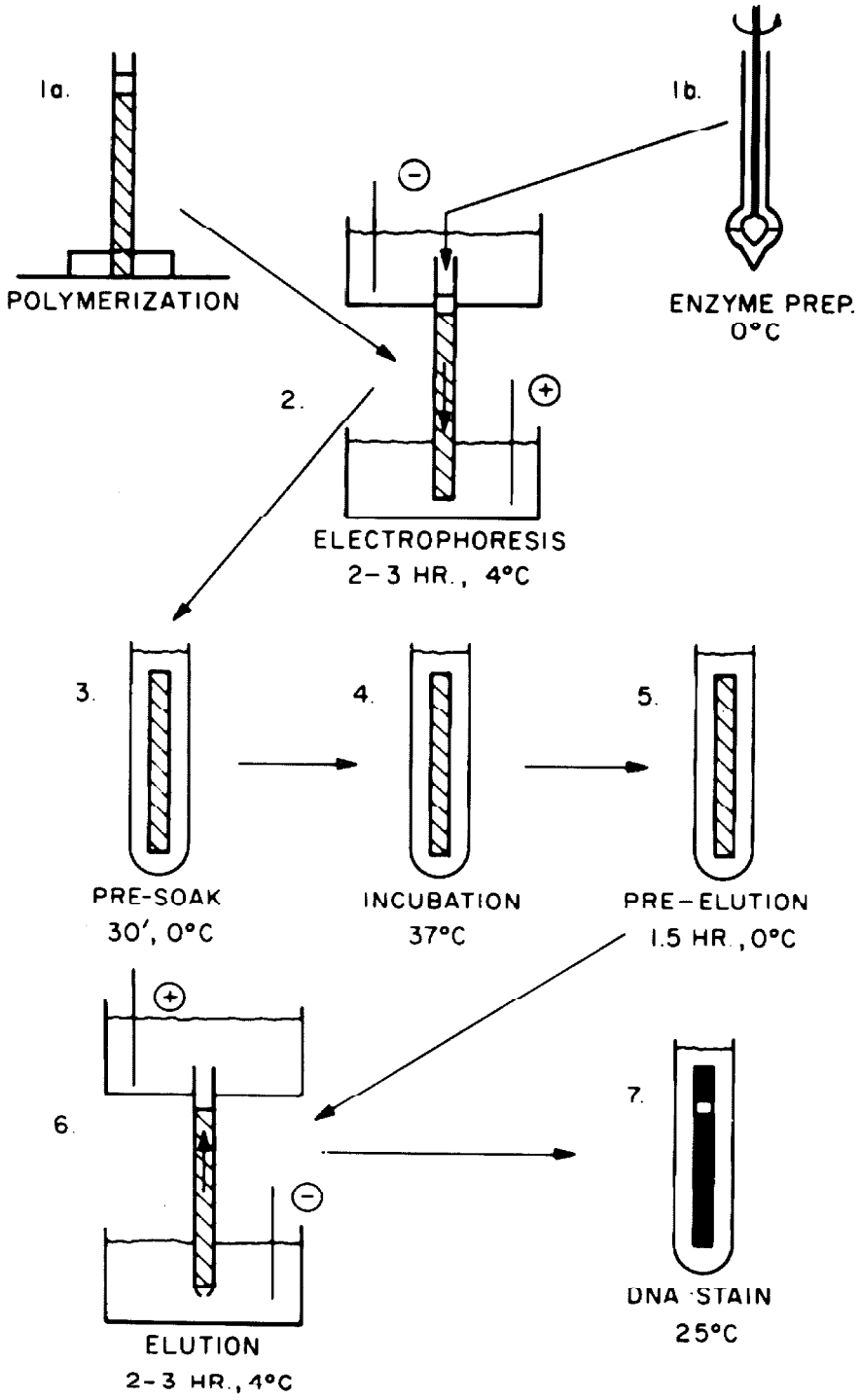


Fig. 1 Schematic representation of the steps involved in detecting DNase activity following electrophoresis of enzymes into an acrylamide gel containing trapped DNA.

TABLE 2

Formulas for Final Gel Solutions

Gels containing native DNA as substrate (1.0 mg DNA/ml gel*)	Gels containing denatured DNA as substrate (0.75 mg DNA/ml gel)	Large-pore spacer gel
2 parts (G)	3 parts (H)	1 part (B)
1 part (C)	1 part (A)	2 parts (D)
1 part (I)	2 parts (C)	1 part (E)
	2 parts (I)	4 parts H_2O

* For good densitometry tracings the gels containing native substrate are made up at a concentration of 0.67 mg of DNA/ml of gel by appropriately diluting (G) with (F) before final mixing.

immediately after mixing. Acid-washed glass electrophoresis columns are filled to a height of 6.2 cm with the gel solution which is in turn overlayered with water. The original rapid water layering technique of Davis (73) can be used conveniently here because of the high viscosity of the DNA solutions.

After polymerization of the DNA-containing gel, 0.2 ml of the spacer-gel solution containing 1 μliter of TEMED/ml is layered on the DNA gel with a 2 ml syringe fitted with a polyethylene delivery tube. This solution is overlayered with water and photopolymerized according to the original instructions of Davis (73).

c. Electrophoresis. All operations between gel formation and staining, with the exception of incubation, are carried out at either 0° C or 4° C. The gels are, therefore, chilled for 15 minutes at 4° C before use. An enzyme preparation of 25 to 100 μliters can be conveniently layered under the buffer solution and over the spacer gel with lambda pipettes (79). Before it is layered, the enzyme solution is generally diluted 1 : 1 with stock solution (J). The BSA in solution (J), in combination with excess brom phenol blue in the upper (cathodic) reservoir, provides a convenient internal marker when it is necessary to run the fast moving brom phenol blue dye-marker off the lower end of the gel. No DNase activity has been detected in the BSA preparation itself. Electrophoresis is performed at 1.5 ma per gel for two to three hours. Under these conditions higher currents should be avoided, because they result in heating which in turn increases the possibility of enzymatic activity.

d. Incubation. At the completion of electrophoresis the gels are removed from the glass columns by forcing a stream of water between the gel and the glass (79). A 10 ml syringe fitted with a blunted 25 guage needle is suitable for this purpose. Before the gels are placed in the 0° C incubation buffer, they are stabbed completely through with a No. 11 scalpel blade at either of the two visible dye markers, and

the spacer gel is discarded. All operations between electrophoresis and elution are performed with each gel remaining in a single test tube (25 ml). Solutions are quickly changed by inverting a rack of tubes over a wire screen.

After the 30 minute preincubation at 0° C the incubation buffer is replaced with 25 ml of fresh cold incubation buffer. These buffers should be about 0.1 M and well buffered in order to overcome the buffer already present in the gel (see discussion). Incubation is commenced by transferring the tubes from an ice bath to a rapidly circulating water bath at 37° C. About five minutes is required for the buffer surrounding the gel to reach 37° C.

e. Elution. Prior to electrophoretic elution the gels are soaked at 0° C for 1.5 hours in the "Gel Soaking Buffer." Halfway through this soaking the buffer is replaced with fresh cold buffer. The gels are then placed with the origin end up in the elution columns. Air bubbles are removed from around the gels after addition of the buffers to the reservoirs by forcing the gels up and down the columns with the aid of a rubber bulb held at the constricted end of the column. Electrophoretic elution is carried out for 2.5 hours at 3 ma/gel and 4° C with the electrodes reversed from the original orientation used for electrophoresis.

f. Staining and Removal of Excess Stain. These and subsequent operations are carried out at room temperature. Gels which contain native DNA as a substrate are stained directly in the methyl green solution for one hour or more. The excess stain is removed by soaking the gels in several changes of 0.2 M acetate buffer (pH 4.0) over a period of one to three days.

Those gels containing denatured DNA must first be soaked in 0.2 M acetate (pH 4.5) for one or more hours before they can be stained. They are then stained in the diluted pyronin B solution for one day. Afterwards the excess stain is removed by again soaking the gels in pH 4.5 buffer. For both stains the solution should be stirred several times while the gel is in the stain in order

to insure an evenly stained background.

g. Photography of Stained Gels. Prior to photography or densitometry the portion of the gel below the scalpel slash is usually broken off and discarded. The end of the gel then serves as an internal mobility marker in the recording. Polaroid 55/PN film 4 x 5 inches is used to photograph gels which have been covered with buffer in an open flat-bottomed glass dish. Movement of the gels in the buffer is prevented by surrounding them with anchors made from a glass rod. The camera is positioned at the shortest distance which still allows proper focusing with a lens of f = 127 mm. Transmitted fluorescent light is the primary source of illumination. By placing a No. 25 A Kodak wratten gelatin filter in front of the camera lens, it is possible to obtain pictures of the methyl green-stained gels which are far clearer than direct observation of the gel. Photographs of pyronin B-stained gels, for which no satisfactory filter has been found, fail to show quite as much detail as can be seen directly with the eye.

h. Densitometry of Stained Gels. Scanning is performed with the Joyce Loebl Microdensitometer as described in Section II, B, 2, h of this thesis. Methyl green-stained gels are scanned using a Joyce Loebl red filter in the light path between the gel and the photocell. A 1.0 OD neutral filter is used with pyronin stained gels. Wedges with optical density ranges of 0.8 and 0.4 are used respectively for scanning methyl green-and pyronin-stained gels. Mobility values can be measured from the tracings relative to the dye marker at which the gel was broken off.

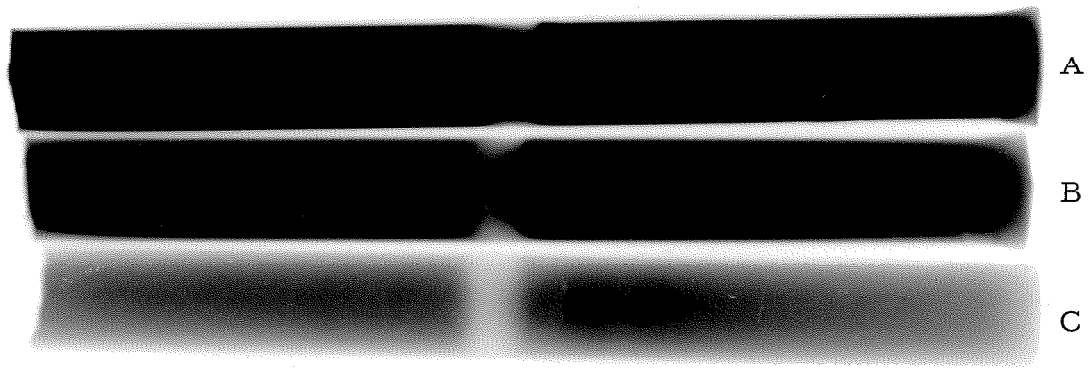
4. Characteristics of the Method

The potentialities of this technique are most clearly demonstrated by its application to a simple test system. Most of the tests reported here, therefore, involve a study of the highly purified enzyme DNase I. This presentation also includes some data obtained from bacterial homogenates which demonstrate a type of application different from that shown by the Drosophila data in Section III, C.

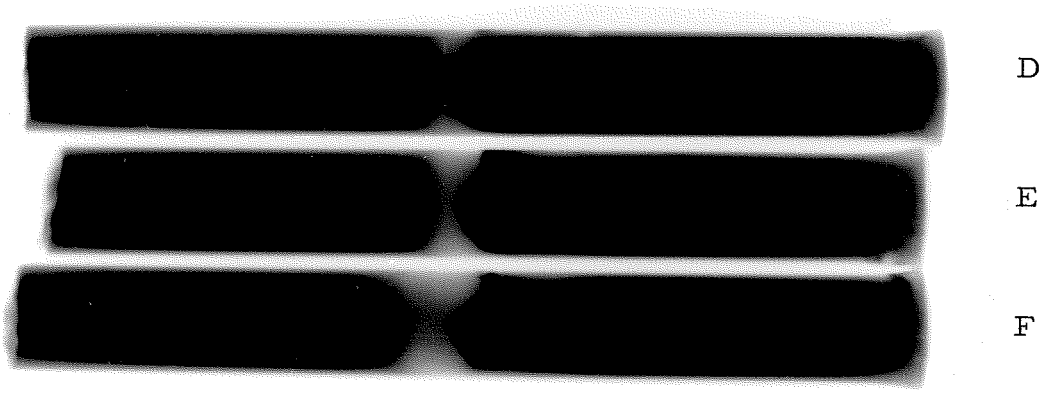
a. DNase I. The two basic methods for recording an experiment are illustrated in Figs. 2 and 3. Densitometry tracings of gels containing either native or denatured DNA are presented in Fig. 3 together with recordings of parallel controls. The only difference between the experiments reported in Figs. 3 and 4 is that the gel used in Fig. 4 had a 10-fold higher enzyme concentration. Although the controls in Fig. 3 did contain DNase I, their densitometry tracings are identical to those of control gels which did not contain any enzyme.

The fact that the controls show no indication of enzymatic activity demonstrates that all such activity occurs during the 37° C incubation. This means that the enzyme does not degrade the DNA through which it passes during electrophoresis at 4° C. These backgrounds are constant enough to permit quantitative measurements of the areas under the peaks obtained from incubated gels. An artifact which is always present at the origin of gels containing native DNA (Fig. 3) can probably be explained by the observations of Davis (75) on the process of polymerization at the origin. He has shown that a thin layer of gel at this position seems to be more porous than the bulk of the gel. Therefore, some of the DNA may be free to move out of the gel leaving a small bleached area at this position. This explanation is substantiated by the fact that the artifact is magnified when acrylamide concentrations less than 7.5% are used to trap DNA. The slope of the background in gels containing denatured DNA may indicate a slight movement of the DNA in the gel during elution. This could be explained by the reduced radius of gyration of the denatured substrate as compared to that of the native material.

