STUDIES ON AMINO ACID INCORPORATION IN THE LARVAE OF DROSOPHILA MELANOGASTER

Thesis by

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ABSTRACT

Amino acid incorporation in the larvae of Drosophila melanogaster was studied by injecting C^{14} labeled amino acids into the hemolymph of the animals. The resulting pattern of incorporation was investigated by direct paper chromatography of the injected larvae and by column fractionation of larval extracts.

It was found that injected amino acids were rapidly incorporated into a large number of components. Many of these were peptides, others contain non-amino acid constituents whose nature is yet unknown. As contrasted to previous reports, the present work indicates a major portion of the amino acids in insect hemolymph occur in bound form. It is felt that the difference is due to the higher degree of resolution of components achieved in the present experiments.

In the hope of obtaining larger amounts of amino acids in non-protein bound forms, attempts were made to inhibit protein synthesis. Of the treatments tested only Metrazol and chlorpromazine were effective in reducing protein synthesis and this reduction was accompanied by a decrease in amount in all radioactive metabolic product. The possible significance of this reduction is discussed.

Material containing bound C^{14} glutamic acid was isolated from larval extracts and reinjected. It was found that radioactivity from the injected material was incorporated into the protein of the larvae but at a slower rate than was the free amino acid. The implications of this finding are discussed.

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INTRODUCTION

Amino Acids in Insects

More than fifty years ago (1) Nazari reported that insects have a higher concentration of amino acids in their blood than do any other animals. This was substantiated by Bishop, Briggs and Ronzoni (2) who, on the basis of amino nitrogen measurements, reported that the non-protein nitrogen concentration in insects is 50 times higher than in mammalian tissue. An idea of the magnitude of the concentration is given by the report of Duval, Portier and Courtois (3) who measured amino acid concentration by the formal titration method and found that it reaches 20 g/l in Saturnia larvae. The high concentration of non-protein amino acids in insect hemolymph is so consistent that Florkin (4) has proposed that it be considered a systematic biological characteristic of the class.

As Buck (5) pointed out in his review on insect blood, until the advent of paper chromatography few of the amino acids in insect hemolymph had been identified. Since this technique has come into widespread use much more information is available. The qualitative distribution of amino acids in various species of insects has been reviewed by Buck (5) and Hinton (6). Since these reviews were written more work on the distribution of amino acids has been done (7,8,9,10,11,

12,13,14,15). On the basis of chromatographic mobility, all of the protein amino acids have been identified as occurring in insect hemolymph. Not all of the amino acids have been found in each species investigated, though this is likely a function of concentration, and the quantitative distribution varies considerably from species to species. Most of the investigations have been made by chromatographing deproteinated hemolymph directly, though Florkin's group has often hydrolyzed the deproteinated hemolymph prior to analysis (9,14,15).

The function of the high concentrations of the supposedly free amino acids in insect blood is poorly understood. Because of their high concentration they do seem to play an important role in maintaining the osmotic pressure of the blood (2,5,16,17). The hemolymph amino acids also seem to serve as substrates for protein synthesis. Several workers have reached this conclusion on the basis of variation in concentration of the amino acids with developmental stage (11,17, 18,19,20,21). During stages of development when growth is especially rapid, and especially when feeding is not possible, the hemolymph amino acid pool is reduced. The studies of Hadorn and his co-workers (22,23) also indicate that the hemolymph amino acids and protein synthesis are related. They found that in the mutant types lethal-translucida (ltr) and lethal-bluter (lbl) an accumulation of amino acids and peptides in the hemolymph is apparently associated with a defect in protein metabolism. More direct evidence is given

by Fukuda and Florkin (24) who have reported the transfer of hemolymph C^{14} glycine to the protein, fibroin, in Bombyx mori. The metabolic pathway by which supposedly free amino acids in the hemolymph are incorporated into insect protein has not been reported.

Bound Amino Acids in Insects

While the emphasis in the work on amino acids in insects has been on the occurrence of the free compounds, there is ample evidence to indicate that a good portion of the amino acids are not present in this form. Wyatt, Loughheed and Wyatt (13) found that the total free amino acids account for only 35 to 55% of the non-protein nitrogen, with other known compounds making up only a small part of the deficit. Finlayson and Hamer (25) made the interesting observation that the hemolymph tyrosine concentration in Calliphora erythrocephila exceeds the solubility of that amino acid by a factor of three. Heller and Moklowska reported (26) that in the residual (non-protein) nitrogen fraction of Deilephila euphorbiae blood there was 120 mg per cent peptide bound nitrogen as compared to 170 mg per cent amino nitrogen.

There are numerous reports of the detection of peptides in insect hemolymph (11,13,19,21,22,23,27,28,29,30,31,32). For the most part no special effort was made to find these compounds. They were observed because of their anomalous chromatographic behavior. There have been few attempts to

determine the amounts of peptides present, although Hadorn and Mitchell (30) reported that a high proportion of the ninhydrin positive material in Drosophila is peptidic and not the free amino acids.