Earlier work (45, 46) which suggested that DNase I can be separated electrophoretically into more than one active component has been confirmed here by the data in Fig. 4. This densitometry tracing shows that several subordinate peaks appear when DNase I is assayed at high concentrations with this technique. Furthermore, when large amounts of DNase I are conducted into gels containing no DNA, protein staining reveals one major and two minor components.



Experiment #1



Experiment #2

Fig. 2 Polaroid photographs of DNase I patterns in gels containing native DNA.

Experiment #1 -- Incubation was carried out for one hour at 37° C in 0.01 M Tris, 0.005 M $MgCl_2$, and at pH 7.0. Gels A, B, and C contained DNA at concentrations of 1.00, 0.67, and 0.33 mg/ml respectively. Each gel contained 10^{-9} g DNase I.

Experiment #2 -- 10^{-9} g of DNase I was conducted into acrylamide gels containing 0.67 mg of native DNA/ml. Incubation was carried out in the same buffer used in Experiment #1. Gels D, E, and F were incubated at 37° C for 0.5, 1.0, and 2.0 hours respectively.

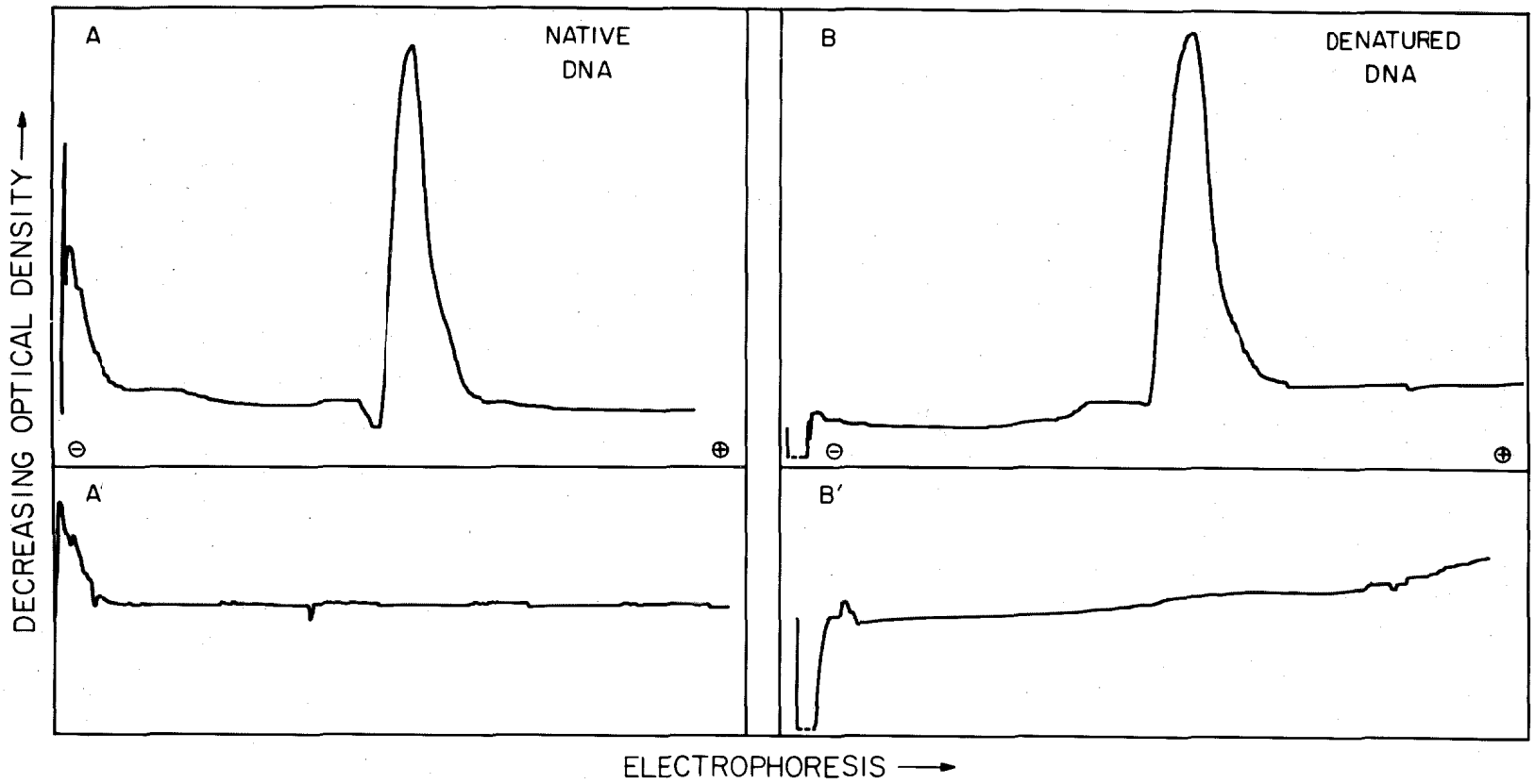


Fig. 3 Densitometry tracings of DNase I patterns in gels containing DNA. Electrode orientations are indicated by + and -. Gel A which contained 0.5×10^{-9} g of DNase I and 0.67 mg/ml of native DNA was incubated for one half-hour. The corresponding control A' was placed in elution buffer at 0° C after the 35 minute preincubation soak and, therefore, received no 37° C incubation. Gel B which contained 3×10^{-9} g of DNase I and 0.75 mg of denatured DNA/ml was incubated for 3 hours. The control B' contained 5×10^{-9} g of DNase I and was treated like A'.

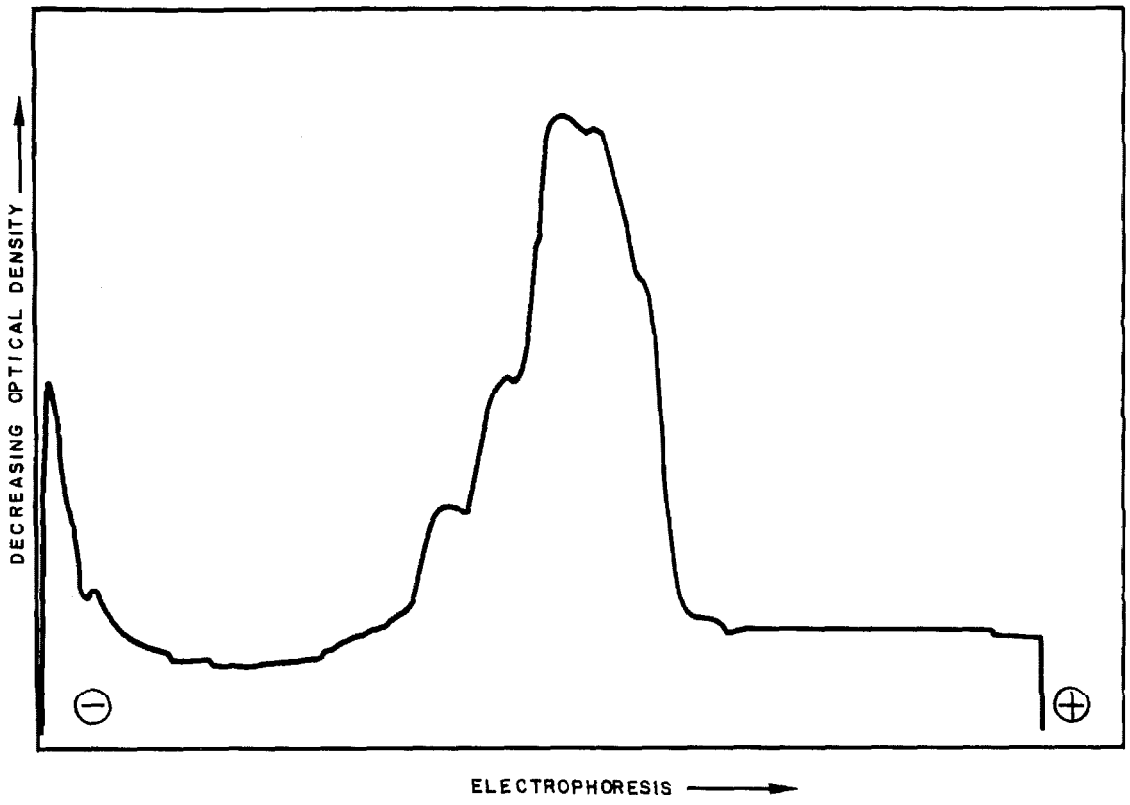


Fig. 4 Enzyme distribution at high DNase I concentration. A densitometry tracing was made from a gel containing 0.67 mg of native DNA/ml of gel and 10^{-8} g of DNase I. Incubation was carried out for 0.5 hour at 37°C in 0.1 M Tris, 0.005 M $MgCl_2$, and at pH 7.0.

As Kaplan (80) has clearly pointed out, however, this type of information by itself is not sufficient to establish whether these components represent the same or different enzymes. Other approaches are required to determine how many peaks represent complexes, aggregates, or isozymes of the same protein. The need for such caution is even greater when complex mixtures of nucleases are being analyzed.

Figs. 5 and 6 were constructed from data obtained by measuring the areas under curves similar to those in Fig. 3. Area measurements with an error of less than one per cent were obtained with a planimeter. Figs. 5 and 6 first of all provide an indication of the sensitivity of the system. Although 0.25 nanograms is the least amount of DNase I yet to be applied to such a gel, it is clear that the sensitivity lies well below this value and that it could be further increased by increasing the time of incubation and decreasing the DNA concentration. Secondly, the data in Figs. 5 and 6 give an estimate of the reproducibility of the method. The average of the percentage errors (coefficient of variation) of seven points (19 gels) above 0.25 nanograms of DNase I and one half-hour incubation is 3.5%. Since the reproducibility of the combined densitometry and planimetry steps is 2.5%, the remainder of the error can be accounted for by pipetting and incubation time errors. Below these concentration and time limits the variation has been considerably higher. This error analysis applies only to data within one experiment. The larger variation which has been encountered between experiments is probably attributable to the instability of the enzyme in the dilute solutions in which it is applied to the gels (81).

The kinetic data presented in Figs. 2 and 6 provide strong support for the contention that the observed gel bleaching is due to a catalyzed reaction rather than to an inhibition of the DNA staining. That the "hole" in the DNA background is due to DNase I activity is further indicated by the failure of the "hole" to appear when a gel containing the enzyme is stained directly after electrophoresis in a solution containing EDTA. A corresponding gel

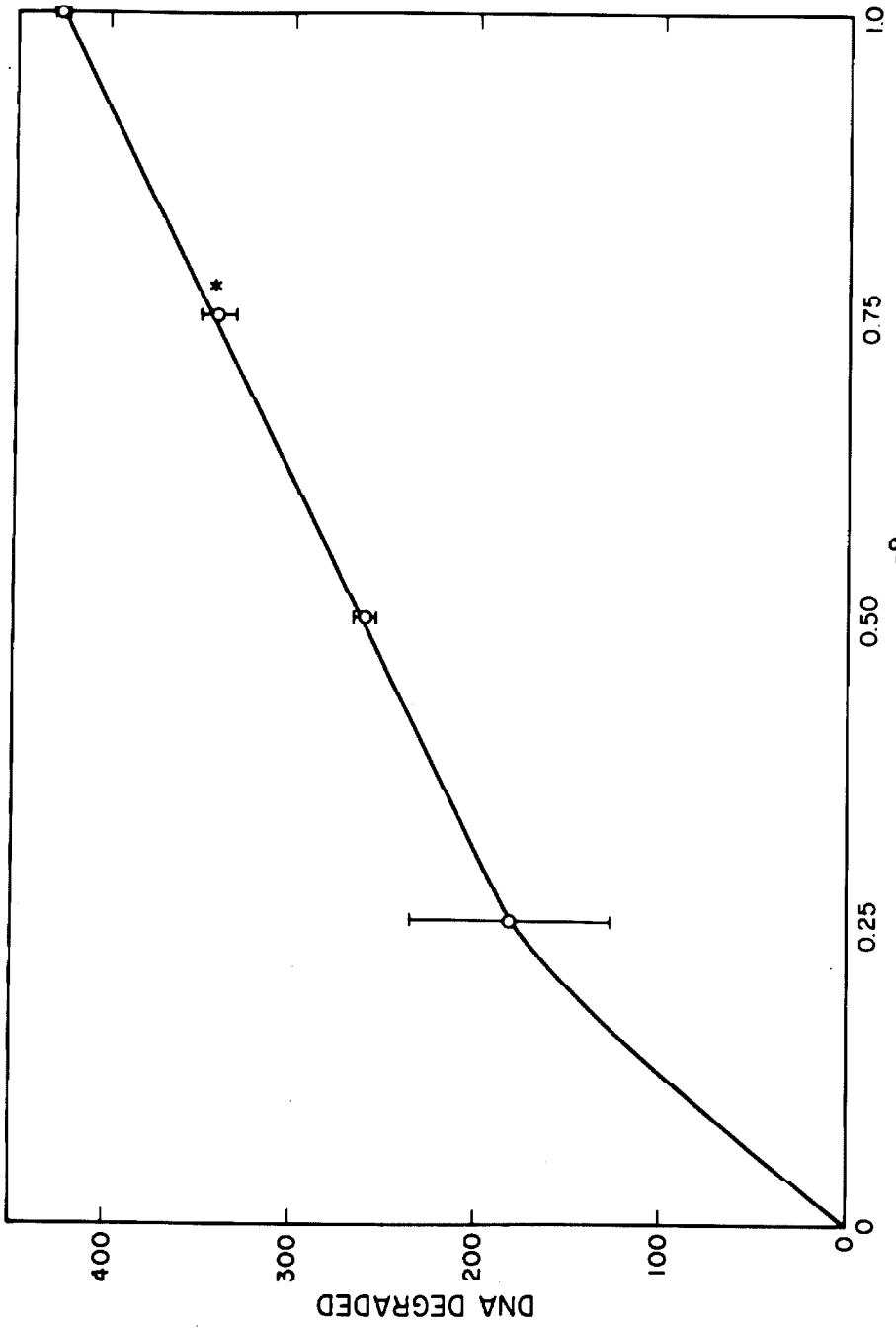


Fig. 5 Native DNA degraded by DNase I during one hour incubation at 37° C. The ordinate represents relative areas measured from the peaks of densitometry tracings. Each point represents the average value obtained from three separate gels unless otherwise indicated. Error range is one standard deviation.
*Average from two gels.