Functions of Peptides

The occurrence of peptides in insects recalls the old question regarding the functions of these compounds in biological systems. Of primary concern has been the possible involvement of peptides as intermediates in protein synthesis. This question has been discussed in recent years, among others, by Campbell and Work (33), Borsook (34,35), Fruton (36), Steinberg, Vaughan and Anfinsen (37), Kamin and Handler (38) and Speigelman (39). While peptides as intermediates are still advocated, the majority of investigators have not favored this hypothesis.

One objection to peptides as intermediate in protein synthesis has been the reported failure to find them in natural sources (33,35,40,41,42). As Meister (43) pointed out, this evidence need not be conclusive since, for example, for many years no intermediates in fatty acid synthesis were found. Actually there is ample evidence for the occurrence of peptides per se in natural sources. This subject has been reviewed by several authors in recent years (44,45,46, 47,48). Additional reports of the occurrence of peptides are not difficult to find. Some are reported as being simply

oligopeptides (49,50,51,52,53), others contain phosphorus (54, 55) or non-amino acid sulfur (56). A number of peptides have been found bound to nucleotides (57,58,59) or to as yet poorly defined constituents (52,60).

Some of the peptides found in natural sources have known functions as hormones, antibiotics or cofactors. The validity of some of the other reported peptides, as Synge (45) pointed out, can be disputed on the basis of faults in the techniques used in isolation. There are, however, a large number of natural peptides whose validity is not disputed and whose function is yet to be determined.

C^{14} Incorporation into Peptides

An interesting recent development has been the report of rapid incorporation of fed C^{14} into peptides in certain microorganisms. Turba and Esser (61) reported finding 40 labeled peptides in Torula following the administration of C^{14} acetate. They found that the specific activity of the peptides increased more rapidly than either the amino acids or the proteins. McManus (62) has confirmed the observations of Turba and Esser. Connell and Watson (63,64) reported similar results after administering C^{14} glucose to Pseudomonas hydrophila. In this case the amino acids had the highest specific activity, but the specific activity of the peptides exceeded that of the proteins. These workers reported the isolation of 28 labeled peptides and determined the amino acid composition of these peptides.

Basis for Present Work

The possibility of studying amino acid incorporation and its relation to the peptides occurring in Drosophila was suggested when, on injecting C^{14} amino acids into Drosophila larvae to follow their incorporation into the lipid fraction reported by Hadorn and Mitchell (30), it was found that a number of chromatographically distinct components became labeled very rapidly. The same report by Hadorn and Mitchell had also noted the presence of a considerable amount of peptide material in Drosophila. Several of the reports cited earlier (11,22,23,31,32) confirm the presence of peptides in Drosophila.

In view of these observations it was decided to find out if the radioactive components observed following injection and the peptides which had been reported were related. If this proved to be true it was hoped that labeled peptides could be isolated from this natural source, and that their possible involvement in protein synthesis could then be investigated.

METHODS AND MATERIALS

Culture of Larvae

The larval third instar of the Oregon R strain of Drosophila melanogaster was the organism used throughout these investigations. Larvae were routinely raised on yeast cakes to which enough of a 2% sucrose solution had been added to make a thick paste. To obtain the large number of larvae used in part of this work approximately 1500 adult flies were placed in each half-pint milk bottle containing the yeast. The adult flies were allowed to remain for six hours and were then removed. Water was added to the bottles as needed in order to maintain a moist paste. Eighty-four hours from the mid point of the laying period the larvae were collected. To collect the larvae water was added to the culture bottles, and after stirring the larvae and yeast mixture was transferred to a large beaker. The yeast was then removed by repeatedly adding water and then decanting after each addition. The larvae were then poured on a silk screen in a Buchner funnel where washing was completed. Finally the larvae were spread out on filter paper and any debris (chiefly parts of adult flies) was removed. By this method from 1 to 2 grams of larvae were obtained from each culture bottle. Larvae grown on yeast were compared with those grown on a standard corn meal-agar-molasses media and

in neither squash experiments or in extracted preparations could differences be detected.

Injection of Larvae

The amino acid whose incorporation was being studied was injected into the hemolymph of the larvae. Larvae to be injected were attached to microscope slides by means of cellophane tape with the sticky side exposed. Injections were carried out by means of an apparatus devised by H. K. Mitchell (65). This device makes it possible to process large numbers of animals in reasonable time intervals. The apparatus delivers a very regular volume (1/5 to 1/20 of a lambda depending on adjustment) when the injection is made into a water drop, but there is about ± 25% variation in the amount received by larvae. This variation is apparently due to back pressure from the larvae. After experiments demonstrated that the incorporation pattern was not affected, larvae were routinely etherized for 75 seconds prior to injection.