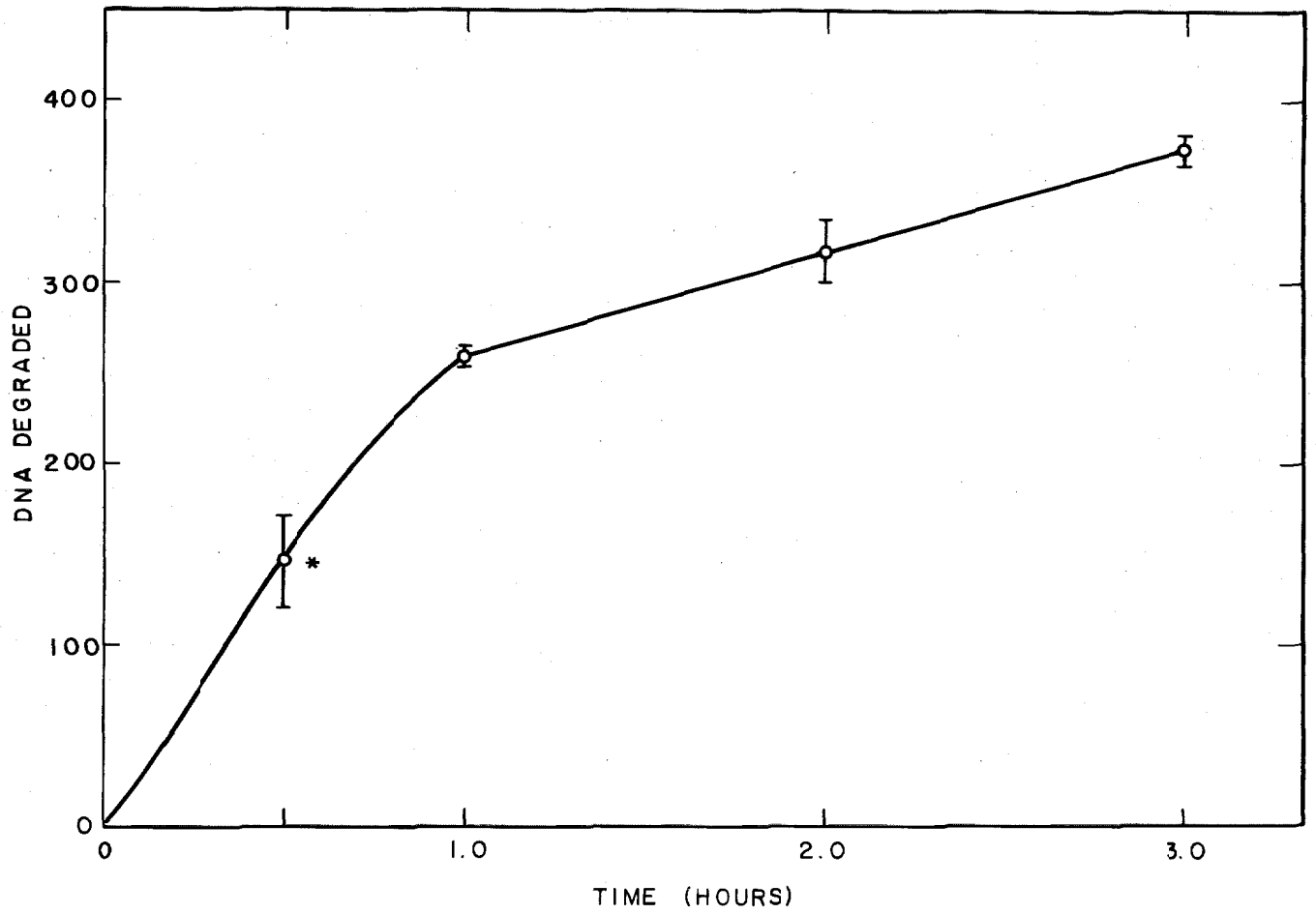


Fig. 6 Native DNA degraded by 0.5×10^{-9} g of DNase I during incubation at 37° C. The ordinate represents relative areas measured from the peaks of densitometry tracings. Each point represents the average value obtained from three separate gels unless otherwise indicated. Error range is one standard deviation.
*Average obtained from two gels.

stained in a Mg^{++}-containing solution develops a steadily widening "hole." However, it is not possible to perform a satisfactory boiled-enzyme control with DNase I in this system. As the gel warms up in boiling water, the enzyme is apparently able to function before it is denatured.

The data in Fig. 5 demonstrate that for DNase I, and probably for other nucleases, it is possible to find a range of activity for which the results are both reproducible and concentration dependent. This then makes it possible to assay quantitatively for one enzyme in the presence of a mixture of enzymes which are separable from each other by disc electrophoresis. It should be pointed out that the concentration dependence exhibited in Fig. 5 is probably due to a concentration-dependent diffusion process. This is implied from the fact that the peaks from which these data were taken are all essentially the same height. The increase in area is due instead to a broadening of the peaks in a manner similar to that shown in Fig. 2. It therefore appears that the first phase of the biphasic curve is a result of a rapid degradation of the substrate at the initial position of the enzyme. This phase is then followed by diffusion of the enzyme into the adjacent substrate. A body of qualitative data, which is not presented here, further indicates that this diffusion is delayed until much of the substrate has been degraded at the initial resting position of the enzyme.

Measurements obtained from the gels shown in Fig. 7 demonstrate that the mobilities of proteins in a crude mixture are retarded by 10% relative to the brom phenol blue dye-marker in the presence of either native or denatured DNA. A similar conclusion is drawn for DNase I from the data in Table 3. In this experiment a four-fold change in the DNA concentration produced a 10% reduction in the relative-mobility of DNase I. The later data do not establish, however, whether this effect on the mobility is entirely due to general effects, such as chromotography, or whether part of the variation is due to an enzyme-substrate interaction occurring during electrophoresis. This is an important question which must be resolved in the case of each enzyme

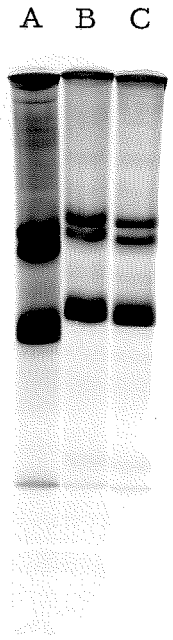


Fig. 7 Effect of DNA on protein mobility. An equal aliquot of crude Drosophila homogenate plus BSA was conducted into each gel. Gel A contained no DNA. Gel B contained 0.75 mg of native DNA/ml and Gel C contained 0.66 mg of denatured DNA/ml. Gels were stained for protein according to Davis (73).

TABLE 3

Effect of DNA Concentration on the Relative-Mobility of DNase I

Concentration of denatured DNA	Mobility relative to brom phenol blue marker	Average relative-mobility
1.0 mg/ml	0.528	0.53
	0.522	
0.5 mg/ml	0.553	0.56
	0.556	
0.25 mg/ml	0.579	0.58
	0.577	

DNase I analysis was performed as described in Methods (B, 3). Gels containing 5×10^{-9} g of DNase I were incubated for one hour at 37°C. Duplicate values were obtained from independently obtained densitometry tracings of the same gel.

for which the substrate specificity is to be determined from an enzyme mixture. It is entirely possible that due to specific interactions the mobility of an enzyme might not be the same in native and denatured DNA. This would introduce problems of enzyme identification and comparison in the two substrates. For DNase I this problem is resolved by the data in Table 4. These data show that the mobilities of DNase I in native and denatured DNA are equivalent when they are normalized to a common DNA concentration. Therefore, if there is any interaction between the enzyme and substrate during electrophoresis, it is of the same magnitude in both substrates.

b. Additional Controls. Raymond and Nakamiki (82) in a preliminary report stated that they have demonstrated the absence of detectable electroosmosis in acrylamide gels. As a result of this they said:

> The absence of electroosmotic flow makes it unnecessary to utilize two marker proteins for the calculation of separation ratios, and permits results to be recorded as the ratio of migration distances (relative migration).

Since DNA-trapping introduces fixed negative charges in the gel matrix, electroosmotic effects are probably present in DNA-containing gels. This statement implies, therefore, that our use of relative-mobilities*(relative migration) in the presence of electroosmosis is not justified. An analysis presented in the Appendix (Section III) shows that Raymond and Nakamiki were probably incorrect in making this statement. A closer study indicates that the use of relative-mobilities to eliminate the problems of variation in absolute electrophoretic mobilities is valid regardless of whether electroosmosis occurs or not.

The data in Table 5 show that, with the exception of acetate buffer at pH 5.3, the 0.1 M buffers are strong enough to maintain the desired pH during incubation. Succinate buffer (0.05M) has proven to be a satisfactory replacement for acetate at this pH. A more rigorous test of the question of the pH change in the gel is shown by the data in Table 6. These preliminary results indicate that

* see Section IV

TABLE 4

Relative-Mobilities of DNase I in Gels Containing Native and Denatured DNA[a].

Substrate	Mobility relative to brom phenol blue marker	Average relative-mobility
Native DNA (0.67 mg/ml)	0.546 0.549 0.549 0.546 0.548	0.548 ± .001
Denatured DNA (0.75 mg/ml)	0.543 0.546 0.544 0.540 0.540	0.543 ± .002
Denatured DNA (0.67 mg/ml)[b].	--	0.549

a. DNase I analysis was performed as described in Methods (II,B.) The data were obtained from densitometry tracings of gels incubated at various times and enzyme concentrations. Errors are one standard deviation.

b. This value was calculated from the data in this table and the data in Table 3.

TABLE 5

Effect of Incubation on the pH of the Incubation Buffer

Buffer composition	Original buffer pH	pH after the 15 minute presoak	pH after the 15 minute presoak and 1 hour incubation
0.1M Citrate	3.9	4.0 4.1	4.0 4.1
0.1M Acetate	5.3	5.7 5.9	6.4 6.0
0.1M Tris	7.2	7.4 7.5	7.4 7.4
0.1M Glycine	9.2 9.0	9.2 9.0	9.2 9.0

The pH of the incubation buffers was measured at room temperature.

TABLE 6

Effect of Preincubation Soaking on the pH in the Gel

pH of the incubation buffer	pH of the gel after 35 min. soaking in incubation buffer at 0°C	pH of the gel after 65 min. soaking in incubation buffer at 0°C
4.0	8.4	8.2
5.3	8.9	8.4
7.0	8.4	8.1
9.0	8.9	8.8

Electrophoresis was conducted without protein in gels containing 0.67 mg native DNA/ml as described in Methods (B.3). After they had been presoaked, the gels were quickly rinsed and placed in about 4 ml of water for 24 hours. The pH of the "water" was then measured at room temperature.

presoaking at 0° C for as long as an hour does not produce the desired pH change in the gel. This question will be considered in more detail in the discussion.

The data in Fig. 8 provide a demonstration of the effect of the preelution-soaking time on the efficiency of elution. In some organisms the proteins can be completely eluted even without a presoaking step. Drosophila homogenates, however, contain a particularly stubborn component which requires 1.5 hours of preelution soaking for its removal.