Following injection the larvae were released from the cellophane tape by flooding with water. They were then rinsed with distilled water to remove any radioactive compounds remaining on the surface of the animals. When large numbers of larvae were required for preparation of extracts, injections were carried out in batches of thirty. At the chosen time from injection the larvae were frozen with either

dry ice or liquid nitrogen. They were then stored at -20°C until a sufficient number had been injected to make the extract. Larvae used in direct squash experiments were kept on moist filter paper for the appropriate time prior to preparation for paper chromatography.

Direct Squash Experiments

Patterns of incorporation of C^{14} labeled amino acids were obtained in direct squash experiments by squashing whole larvae on the origin of paper chromatograms at various intervals from the time of injection. Three animals were usually used at each spot. Drying of spots was speeded by an air stream pulled through the area of the spot by an aspirator. The chromatograms were developed in n-propanol:1% aqueous NH_3 (2:1). Following development, the chromatograms were scanned for radioactivity by the Actigraph II with a gas flow, micromil end window Geiger tube (Nuclear-Chicago). Quantitative information was obtained by measuring the area under radioactive peaks with a planimeter. Under the conditions used 1.0 on the planimeter scale equaled approximately 0.05 μC of radiation. Since it was not possible to inject the same amount of amino acid into each animal the radioactivity of a given spot was computed as the percentage of the total activity present. Little of the injected radioactivity was lost from the larvae during the course of experiments and the variation in amount injected did not

detectably affect the pattern observed. A greater difficulty with the method is the inability to be certain that the same compound or compounds are being measured at a given location at different time intervals.

The occurrence of the injected amino acid in any given spot was determined by eluting the spot, taking the eluent to dryness over P_2O_5 in vacuo, then hydrolyzing in sealed tubes with 6N HCl at 105°C. Following hydrolysis the samples were taken to dryness over P_2O_5 and NaOH in vacuo. After addition of water the samples were chromatographed and radioactive spots again determined. Chromatograms not eluted for hydrolysis and those prepared from hydrolyzed fraction were treated with ninhydrin (66) following counting, and the results were compared with known amino acids developed on the same sheet.

Attempts were made to alter the incorporation pattern by the use of various inhibitors. In separate experiments the possible inhibitors were injected at the time of, and prior to the time that the labeled amino acids were injected.

Preparation and Purification of Extracts

A mixture of 100 μC of C^{14} L-glutamic acid and 50 μC of C^{14} L-leucine was dissolved in 0.5 ml of distilled water. Approximately 7,000 larvae weighing 5 grams were injected with this mixture. Forty-six grams of uninjected larvae were added to the labeled animals and all were then ground

with dry ice at -80°C (67) in an Omni-Mixer (Servall). The resulting powder was placed on a sintered glass funnel and extracted with 900 ml of chloroform:methanol (2:1) at -20°C. The extract was washed with water according to Folsch's procedure (68) and after removal of chloroform and methanol the aqueous phase was retained. The residue from the chloroform:methanol extract was dropped into boiling water while still frozen and was homogenized for 5 minutes at 100°C. The homogenate was then centrifuged at 15,000 RPM for 20 minutes in the 30 rotor of the Spinco Model L ultracentrifuge. The supernatant was decanted and the water extraction of the residue was then twice repeated. The combined water extracts (170 ml) were added to the aqueous phase from the wash of the chloroform:methanol extract and the whole was placed on an ion exchange column for fractionation.

Fractionation was carried out on a Dowex 50-X4 22 mm x 45 cm column. The resin was prepared according to the method of Hirs, Stein and Moore (69). Since purified fractions were to be used in further studies a system of gradient elution using volatile buffers was adapted from the work of Thompson (70). The column was jacketed and the temperature was stabilized by the circulation of cold tap water. Prior to the separation of the radioactive larval extract the behavior of known amino acids and of other larval extracts had been determined using identical conditions. Fractions of 15 ml were collected from the column. A 25 lambda aliquot

was taken from each fraction, plated on an aluminum planchet and counted with a thin window, gas flow Geiger counter (Nuclear-Chicago). The remainder of the sample was lyophilized and the buffer was then removed by sublimation at 65°C in a vacuum oven. Ion free water (0.3 ml) was added to each fraction. Three lambdas of the resulting solution were spotted for chromatography and 10 lambdas were hydrolyzed with 6N HCl. Following the removal of the HCl from the hydrolyzed sample, the sample was taken up in 10 lambdas of water and 3 lambdas of this solution were spotted next to the unhydrolyzed sample. A mixture of six amino acids of known concentration was chromatographed on each sheet. The propanol-NH_3 solvent was used for development. Following chromatography those fractions having sufficient radioactivity were scanned with the strip counter. All strips were then treated with 1% ninhydrin in acetone (w/v). Color development was enhanced by heating for 60 minutes at 65°C in vacuo. Total ninhydrin color before and after hydrolysis was determined by means of the Spinco Analytrol Recording Densitometer. Concentrations were determined by reference to the known amino acids which were placed on each sheet.