Densitometry measurements of stained gels were used to test the stability of the staining solutions. After extensive use the staining capacity of the methyl green solution is decreased by only 6%, while that of the pyronin B solution is reduced by 9% after only one use. This difference is probably due to the large difference in concentration between the two solutions. Data obtained in this same manner have shown that gels containing native DNA are maximally stained in less than one half-hour at room temperature. Gels containing denatured DNA, on the other hand, continue to absorb stain after 12 hours.

c. DNase II. Unlike DNase I, DNase II will not move as a discreet band through a gel containing native DNA. It apparently interacts strongly with DNA under the conditions of electrophoresis and binds to the DNA at the origin. At higher enzyme concentrations it progressively clears the gel of DNA from the origin downward. This behavior of an important DNase does not limit studies of crude enzyme mixtures as much as it might appear. In all three of the organisms which have been investigated only a small percentage of the total enzymatic activity behaves in this manner.

d. Applications to Biochemical Genetics. Histochemical localization of enzymes after their separation by gel electrophoresis has found application in biochemical genetics because of the sensitivity and speed of this technique (71, 83-87). These two factors make it possible to scan a large number of mutants suspected of having a deficiency of alteration of a normal enzyme. The data in Fig. 9

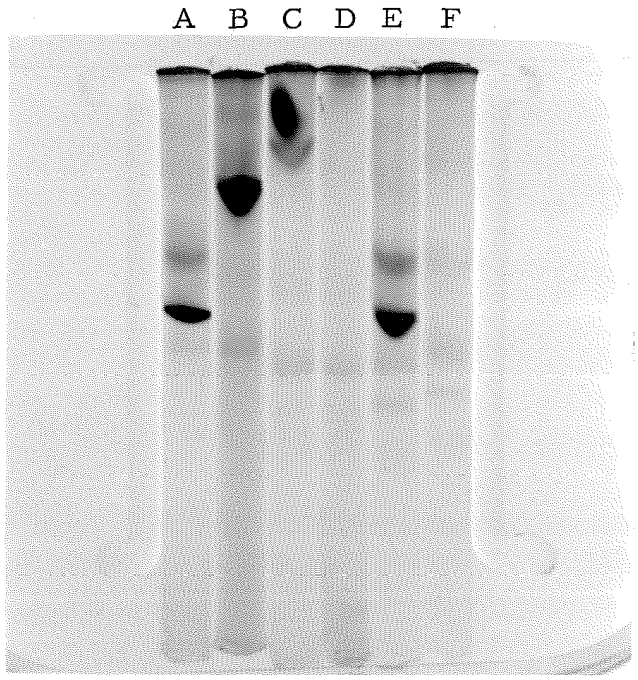


Fig. 8 Effect of preelution-soaking time on protein elution. Homogenates of Drosophila flies were subjected to disc electrophoresis in gels containing 0.67 mg of native DNA/ml. With the following exceptions the standard DNase detection procedure was followed using a one hour 37° C incubation. Gels A-D were incubated at pH 7.0, and Gels E and F at pH 4.0. Before the two hour elution Gels A, B, C, and D received 0.0, 0.5, 1.0, and 1.5 hours preelution soaking respectively. Gel E was presoaked 0.5 hours and Gel F 1.5 hours. All Gels were stained for protein according to Davis (73).

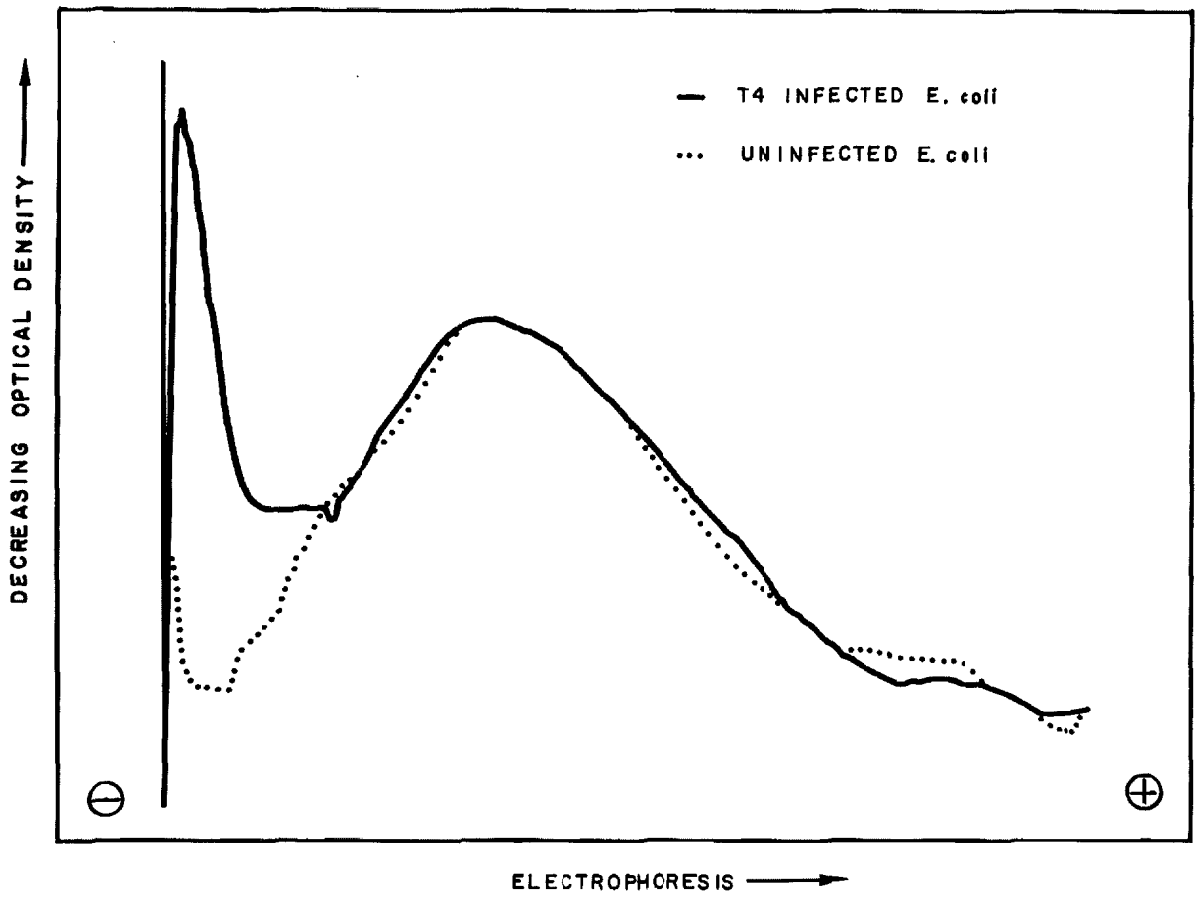


Fig. 9 Partial DNase patterns of E. coli S/6/5 infected and uninfected by phage T4. Cells were chilled, isolated, washed, and sonicated for 15 minutes after infection at 37° C. The sonicate was incubated for one hour at room temperature, centrifuged at 60, 000 g for 15 minutes, and the supernatent was diluted and subjected to electrophoresis in 7. 5% acrylamide containing 0. 67 mg of native DNA/ml. Incubation was carried out at pH 9. 5 in 0. 05 M Tris, 0. 05 M ethanolamine, 0. 005 M mercaptoethanol, and 0. 005 M $MgCl_2$ according to Weissback and Korn (31).

indicate the basis for a study of this type, involving a nuclease associated with the infection of Escherichia coli by the phage T4. In this experiment some of the nucleases have been run off the end of the gel in order to expose more clearly the additional peak which appears at the origin. The quick sensitive demonstration of DNase by this method should facilitate this type of study.

5. Discussion of the Method

The general procedure described here has been designed to detect the majority of DNase activities which might be found in a crude homogenate. Since no one set of conditions will be optimum for all enzymes in such a wide spectrum of activities, the procedure must frequently be modified to suit a particular problem. For example, the conditions presented here give good results with Drosophila homogenates but are not the best for similar preparations from microorganisms. The following discussion defines the parameters of the method in more detail in order to indicate possible approaches for improvement in a given situation.

a. Trapping the DNA. As long as the DNA is not dissolved in a stock solution containing the acrylamide monomers, it appears to have little effect on the polymerization of the gel. Because of age and other factors, however, the gel may not always polymerize to the same extent. This variable was usually monitored in these experiments by using one of the 32 gels, which were run at one time, to measure the mobility of a protein whose mobility in fresh gel is known. It is also advisable to test the quality of each new DNA sample, because in the course of this work one batch was encountered which was insufficiently polymerized to be held firmly in a 7.5% gel matrix. With the stains described here it is best to keep the DNA concentration at 0.5 mg/ml of gel or higher. A concentration of 1.0 mg/ml is used for photographic recordings (see Fig. 2) while a level intermediate between these two is most suitable for densitometry.

It is often useful to run the proteins of low mobility further into the gel in order to attain better resolution. This can be accomplished

either by decreasing the gel concentration or by running the electrophoresis for a longer period of time. The latter approach is preferable because of the possibility that the DNA will migrate along with the protein when the acrylamide concentration is decreased.

b. Incubation pH. The question of the efficiency of buffer replacement by the half-hour preincubation period described here has not been investigated extensively. However, it may be noted that a fairly rapid exchange of ions between gel and surrounding buffer probably occurs since some enzymes which are active in Mg^{++} -containing buffer show no activity in buffer containing EDTA. Furthermore, the data in Fig. 8 demonstrate that the preelution soak at 0° C has a decided effect on the mobility of a given protein. This also presumably indicates that the pH within the gel is changing. On the other hand, the data in Tables 5 and 6 show that although the incubation buffers maintain their pH, the pH in the gel is not quickly altered at 0° C. A similar result was found for the preelution soak. Again the external buffer maintains its pH during the last 45 minutes of soaking, but the pH in the gel appears to remain about what it was during incubation. Since the proteins travel into the gel in doughnut-like rings, however, the pH in the vicinity of the protein may reach that of the buffer before the entire gel has been influenced. Clearly the problem of changing the gel pH has not been fully solved and needs to be more carefully considered in those cases in which the pH optimum of an enzyme is to be determined.

c. Elution. There are three principal reasons for eluting the gels following incubation. Firstly, removal of the oligonucleotides, which have been freed from the gel by enzymatic action, greatly increases the sensitivity of the method. Secondly, protein removal provides a superior background, because the DNA stains also stain protein to some extent. And finally, the nucleases must be removed as they often continue to act in the stain solution, eventually clearing the entire gel of DNA.

As the results shown in Fig. 8 demonstrate, the elution procedure has been designed to remove greater than 95% of the protein in a crude homogenate from gels which have been incubated in the range pH 4 - 10. Although this requires that the conditions of the presoak and electrophoresis steps be excessive for gels incubated at higher pH, it does make it possible to handle all such gels in a single operation. It is particularly important that the pH in the upper reservoir (anodic) does not drop during the course of elution. When this occurs the gels swell and split prohibitively.

d. Staining. Of the several stains which have been tried, including the Feulgen stain, methyl green and pyronin B have given the most reproducible results and are the easiest to use. Under standard staining conditions neither stain binds to acrylamide itself as is indicated by a complete leaching of color from gels which contain no DNA. Neither, however, is the binding of the dyes to DNA absolutely permanent. It is, therefore, recommended that the nuclease patterns be recorded sometime in the month following staining. An additional advantage in using methyl green is that it is fairly specific for highly polymerized native DNA(76). This fact, which has been substantiated in these studies with densitometry measurements, eliminates any ambiguity which might arise from contamination of native DNA with denatured DNA. Since pyronin B and methyl green are not the most densely staining compounds which have been used, other stains could probably be utilized to permit the use of lower DNA concentrations.

e. Recording. While photography provides a rapid method for qualitatively scanning a large number of gels, the densitometer has proven itself invaluable as a means of obtaining finer resolution and semi-quantitative data. These data are not limited by the error which results from the different affinities of proteins for a given stain in protein stained gels. Nevertheless, much work remains to be done with enzymes having different mechanisms of action before the significance of quantitative nuclease determinations can be fully evaluated.

The data presented in Tables 3 and 4 demonstrate that the relative mobilities measured from densitometry tracings can be extremely reproducible. The data obtained from Drosophila homogenates, which is presented in Section III, C, show that this reproducibility is reduced in complex mixtures. Nevertheless, the precision remains high enough to be very valuable for identifying individual activities in such mixtures.

f. Background Considerations. It is important to realize that unlike DNase I, which acts only during the 37° C incubation, many enzymes exhibit considerable activity during the soakings at 0° C. Depending upon the enzymes under study, this "background" can be eliminated or reduced by certain procedural modifications. These include decreasing the enzyme concentration and increasing the incubation time or adding specific inhibitors before the completion of incubation.

g. Applications. The results shown here and in Section III, C demonstrate the potential of this method for the rapid preliminary identification and study of DNases. The simplicity of disc electrophoresis makes it possible to scan large numbers of organisms or mutants under varying physiological and developmental conditions. Quantitative aspects of the procedure should facilitate isolation of enzymes once they have been identified. In addition, it may be possible to characterize DNases in crude mixtures with respect to their pH optimums, substrate specificities, and even their mechanisms of action.

C. A Study of DNases in Drosophila melanogaster

1. Results

a. Relative-Mobility (Rm)* and Peak-Height Determinations. A preliminary investigation of the major DNase activities present in several developmental stages of Drosophila melanogaster has been carried out using the method outlined in Section III, B. The primary purpose of this work was to obtain an indication of the number of enzymes which are present together with some suggestions of their functional significance in development. A representative selection of the primary data is presented in Fig. 10. These densitometry tracings demonstrate the great variety and fluctuation of enzymatic activities which are found during the development of this insect.

An analysis of these patterns of activity has been made using two types of measurements taken from such tracings. The electrophoretic mobilities of the activities have been measured relative to the bovine serum albumin marker in order to facilitate comparisons between patterns obtained with different substrates, buffers, and animal ages. These relative-mobility measurements are referred to as Rm values. The heights of the peaks above background have also been measured as a rough quantitative estimate of enzymatic activity. A summary of these data is presented in Figs. 11-14 and in Tables 7-9. In order to eliminate possible variations in gel polymerization, most of these data were taken from one experiment carried out over a period of three days. The only exception is the data from one hour pupae which were taken from another experiment. The two experiments have been shown to be comparable by a comparison of additional controls which were common to both experiments. The age of the animals was determined as described in Methods (III, B). Homogenates were prepared within the hour preceding electrophoresis in order to reduce the slow enzyme destruction which has been observed after repeated freezing or long storage at 0° C. Except where indicated, all samples were prepared and treated identically.

*See Appendix

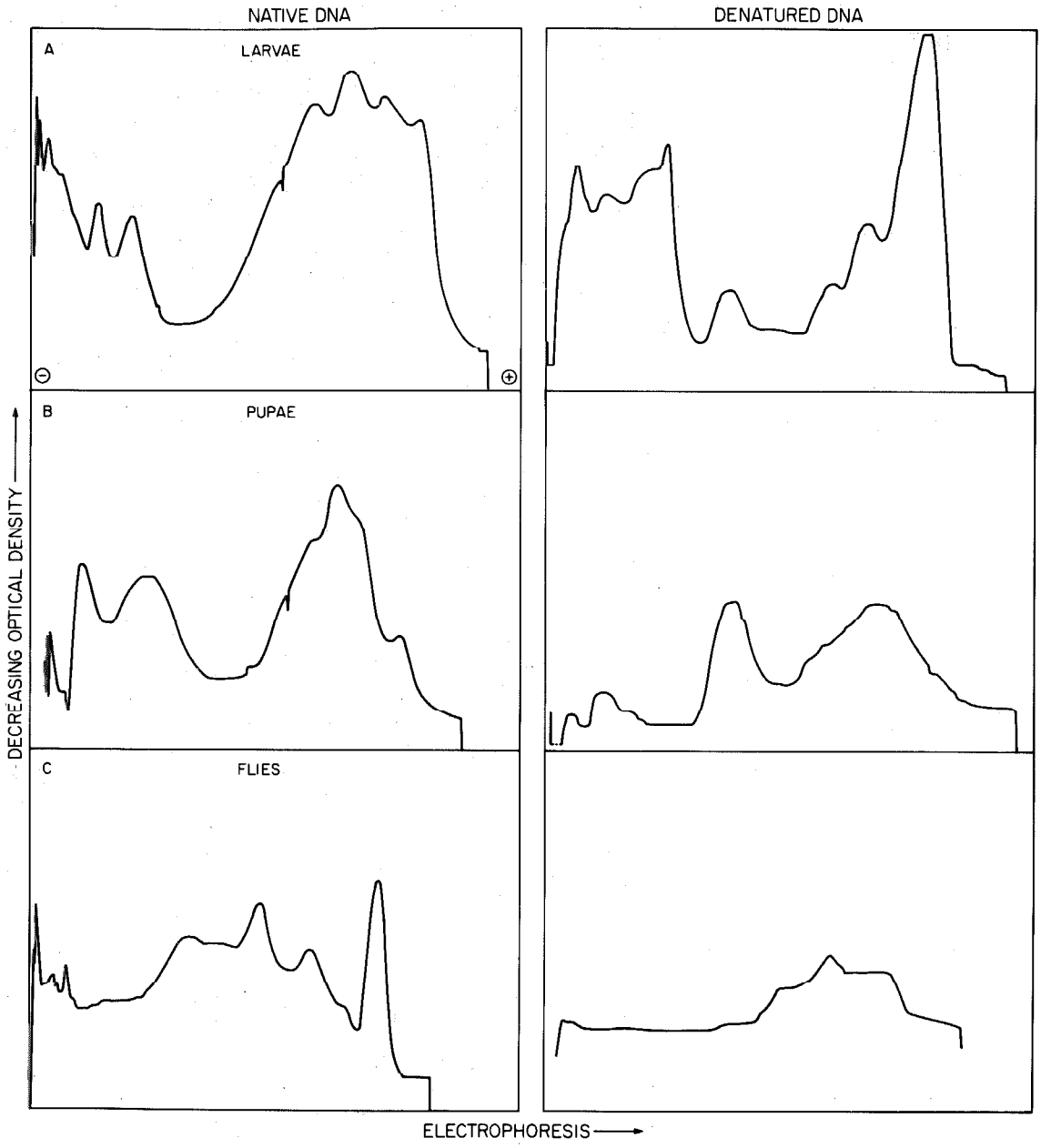


Fig. 10 Densitometry tracings of DNase patterns obtained from homogenates of Drosophila melanogaster. Larvae were collected 61 hours after egg-laying, pupae 55 hours after they floated on water (78), and flies less than a day after emergence. Incubation of the gels was conducted for 35 minutes at 37° C in 0.1 M acetate, 0.005 M $MgCl_2$, and at pH 4.0.

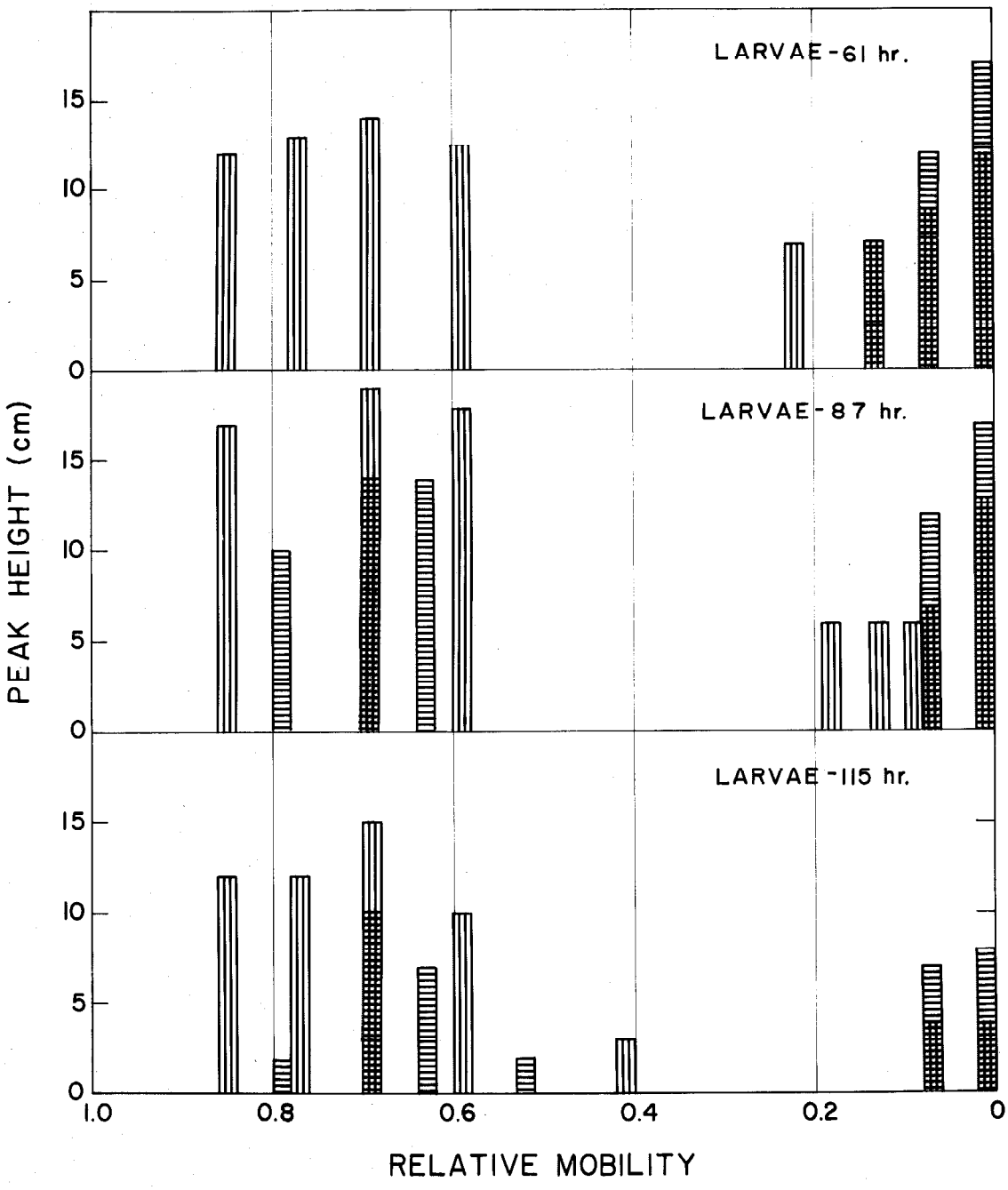


Fig. 11 Disc electrophoresis patterns of DNases obtained from Drosophila melanogaster. Gels containing native DNA were incubated at pH 4.0. The two buffers used were: 0.06 M sodium citrate, --0.005 M EDTA ▤ and 0.1 M sodium acetate --0.005 M $MgCl_2$ ▥. The height of each bar represents an average obtained from two duplicate gels except in the case of one-hour-pupae where only a single value was used. The position of each bar represents the average Rm of similar peaks obtained at a given substrate and incubation. Further details are provided in the text.

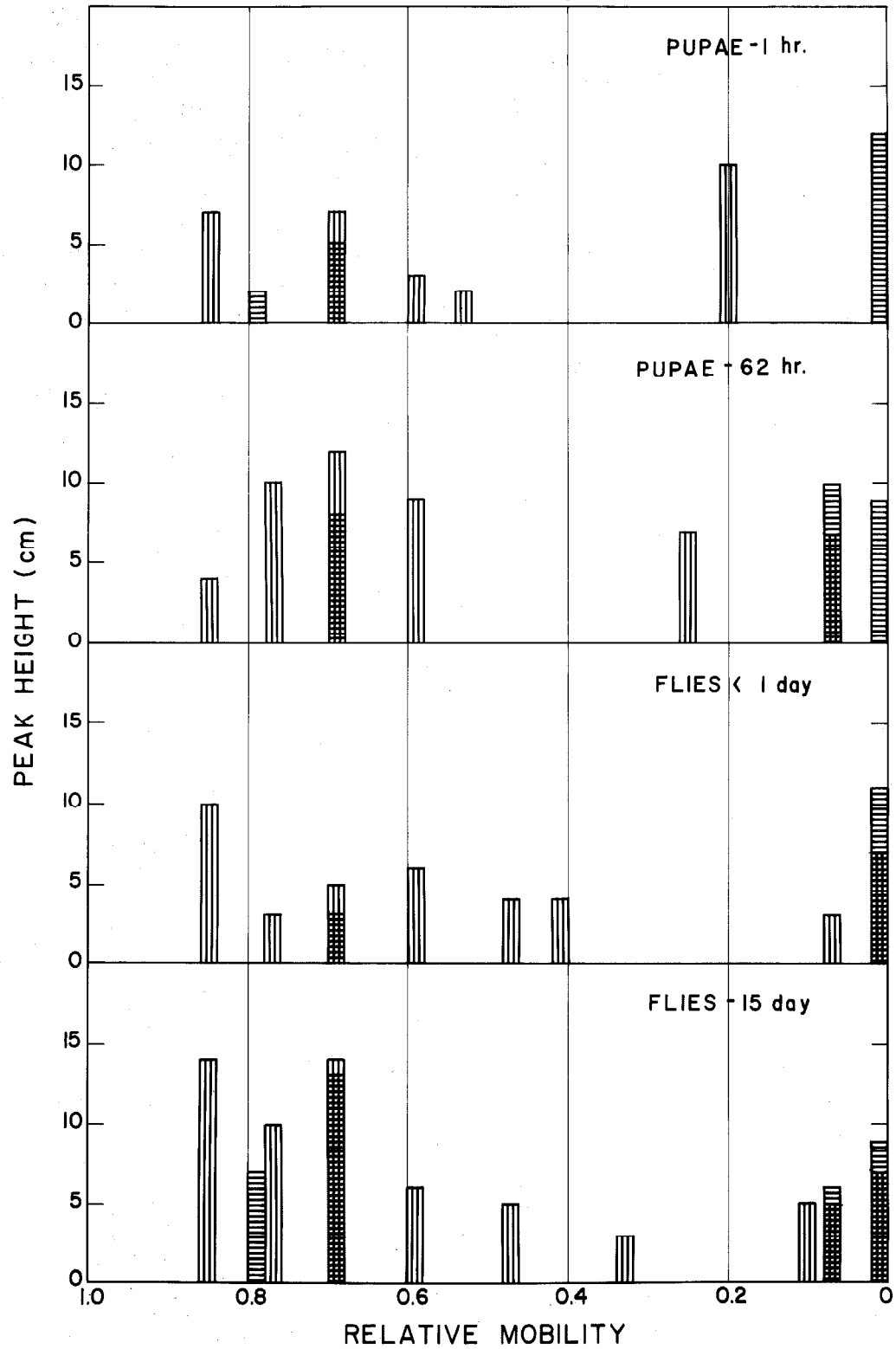


Fig.12 Disc electrophoresis patterns of DNases obtained from Drosophila melanogaster. Gels containing native DNA were incubated at pH 4.0. The procedure was identical to that described in Fig. 11. Mg^{++} incubation ▥. EDTA incubation ▤.