Revised Extraction Procedure

Recent work (71) has indicated that Drosophila contains amino acids bound in linkages that are labile to heat and to acidic and basic conditions. To investigate these compounds,

as well as to obtain an additional supply of radioactive peptides, a new extraction procedure was devised. Larvae were ground with dry ice as before. The resulting powder was then extracted on a sintered glass funnel with 100% methanol (50 volumes) at -20°C followed by 50% aqueous methanol (50 volumes) at the same temperature. These combined extracts were defatted with chloroform and designated as Fraction 1. The residue was then extracted with chloroform: methanol (2:1) (50 volumes) at -20°C and this extract was treated with water at 0°C (68), the separated water phase being Fraction 2. The residue was then homogenized with 10 volumes of ice water in the Omni-Mixer. The homogenate was centrifuged at 28,000 RPM for 30 minutes in the 30 head of the Spinco preparative ultracentrifuge. The resulting supernatant (Fraction 3) was decanted from the residue. The process was then repeated on the residue to give Fraction 4. The same technique was then applied using water at 60°C (Fraction 5) and finally with boiling water (Fraction 6).

Using this procedure approximately 3000 larvae that had been injected with a total of 50 microcuries of C^{14} glutamic acid and then frozen 12-1/2 minutes from the time of injection were processed. Fractions 1 and 3 were further resolved by means of paper chromatography. Propanol:water (3:1) was used and the solvent was allowed to ascend the paper three times, the paper being dried between each ascension. Radioactive areas resolved in this manner were eluted with water

and were checked for bound glutamic acid content by means of radioactivity and ninhydrin patterns before and after hydrolysis. Quantitative ninhydrin determinations before and after hydrolysis were also made.

Reinjection of Fractions Containing Bound Glutamic Acid

Fractions from the Dowex column showing similar ninhydrin and radioactivity patterns were combined. Additional purification was then carried out by means of paper electrophoresis at 10.7 volts/cm for 4 hours using a pyridine:acetic acid:water (1:9:90) buffer. Fractions eluted from electrophoresis sheets were further purified by means of paper chromatography. The chromatographic behavior of the radioactive constituent was determined before and after hydrolysis as was the ninhydrin pattern. Only radioactive fractions having chromatographic behavior different than glutamic acid prior to hydrolysis, but showing glutamic acid as the only radioactive component after hydrolysis were used. All fractions showed more than one ninhydrin positive spot following hydrolysis. Some of the fractions were not ninhydrin positive prior to hydrolysis.

Preparations obtained from Fraction 1 of the revised extraction procedure were also used for reinjection. No attempt at purification beyond chromatography in propanol:H_2O was made. The preparations used contained glutamic acid as the only radioactive component and showed increased ninhydrin

reactivity, both in concentration and number of spots, following hydrolysis.

For determination of incorporation of radioactivity into protein 10 larvae were injected for each time interval. At the appropriate time from injection the larvae were frozen with dry ice. When an injection series was completed the larvae were homogenized in a glass cone grinder in 1.0 ml of hot 80% ethanol. The homogenate was then refluxed for 10 minutes. After cooling, the samples were centrifuged in a clinical centrifuge and supernatant liquid was then decanted. The residue was then extracted with 1.0 ml of ether which was decanted after centrifugation. Two washings with 1.0 ml of 80% alcohol then followed, and the supernatants from these washings were combined with the first alcohol supernatant. The residue was then treated with 0.5 ml of 0.1N NaOH for 30 minutes. After centrifugation, the NaOH supernatant was decanted and 50% trichloroacetic acid (TCA) added to it to give a final concentration of 10% TCA. The precipitate resulting from this treatment was centrifuged and the supernatant decanted. The precipitate was then washed with 0.5 ml of 80% alcohol. The resulting protein preparation was dissolved in 0.1 ml of 0.1N NaOH. Duplicate determinations of protein concentration were made by the method of Folin et al (72) using 10 lambda aliquots. The radioactivity of 75 lambdas of the solution was determined using the thin window

counter. The radioactivity of all of the solutions used in the washing procedure was also determined.

Miscellaneous Methods and Special Materials

In addition to the ninhydrin reagent, the -NH- chlorination method of Smith (73), the periodate method of Metzenberg and Mitchell (74) and the phosphate method of Bandurski and Axelrod (75) were used to look for compounds of interest on paper chromatograms. Chromatograms were also examined for absorption and fluorescence under ultraviolet lamps. Paper washed by elution with an NH_4OH solution was used in these, as well as in all other operations involving the use of filter paper.