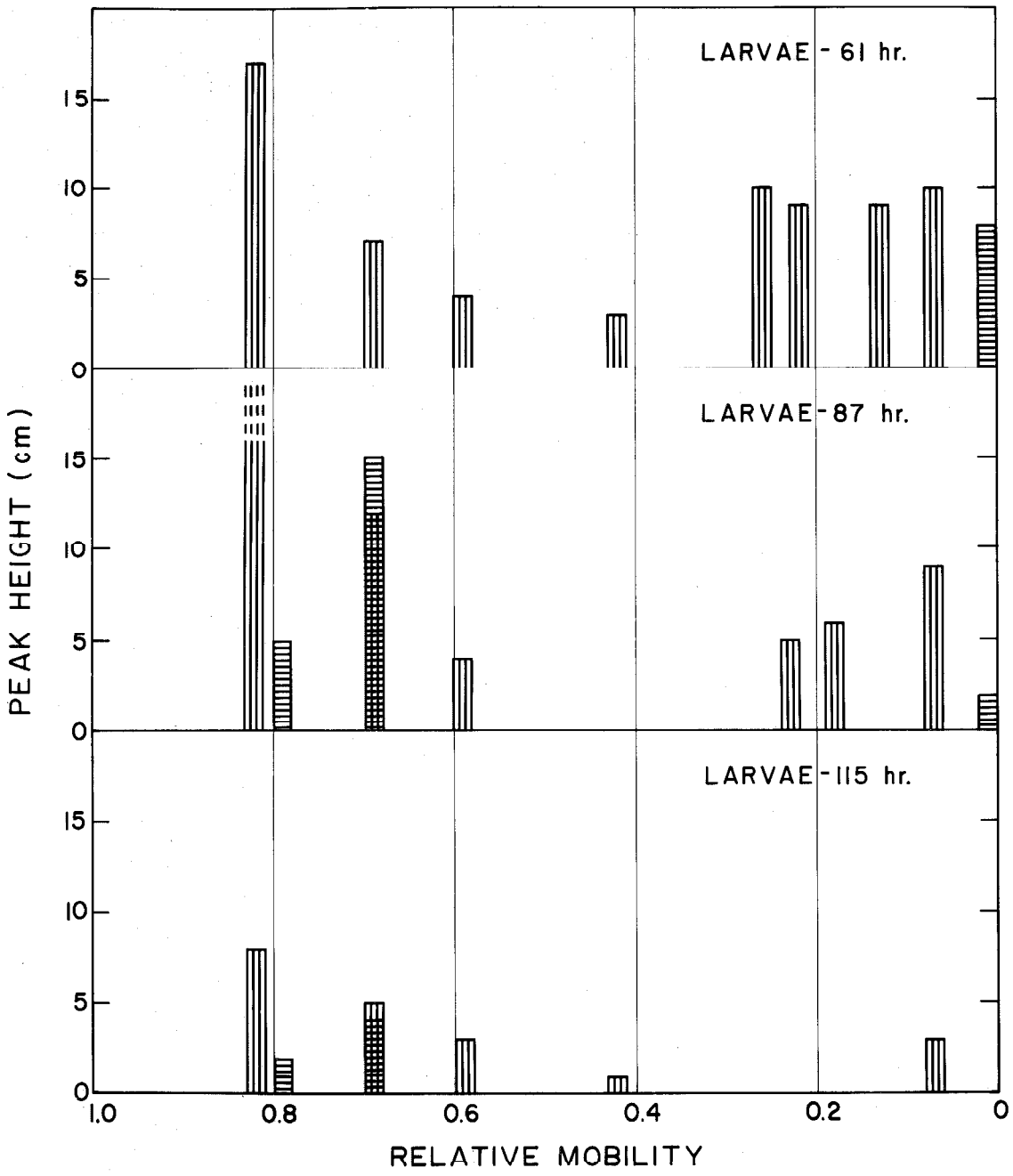


Fig. 13 Disc electrophoresis patterns of DNases obtained from Drosophila melanogaster. Gels containing denatured DNA were incubated at pH 4.0. The procedure was otherwise identical to that described in Fig. 11. Mg^{++} incubation ▥ EDTA incubation ▤

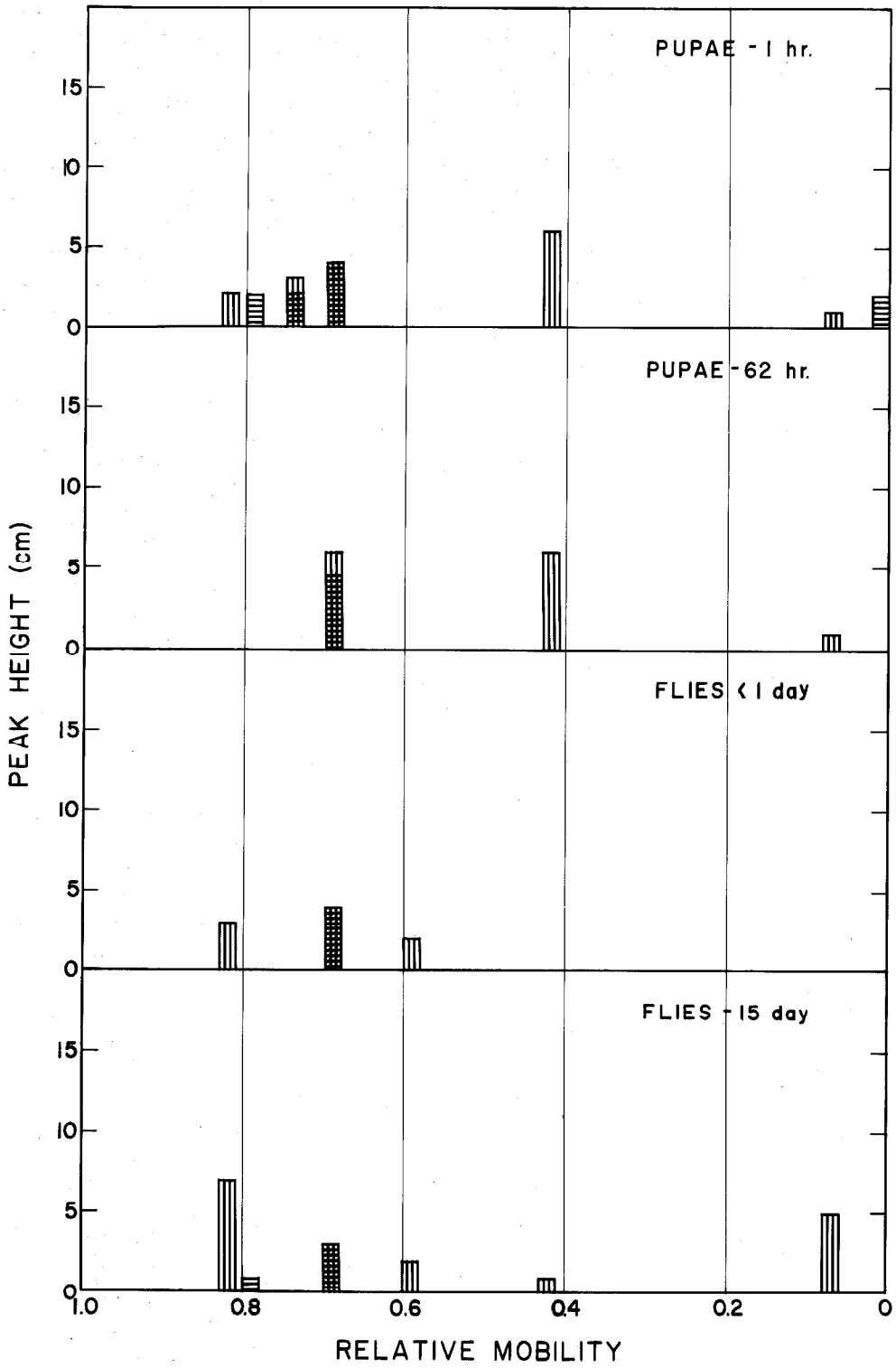


Fig. 14 Disc electrophoresis patterns of DNases obtained from Drosophila melanogaster. Gels containing denatured DNA were incubated at pH 4.0. The procedure was otherwise identical to that described in Fig. 11. Mg^{++} incubation ▥ EDTA incubation ▤

TABLE 7

Relative Electrophoretic Mobilities of DNases from Drosophila melanogaster --Incubation at pH 4.0 in the Presence of EDTA*

Native DNA

Animal Age	Relative Mobility						
61 hr. Larvae					(0.13)	(0.06)	0.0
87 hr. "	0.79	0.68	0.62			0.07	0.0
115 hr. "	0.78	0.69	(0.64)	(0.52)		(0.05)	0.0
1 hr. Pupae	0.80	0.70	(0.61)			(0.04)	0.0
62 hr. "		0.70				0.09	0.0
1 day Flies		0.70					0.0
15 day "	0.79	0.69				0.07	0.0

Denatured DNA

Animal Age	Relative Mobility						
61 hr. Larvae							0.0
87 hr. "	0.79	0.69					0.0
115 hr. "	0.78	0.68					
1 hr. Pupae	0.79	0.73					
62 hr. "		0.70					
1 day Flies		0.69					
15 day "	0.80	0.69					0.0

* Buffer: 0.06 M sodium citrate --0.005 M EDTA

TABLE 8

Relative Electrophoretic Mobilities of DNases from Drosophila melanogaster--Incubation at pH 4.0 in the Presence of Mg^{++} a.

Native DNA

Animal Age	Relative Mobility											
61 hr. Larvae	0.85	0.77	0.70	C.61			0.22	0.14		(0.07)	0.0	
87 hr. "	0.86		0.65	C.58		0.30	0.18	0.13	0.09	0.08	0.0	
115 hr. "	0.84	(0.76)	0.68	(C.58)		(0.39)				0.08	0.0	
1 hr. Pupae	0.82		0.68	C.56		0.28		0.11				
62 hr. "	0.85	(0.74)	0.69	(C.64)			0.25			0.09		
1 day Flies	0.87	(0.78)	0.70	C.59	(0.49)	0.42				0.09	0.0	
15 day "	0.85	(0.79)	0.69	(C.56)	(0.45)	(0.33)			0.10	0.07	0.0	

Denatured DNA

Animal Age	Relative Mobility									
61 hr. Larvae	0.83		0.70	0.62	0.40	0.26	0.22	0.13		0.07
87 hr. "	0.83		0.69	0.58			0.23	0.18		0.07
115 hr. "	0.79		0.68	(0.57)	(0.45)					0.08
1 hr. Pupae	0.82	0.74	0.69		0.43					
62 hr. "			0.70		0.39					0.05
1 day Flies	(0.82)		0.69	(0.57)						
15 day "	0.84		0.69	0.60	0.45					0.06

a. Buffer: 0.1 M Acetate -0.005 M $MgCl_2$

TABLE 9

Relative Electrophoretic Mobilities of DNases from Drosophila melanogaster--Incubation at pH 7.0 in the Presence of Mg^{++} a.

Native DNA

Animal Age	Relative Mobility									
61 hr. Larvae	0.86	(0.75)	0.69	0.61			0.22	0.14	0.06	0.0
87 hr. "	0.86		0.68	———	0.53[b.]			0.12	0.07	0.0
115 hr. "	0.84	0.73	0.69	0.57		(0.31)			0.08	0.0
1 hr. Pupae	0.84			0.58		0.32	0.17	0.15 0.12	0.07	
62 hr. "	0.86			0.62			0.23		0.10	
1 day Flies	0.87	0.79							(0.09)	0.0
15 day "	0.85	(0.78)	0.73						0.10	0.0

Denatured DNA

Animal Age	Relative Mobility								
61 hr. Larvae	0.81	0.69	0.61			0.24	0.20	0.12	0.07
87 hr. "	0.80	0.69	0.57			0.23	0.17		0.06
115 hr. "	0.81		(0.60)	0.44					0.08
1 hr. Pupae	0.83			0.44					
62 hr. "			0.60	0.39			0.15		
1 day Flies	0.83		0.57						
15 day "	0.83		0.58				0.14		0.06

a. Buffer. 0.1 M Tris --0.005 M $MgCl_2$.

b. Area of the gel in this mobility range was saturated with activity.

This included homogenization at a concentration of 75 mg of Drosophila per ml of electrophoresis buffer. In all cases incubation was carried out at 37° C for 35 minutes. With the exception of the data obtained from one hour pupae all recorded results represent the averages of values obtained from two identically treated gels.

Brackets are used in Tables 7-9 to indicate that the Rm value within the brackets was obtained from a shoulder of a larger peak and that the slope of the curve at the shoulder did not change sign. Inspection of the relative-mobility data in these tables reveals that the precision of the results is less than that previously encountered with purified DNase I. In order to simplify Figs. 11-14, most of the peak-height data have been plotted at Rm values averaged from all developmental stages. Each of these averages was calculated from those Rm values which were judged to be the result of one enzyme. The Rm values of activities which appear in the range Rm 0.1 - 0.4 have not been averaged because of their failure to satisfy any clear pattern.

b. Error Analysis. Part of the data of two identical but separate experiments is combined in Fig. 15 in order to indicate the error in both the relative-mobility and peak-height measurements. The peak-height data are seen to provide a very rough but adequate basis for comparison of relative enzyme concentrations. The highest peak-heights provide only a minimum estimate of enzymatic activity, because most of the DNA has been removed from the gel at these positions. Experiments in which the enzyme concentration was varied suggest that the peak-heights of some of the lower peaks also do not change. This implies that the specificities of the enzymes giving rise to these peaks do not permit complete destruction of the DNA. Rather surprisingly, the difference between peak-heights obtained from identically treated gels appears to be independent of the peak-height or the Rm of the peak. This permits an approximate error to be calculated by averaging several

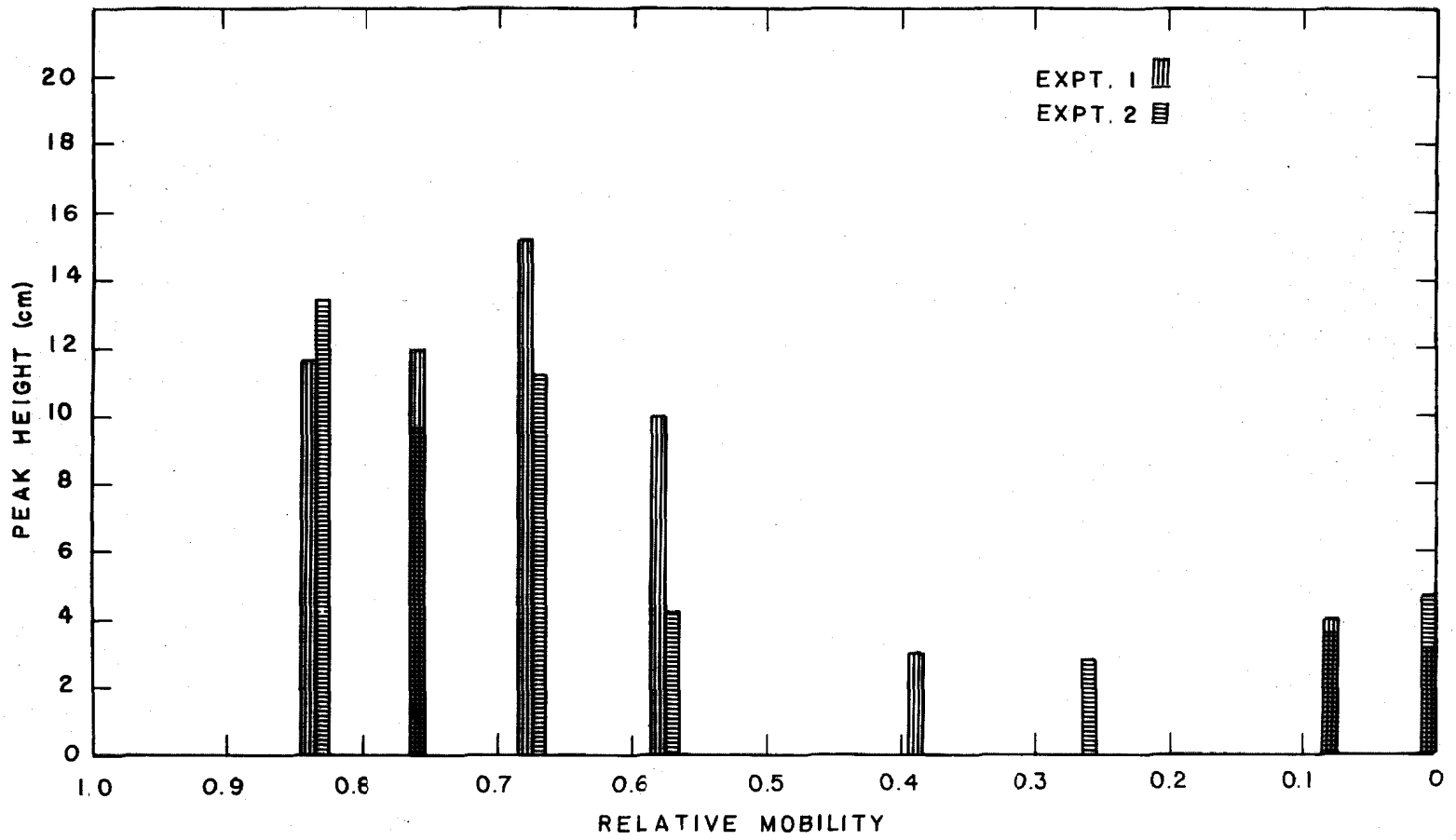


Fig. 15 Reproducibility between duplicate experiments. Half of these data are taken from Fig. 11 and Table 8. All results were obtained with 115 hour larvae and gels containing native DNA. The data from Experiment 1 represent the average obtained from two identical gels, while those from Experiment 2 were taken from one gel.

such differences. Average differences calculated from the data obtained from two larval ages are presented in Table 10. Although the absolute error is only slightly higher in denatured DNA than it is in native DNA, the percentage error is significantly higher, because the peak-heights are usually lower in denatured DNA.

Inspection of Fig. 15 and Tables 7-9 reveals that the reproducibility of the Rm values is good enough to be used for identification of all bands except those in the region 0.1 - 0.4. In addition to the poor reproducibility of the data between experiments in the region Rm 0.1 - 0.4, the variability of the data between duplicate gels within an experiment is also much greater in this region. Qualitative observations of gels which have received different concentrations of enzymes suggest that the instability of the Rm values in this region is at least partially due to a concentration dependent aggregation of the enzymes. In an experiment in which enzymes from flies were assayed with denatured DNA, two bands that were present in this region disappeared as the enzyme concentration was raised. If the hypothesis of aggregation is correct, they presumably appeared elsewhere at the position of one of the larger more stable bands.

A more detailed analysis of the error in the Rm data for bands appearing outside the 0.1 - 0.4 region is given in Table 11. Again the assumption has been made that the error has a similar distribution in all bands. Calculation A in Table 11 shows that the precision within one experiment may be as high as was found for DNase I. Calculation B points to a slightly higher variation in the Rm values obtained from different experiments. This is to be expected since the degree of gel polymerization may vary slightly with the age of the gel stock solutions. A still higher variation is found between the Rm values of peaks obtained from homogenates of different age animals. Calculations C and D, which were made from some of the data in Tables 7 and 8, both show this error. In

TABLE 10

Error Analysis of the Peak-Height Data

	Native DNA*	Denatured DNA
Average difference in the peak-heights of corresponding peaks obtained from duplicate gels	0.8 cm	1.0 cm
Number of measurements compared	31 pairs	23 pairs
Range of the differences	0-2.5 cm	0-3.6 cm

* Errors in native and denatured DNA-containing gels cannot be directly compared, because different stains, filters, and densitometer settings were used on gels containing the two substrates.

TABLE 11

Error Analysis of the Relative-Mobility Data

Level of error determination	Native DNA		Denatured DNA	
	Standard deviation or difference	Average standard deviation or difference	Standard deviation or difference	Average standard deviation or difference
A. Difference between duplicate gels.[a]		<0.01 Rm (38 pairs)[e]		0.01 Rm (35 pairs)
B. Differences between duplicate experiments.[b]	0.00(Rm.84) 0.03(Rm.76) 0.00(Rm.69) 0.01(Rm.58) 0.01(Rm.08)	0.01 Rm (5 pairs)		
C. Differences between animal ages.[c]		0.02 Rm (14 pairs)		0.02 Rm (11 pairs)
D. Standard deviation for different animal ages.[d]	0.01(Rm.85) 0.02(Rm.77) 0.02(Rm.70) 0.03(Rm.61) 0.01(Rm.08)	0.02 Rm (30 averages)	0.01(Rm.83) 0.01(Rm.69) 0.02(Rm.59) 0.03(Rm.42) 0.01(Rm.07)	0.02 Rm (22 averages)

a. The differences in Rm values of corresponding activities in duplicate gels were averaged from data obtained from three larval ages.

b. Average Rm values were obtained from duplicate gels used to assay 115 hour larvae at pH 7.0. The difference between the averages from parallel experiments are presented here. These data complement those presented in Fig. 15.

c. Two values were selected at random from a column of figures in Table 9 (pH 4.0 + Mg^{++}). The difference between these two values was determined and averaged with similar differences. No value was used twice.

d. The standard deviation was calculated for a column of figures in Table 9. The data from one hour pupae have not been included in any of these calculations.

e. Number of values analyzed.

most cases the Rm values tend to group together well enough to suggest that the variation is not due to the appearance of new enzymes.

A further source of error which has been observed is the complete fusion of several peaks into one due to poor separation or high enzyme concentration. The existence of this type of error means that the apparent absence of a peak cannot be given much significance when its position is surrounded by other high peaks. This difficulty together with the problem of detecting minor components can only be solved by future experiments employing additional criteria of separation.

c. Supplementary Controls. In Section III, B. the point was made that in certain cases considerable DNase activity may occur during those steps of the procedure in which the samples are kept at 0° C. The densitometry tracings in Fig. 16 demonstrate that this is true for some of the enzymes of Drosophila. These tracings are comparable to that presented in Fig. 3 A' as background for the DNase I analysis. A comparison of the two sets of data shows that some Drosophila enzymes lower the activation energy for phosphodiester bond hydrolysis considerably more than does DNase I.

The data in Fig. 16 also assist in discounting the existence of a potential artifact which might arise from any protein remaining in the gel. Since the DNA stains also stain proteins to some extent, any residual protein will contribute a region of fixed stain which is resistant to DNase activity. If such a protein should occur in a DNase band, this procedure would register a split band, and a single enzyme would be erroneously recorded as two activities. For several reasons this is not a likely occurrence under the present conditions. Firstly, an inspection of the gels pictured in Fig. 8 reveals that over 95% of the protein has been removed by the elution procedure. Secondly, the neighboring peaks of enzymatic activity in patterns which have been obtained from

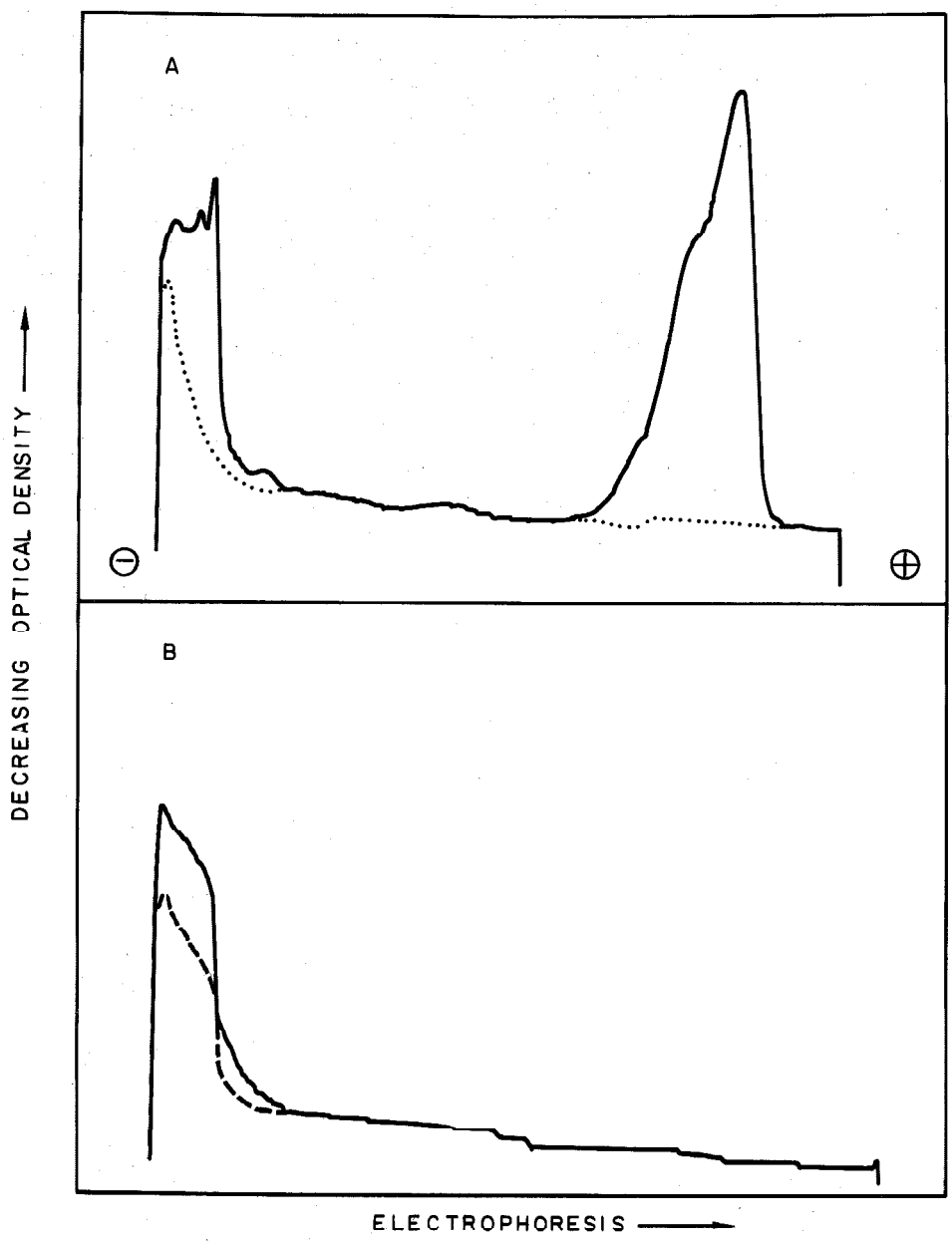


Fig. 16 Densitometry tracings representing enzymatic activity occurring at 0° C. Two-thirds of the standard quantity of homogenate from one day old flies was subjected to electrophoresis in gels containing native DNA. The standard DNase detection procedure was followed except that the 37° incubation was replaced by an extended preincubation soaking of 60 minutes at 0° C in pH 4.0 buffer. The preincubation buffers for gels A and B (heavy lines) contained $MgCl_2$ and EDTA respectively. These buffers are described in Tables 7 and 8. A densitometry tracing of a gel for which the preincubation soaking was omitted entirely is indicated by ---. A gel which had received the complete treatment but contained no enzyme gave the pattern shown by . . . .

various stages of Drosophila development have been found to vary in intensity independently of one another. This variation suggests that such neighboring activities are not the result of a single protein. The best evidence against this type of artifact is found in Fig. 16. In a region of a gel where there is no DNase activity, the presence of any significant amount of protein would be revealed by a positively staining area in the DNA background. No such area is found in Fig. 16, nor has one been seen in densitometry tracings obtained from any other gel from which the protein has been adequately eluted. This observation has been made in gels containing both denatured and native DNA. The elution procedure is, therefore, adequate to eliminate this type of protein band interference.