The following radioactive amino acids were used:

DL-Alanine $2C^{14}$, Specific Activity (Sp Ac) 1 mC/mM, California Foundation for Biochemical Research

L-Aspartic Acid 2,3 C^{14} Sp Ac 2 mC/mM, California Foundation for Biochemical Research

L-Glutamic Acid (UL) C^{14}, Sp Ac 4.167 to 8.96 mC/mM, Nuclear-Chicago

Glycine (UL) C^{14}, Sp Ac 3.66 mC/mM, Nuclear-Chicago

L-Leucine (UL) C^{14}, Sp Ac 8.78 mC/mM, Nuclear-Chicago

L-Valine (UL) C^{14}, Sp Ac 11.8 mC/mM, Nuclear-Chicago.

RESULTS

General Pattern of Amino Acid Incorporation

The incorporation of aspartic acid, glycine, alanine, leucine, glutamic acid and valine, each labeled with C^{14}, was studied by chromatography of injected animals. In each case the labeled amino acid was rapidly incorporated into a number of radioactive fractions which could be separated by means of paper chromatography. The fractions so separated, when eluted, hydrolyzed, rechromatographed and recounted, for the most part yielded the injected amino acid, though this was not always the only radioactive component. Many of the eluted fractions showed only one ninhydrin positive spot prior to hydrolysis, and its chromatographic mobility was different than that of the injected amino acid. The eluted fractions, when hydrolyzed, gave several ninhydrin positive spots following chromatography, with one of the spots corresponding to the injected radioactive amino acid.

On the basis of incorporation patterns the injected amino acids could be divided into two classes, with leucine and valine belonging to one class and the non-essential amino acids to the other. While radioactivity from the injected amino acid was incorporated into a number of components other than protein when either essential or non-essential amino acids were used, the rate of increase of radioactivity in such components was much slower with the essential amino

acids, after a brief initial period following injection, than with the non-essential amino acids. The rate of incorporation of radioactivity into protein is also quite different with the two classes of amino acids. Figure 1 shows the rates of incorporation of glutamic acid and leucine. While the data in Figure 1 were obtained by determining specific activity of protein extracts, a similar difference could be seen in the results of the direct squash experiments. Figure 1 also shows that the simultaneous injection of essential amino acids enhances incorporation of the labeled amino acid into the larval protein.

Glutamic Acid Incorporation in Direct Squash Experiments

The results recorded in Table 1 and Figure 2 are typical of the pattern found in seven different series in which glutamic acid was injected. At the earliest time intervals the major radioactive peak matched the chromatographic position of known glutamic acid very closely. With increasing time from injection there was a marked tendency for this area of greatest radioactivity to shift to higher R_F. When the area was eluted, hydrolyzed and rechromatographed it once more closely matched known glutamic acid in position. It would thus seem that the major chromatographic peak is multicomponent in nature, and that the proportion of the various components change with time. All of the radioactive areas showed corresponding ninhydrin positive reactions, but since

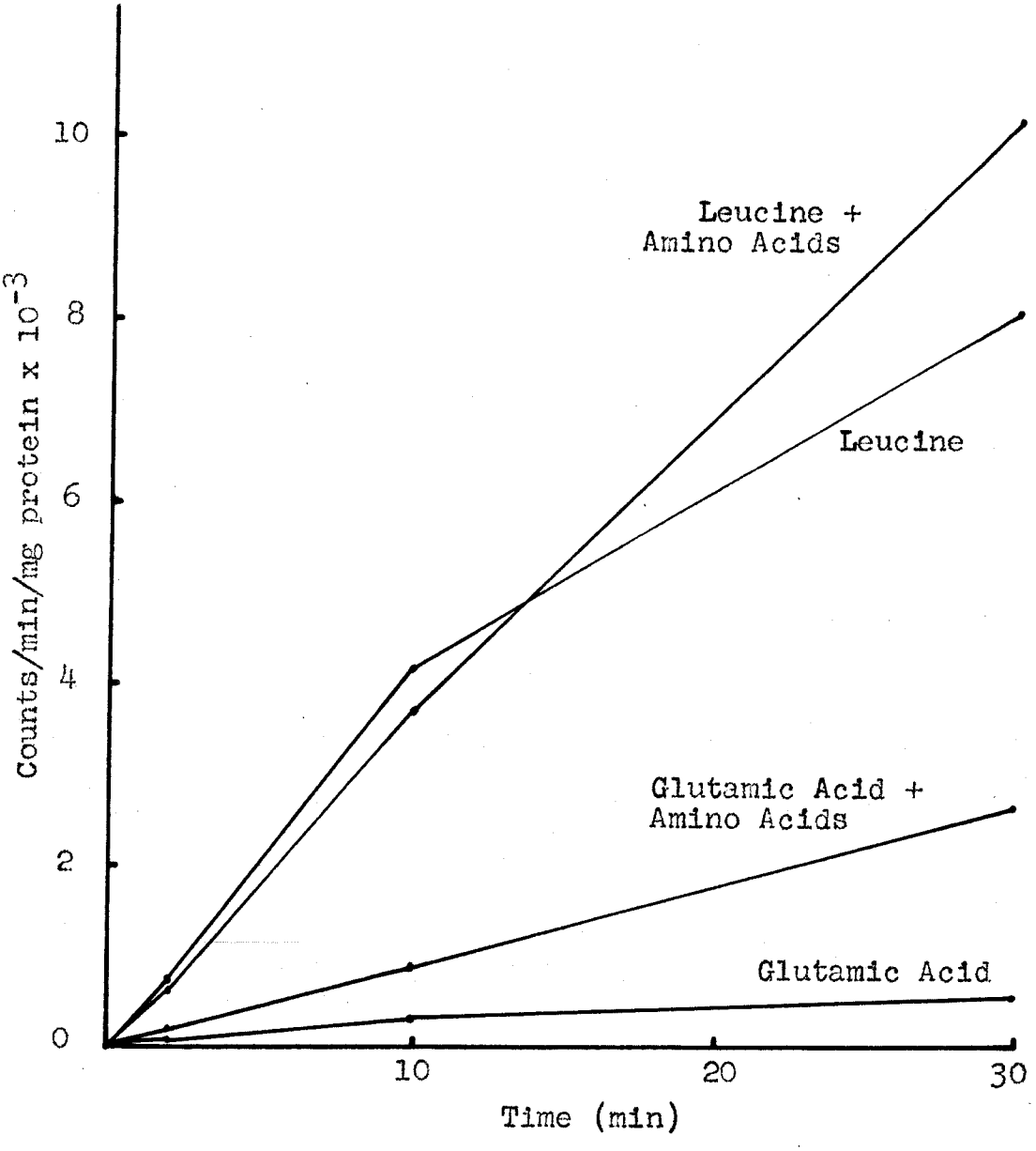

Figure 1

Eighty-four hour larvae were injected with a solution (3 μC/10 λ) of the radioactive amino acid, with and without added essential amino acids. Ten larvae were injected for each time interval and the specific activity of an extracted protein preparation was determined using the method given in the Methods and Materials Section.