Within the experimental error of the data in Table 12 the Rm values of the individual enzymes do not change when the DNA concentration is reduced by one-half. Thus, at this resolution any interaction between these nucleases and the supporting matrix does not represent enzyme-substrate interaction, because it is equally expressed between the BSA reference and the matrix. On the other hand, if Mg^{++} is incorporated into the original gel together with native DNA, strong interactions probably occur during electrophoresis. In this situation none of the activity progresses past Rm 0.57, and most activity is expressed as a smear near the origin. The conclusion is, therefore, that most of the Drosophila DNases do not interact specifically with the DNA in the gel during electrophoresis, although such interaction can be induced by the presence of Mg^{++}.

The conflict over the effect of DNA concentration on the relative mobilities of DNase I and Drosophila DNases (Tables 3 and 12) is probably a result of the fact that two different reference markers were used in these experiments. Since the results in Fig. 8 show that DNA generally reduces the mobility of proteins relative to that of brom phenol blue, it is to be expected that different results would

TABLE 12

Effect of DNA Concentration on the Relative-Mobilities of Some *Drosophila* DNases[a].

DNA Concentration	Relative-Mobilities				
		Native DNA			
0.67 mg/ml	0.94	0.77	0.59		0.11
0.50 mg/ml	0.93	0.78	0.60		0.09
0.33 mg/ml	0.93	0.78	0.60		0.09
		Native DNA plus 0.001 M $MgCl_2$ [c]			
0.67 mg/ml			0.57	smear	
		Denatured DNA			
0.75 mg/ml	0.80	0.57	0.20	0.14	0.04
0.50 mg/ml	0.83	0.59	0.22	0.15	0.05
0.37 mg/ml	0.81	?	0.21	?	0.05

a. Two-thirds of the usual quantity of a homogenate from one week old flies was assayed for DNase by the standard procedure. All gels were incubated at pH 7.0 in 0.1 M tris-chloride --0.005 M $MgCl_2$.

b. Measured from photographs relative to the BSA marker.

c. Gel polymerized from a solution containing $MgCl_2$.

be obtained when BSA and brom phenol blue are used as reference markers. The difference between the behavior of the markers, however, does not nullify the conclusion that any DNA-DNase interaction is nonspecific.

2. Discussion

a. Data Analysis. There are four major ways in which the DNase data (Figs. 11-14 and Tables 7-9) may be analyzed. These include comparisons of the data in which the following factors were studied under otherwise constant conditions: 1. the presence of Mg^{++} or EDTA in the incubation buffer; 2. different stages of the life cycle; 3. variation in the substrate; and 4. variation in the pH of the incubation buffer. Comparisons between data in which the Mg^{++} concentration or the age of the animals were varied are the most significant of the four. In both of these situations the relative-mobility of an enzyme has been shown to be a satisfactory criteria of identification. Thus it is possible to follow the effect of these two variables on specific enzymes which can be identified by their appearance at a given position in the electrophoretic pattern.

A third potentially valuable comparison of the data can be made between identical samples which have been assayed with different substrates. To identify a given enzyme in both substrates by means of its relative-mobility, it is first necessary to show that specific DNase-substrate interactions have not affected the enzyme mobility during electrophoresis. Preliminary experiments with both DNase I (Table 4) and Drosophila DNases (Table 12), which were performed to test the possibility of such interactions, have been negative. This strongly implies that a given Rm value should serve to identify an enzyme in both native and denatured substrate. The Drosophila data (Figs. 11-14) have, therefore, been analyzed by comparing the results obtained with the two substrates. A tabulation of six activities which appear to have a strong substrate

preference is presented in Table 13.

Because these data were obtained from gels in which the pH was not precisely known, it is not possible to make a satisfactory estimate of the pH optimum of any of the enzymes studied. Thus the fourth and final type of comparison has not been exploited in these studies.

b. Estimated Number of Drosophila DNases. The collected DNase data in Figs. 11-14 and Tables 7-9 reveal that a very large number of peaks of enzymatic activity have been detected. This does not mean, however, that each peak represents a separate protein. On the contrary there exists a strong possibility that several peaks represent isozymes, complexes, or aggregates of a single protein (88). Although limitations in the data make it impossible to decide precisely where this is true, an estimate of the minimum number of distinctive activities may be obtained by first assuming that all activities represent aggregates of a single protein. An attempt is then made to single out those activities which disprove this assumption by possessing significantly different properties from one another. This analysis has been performed on only the prominent activities which appear in more than one animal age, ie have been detected in at least 4 gels. The following assumptions are implied in the discussion:

1. No DNA-DNase interactions occur except for those activities appearing at the origin.
2. There is no aggregation with non-enzymatic age-specific proteins.
3. Aggregates have enzymatic properties similar to the primary enzyme.

Within the mobility range Rm 0.4 - 1.0 it is clear that all activities requiring Mg^{++} are distinct from those requiring EDTA. (Figs. 11-14). This conclusion is made possible by data from 61 hour larvae in which the EDTA activated components are absent. Since the Mg^{++} activated components are present in high concentration at

TABLE 13

Tabulation of Drosophila DNase Activities Exhibiting a Strong Substrate Specificity

Relative mobility	Substrate Preference	Activating Ion
0.0	Native DNA	EDTA
0.0	" "	Mg^{++}
0.07	" "	EDTA
0.62	" "	EDTA
0.72	" "	Mg^{++}
0.42	Denatured DNA	Mg^{++}

this age, the two types of activities cannot be due to the same protein even though both types of activity are found together at other ages. Thus, these two sets of activities may be considered separately. At least three EDTA activated components are present in this area, one of which (Rm 0.62) is different from the other two by virtue of its inactivity in denatured DNA. The data, therefore, suggest the presence of at least two classes of EDTA activated enzymes in this mobility range.

The data in Figs. 11-14 further show that six Mg^{++} activated components also appear within the range Rm 0.4 - 1.0. Two of these (Rm 0.77 and 0.47) are different from the others, again because of a failure to show activity in denatured substrate. Furthermore, these two are probably not aggregates of a single protein, because the Rm 0.47 activity is present only in flies and not in younger animals in which the Rm 0.77 activity is very high. Thus, a minimum of three classes of Mg^{++} activated enzymes are also present in the range Rm 0.4 - 1.0.

Observations taken from analyses of other organisms strongly suggest that at least some of the activities appearing at the origin are characterized by a strong interaction with the substrate. Drosophila pupae (Figs. 12 and 14) produce at least one such enzyme which is active only in EDTA. Since other developmental stages of the organism possess activity which appears at the origin in both Mg^{++} and EDTA, at least two enzymes are present at this position over the total course of development. Although the activities in the range 0.0 - 0.1 are usually too numerous to be analyzed, at least one additional EDTA activated enzyme which is different from any of the others that have so far been included is found in this range.

Estimation of the maximum number of activities present is simplified by the assumption that components active in EDTA are distinct from those active in Mg^{++}. This supposition has been

supported in Drosophila for all but two activities which appear near the electrophoretic origin and is upheld by our knowledge of the characteristics of DNases which have been isolated from other organisms. In all the ages tested there are about 19 separate peaks of DNase activity as judged by the above criteria. Additional considerations suggest that some peaks counted as single activities may in fact be the result of more than one type of enzymatic activity. Thus further studies employing techniques with improved resolution would be expected to raise both the maximum and minimum estimates of the number of Drosophila DNases.

A summary of the maximum and minimum estimates follows:

Relative-mobility	0.4-1.0	0.1-0.4	0.0-0.1	Total
Maximum estimate	10	4	5	19
Minimum estimate	5	0	3	8

With the assumptions which were given initially, it is therefore concluded that of the great number of activites observed, at least eight probably represent distinct enzymatic types. The clues which this analysis has provided to the character of these enzymes should also serve as useful guides for future studies.

The possibility that some of these activities are derived from contaminating yeast has not been rigorously excluded, although it appears very unlikely at present. Pupae probably do not contain any yeast at all, because they lack a functional digestive tract. Drosophila ingest yeast in both the larval and fly stages, but the weight of microorganisms present at any one time is a very small percentage of the total weight of the animal. Furthermore, disruption of intact yeast generally requires much stronger forces than have been employed for homogenization of Drosophila (89).

The data presented here probably show as extensive a variety of enzymatic activity as can be revealed in whole animals with this method. Gels have been incubated in buffers ranging from pH 3 to 10 without the appearance of any peaks not already seen at pH

4 or 7. Some attempt has been made to look for enzyme activation by preincubation of homogenates at room temperature, but no such phenomenon has so far been found. No major qualitative difference between DNase patterns obtained from the sexes has been found, either, although this work was not performed with the system at its present resolution.

c. Nuclease Patterns in Development. A number of striking changes in the DNase patterns obtained from Drosophila homogenates have been recorded during the course of the insect development. This information is valuable in selecting the developmental stage for studying a particular enzyme as well as for the suggestions it provides about the function of the enzymes. One of the most dramatic trends found in this study is the significant decrease in total enzymatic activity which is observed during larval development (Figs. 11 and 13). In the period between 60 and 115 hours of larval life, which was studied here, an individual animal more than doubles its weight. Since all homogenates were prepared on the basis of equal animal weight, the data show that the amount of enzymatic activity per animal is more constant than is the activity per tissue weight. Therefore,most of these enzymes may be important for cellular activities which are not a function of all the tissue.

Certain enzyme patterns have appeared which are characteristic of specific developmental stages of Drosophila. The paucity of EDTA activated enzymes in 61 hour larvae has been noted previously and may reflect the strong emphasis of this tissue on growth at the expense of differentiation. Another abrupt break in the DNase pattern is the disappearance from pupae of the Mg^{++} activated enzyme which is ordinarily present at the origin of gels containing native DNA. Since pupae lack a functional digestive system, this enzyme might be expected to exist in the digestive system of the feeding forms of the organism.

Observations of interest for further studies include peaks which are characteristic of certain developmental stages such as Rm 0.45 (Mg^{++}) in flies and Rm 0.62 (EDTA) in older larvae (Figs. 11 and 12). In pupae there is a conspicuous absence of the peak at Rm 0.58 (Mg^{++}) while the Rm 0.42 (Mg^{++}) peak becomes more predominent at this stage of development (Figs. 13 and 14). In addition, the high activity exhibited by young larvae in the region Rm 0.1 - 0.4 is lacking in flies (Figs. 11-14).

3. Conclusion

The stated purpose of this work, to identify and partially characterize the DNase patterns of Drosophila, has been accomplished by the discovery of a large number of enzymatic activities which include at least eight distinct enzymatic types. This work has also shown that disc electrophoresis provides a rapid method for the preliminary characterization of DNases. At the present stage in the study of these enzymes this is particularly useful, because it provides a potential for identifying new enzymes in a complex mixture.

Investigations of this type, in insects like Drosophila with holometabolous development, offer the advantage that the processes of growth and differentiation are sharply segregated in different tissues and in different stages of development. At the present time only one DNase has been carefully studied in this type of insect (90-93). In this case the advantage of having growth and differentiation separated has not been exploited. Homogenates of whole animals, such as were used here, necessarily limit the detail to which an enzymatic study can be carried. Future studies of specific tissues, such as those that have lost all capacity for cell division, offer a real hope for the eventual determination of the function of more of the DNases.

Appendix

Justification for the Use of Relative Mobility Measurements When Electroosmosis is Involved

The following expression defines the electrophoretic mobility of an unknown in the presence of a reference substance of known mobility (94).

$$U = \left(\frac{d - d_o}{\overline{d} - d_o} \right) \overline{U}$$

d = distance traveled by the unknown from the origin

$\overline{d}$ = distance traveled by the reference from the origin

d_o = distance traveled by a given body of fluid under the same conditions used for $\overline{d}$ and d.

U = mobility of unknown

$\overline{U}$ = mobility of reference

In order to calculate the unknown mobility it is necessary to find d_o with the use of a second reference material. This information is not necessary, however, when only the ratio of the mobilities* is to be determined.

$$\frac{U}{\overline{U}} = \frac{d - d_o}{\overline{d} - d_o} = \frac{X}{\overline{X}}$$

X and $\overline{X}$ are the distances traveled by the two substances in the absence of fluid flow.

Since,

$$X = KE$$

$$\overline{X} = K'E$$

$$d_o = K''E$$

Then,

$$d_o = CX = C(d - d_o)$$

$$d_o = C'\overline{X} = C'(\overline{d} - d_o)$$

* See Section IV

where K, K', K'', C and C' are constants, and E is the field strength. Solving for d_o gives:

$$d_o = \frac{Cd}{C+1} \quad , \quad d_o = \frac{C'\bar{d}}{C'+1}$$

Equating d_o and rearranging gives:

$$\frac{d}{\bar{d}} = \text{constant}$$

This last equation implies that regardless of how long the electrophoresis is run, the ratio of the distance traveled by the unknown to the distance traveled by the reference is a constant. Since small variations in absolute electro-migration rates are difficult to avoid (82), this ratio represents a useful means of comparing different electrophoretic patterns whether or not electroosmosis has occurred.

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IV. Abbreviations and Definitions

Bis	-	N, N'-Methylenebisacrylamide
BSA	-	Bovine Serum Albumen, Fraction V Powder
cpm	-	counts per minute
dpm	-	disintegrations per minute
EDTA	-	Ethylenediaminetetraacetic acid, disodium salt
ΔF°	-	standard free-energy change
Mobility	-	velocity relative to the gel
PTA	-	Phosphotungstic acid
Relative mobility	-	$\frac{\text{distance traveled by nuclease}}{\text{distance traveled by marker}}$ - (Rm)
sRNA	-	soluble ribonucleic acid
TEMED	-	N, N, N', N'-Tetramethylethylenediamine
Tris	-	Tris(hydroxymethyl)aminomethane