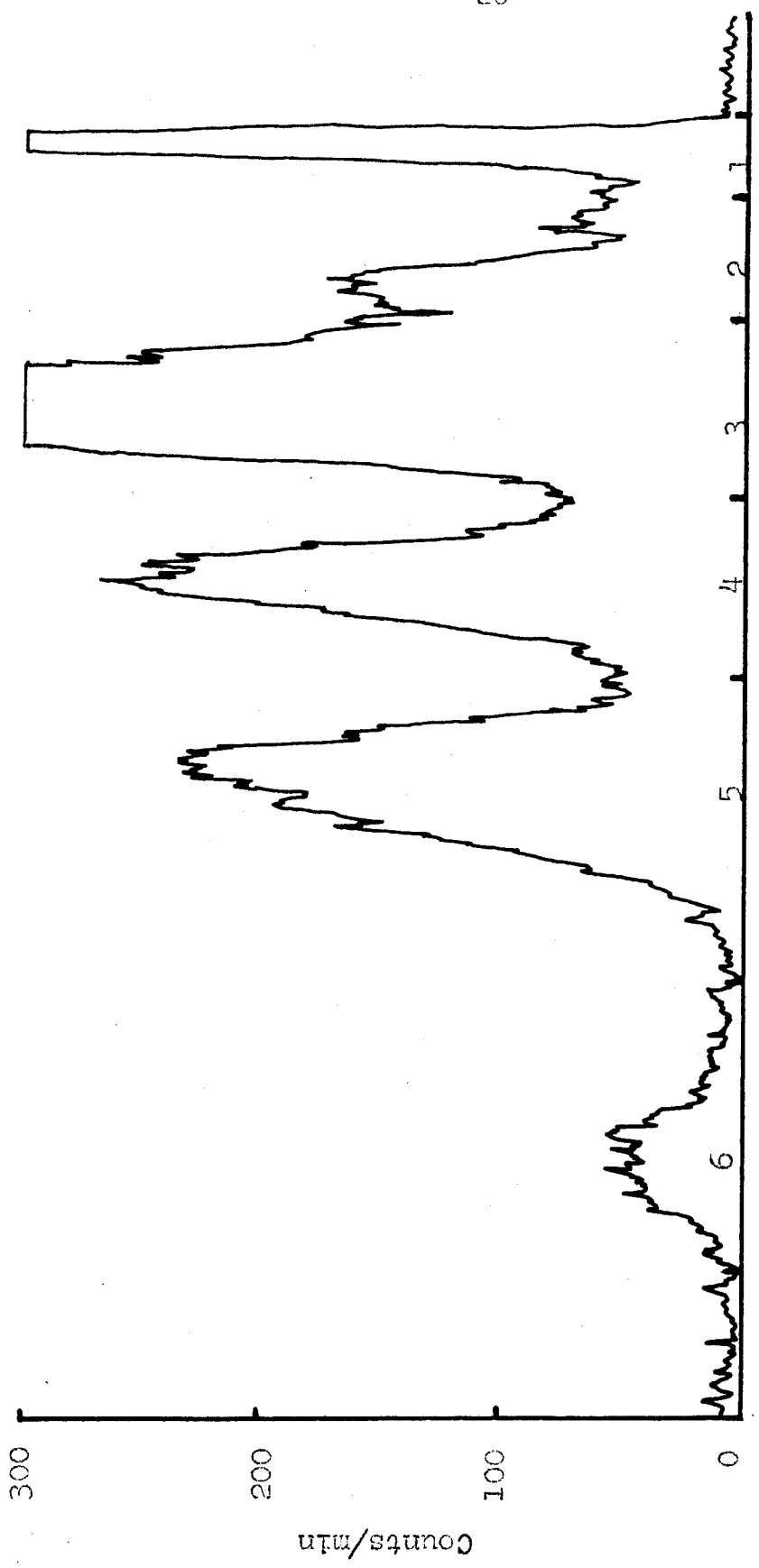

Figure 2

Distribution of radioactivity resulting from the paper chromatography of squashed whole animals injected with C^{14} glutamic acid (4 μC/15 λ). Numbers indicate areas measured by planimeter with 1 being the chromatographic origin and 3 most closely matching the position of known glutamic acid.

Table 1

Eighty-four hour larvae were injected with a solution of 4 μC/15 λ of L-glutamic acid and 3 larvae were squashed on Whatman #1 paper at the time from injection indicated. After chromatography in the propanol-NH_3 solvent the chromatograms were scanned with the strip counter and the areas determined by planimetry. Radioactivity is reported as the per cent of total radioactivity present. Areas refer to those designated in Figure 2.

Time Min	Total Area	% of Total Radioactivity at Area 1	2	3	4	5	6
1	1.855	1.5	5.7	77.8	8.3	6.9	0.0
5	1.955	2.5	9.2	62.0	11.8	12.3	2.4
15	3.885	9.6	9.7	42.7	16.6	17.4	3.9
30	.940	10.0	10.0	32.4	23.1	16.8	7.8
60	2.069	13.5	7.2	30.0	22.6	22.0	4.7

the ninhydrin pattern from whole animals is very complex it would be a mistake to take this as evidence that all of the radioactive components are actually ninhydrin positive.

On hydrolysis all of the radioactive components yielded glutamic acid, though this was not always the only radioactive component found. All of the radioactive areas, when eluted and hydrolyzed, yielded more than one ninhydrin positive spot following chromatographic separation.

It was found that four hours of starvation prior to the injection of the C^{14} glutamic acid did not appreciably alter the pattern of radioactivity found following chromatography. Increasing the amount of radioactive amino acid injected 4-fold likewise failed to alter the pattern observed.

Leucine Incorporation

The results given in Table 2 and Figure 3 are an example of those obtained in three experiments in which animals injected with leucine were squashed and chromatographed. As was the case with glutamic acid, the pattern of radioactivity resulting from the injection of leucine was very complex. It differs from glutamic acid in the more rapid incorporation of radioactivity into the origin material. Hydrolysis of material from various areas of the chromatograms resulted in the recovery of material which corresponded chromatographically to leucine, plus other ninhydrin positive material. However, at 30 minutes only about half as much of the non-origin

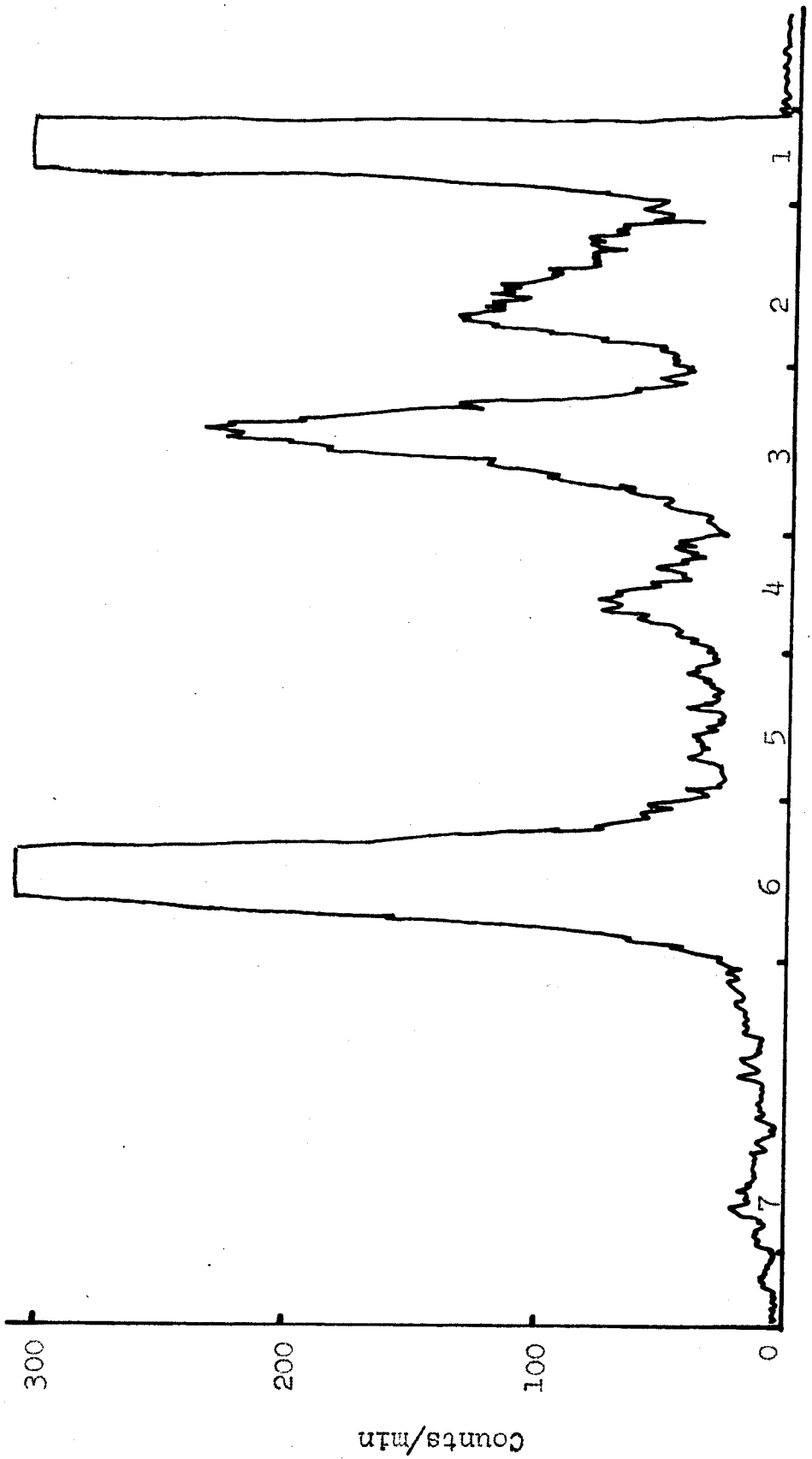

Figure 3

Distribution of radioactivity resulting from the paper chromatography of squashed whole animals injected with C^{14} leucine (10 μC/20 λ). Numbers indicate areas measured by planimeter with 1 being the chromatographic origin and 6 most closely matching the position of known leucine.

Table 2

Eighty-four hour larvae were injected with a solution of 10 μC/20 λ of L-leucine and 3 larvae were squashed on Whatman #1 paper at the time from injection indicated. After chromatography in the propanol:NH_3 solvent the chromatograms were scanned with the strip counter and the areas determined by planimetry. Radioactivity is reported as the per cent of total radioactivity present. Areas refer to those designated in Figure 3.

Time Min	Total Area	% of Total Radioactivity at Area 1	2	3	4	5	6	7
1.5	5.184	0.8	0.7	1.6	1.1	2.3	92.8	0.9
3	5.349	2.1	2.0	4.7	3.9	7.1	79.3	1.1
5	3.886	4.3	1.8	1.4	1.5	6.3	83.1	1.6
10	3.86	11.7	1.2	3.1	3.0	6.4	71.2	3.4
30	4.01	19.4	10.7	4.2	5.7	4.6	53.6	1.8
60	5.985	23.9	3.3	4.6	3.0	6.4	57.7	1.2
140	3.909	54.8	8.3	10.5	3.4	3.2	18.2	1.6

radioactive material hydrolyzed to the amino acid as was the case when glutamic acid was used. On the basis of the direct squash chromatograms of animals which have been injected with leucine it is difficult to draw any conclusions regarding the homogeneity of a given radioactive area. At some stages of the time series recorded in Table 2 there were well defined peaks at the chromatographic positions indicated. At both earlier and later times these same areas could not be distinguished from adjacent areas on the basis of radioactivity. It seems probable that different compounds were being measured at different points in the time series. This supposition is supported by the tendency for the point of greatest radioactivity to shift with time, even though it occurs in the same general area.

The leucine pattern is marked by the rapid formation of radioactive material which, with the exception of the material at the origin, shows little quantitative change with time. It is also noteworthy that even over a period of 140 minutes, during which the larvae were not fed, there was not a great loss in the total amount of radioactivity present.

Valine Incorporation

The results given in Table 3 and Figure 4 are representative of those found in five injection experiments using C^{14} valine. At no point during the time series was it possible to distinguish distinct peaks of radioactivity other than at

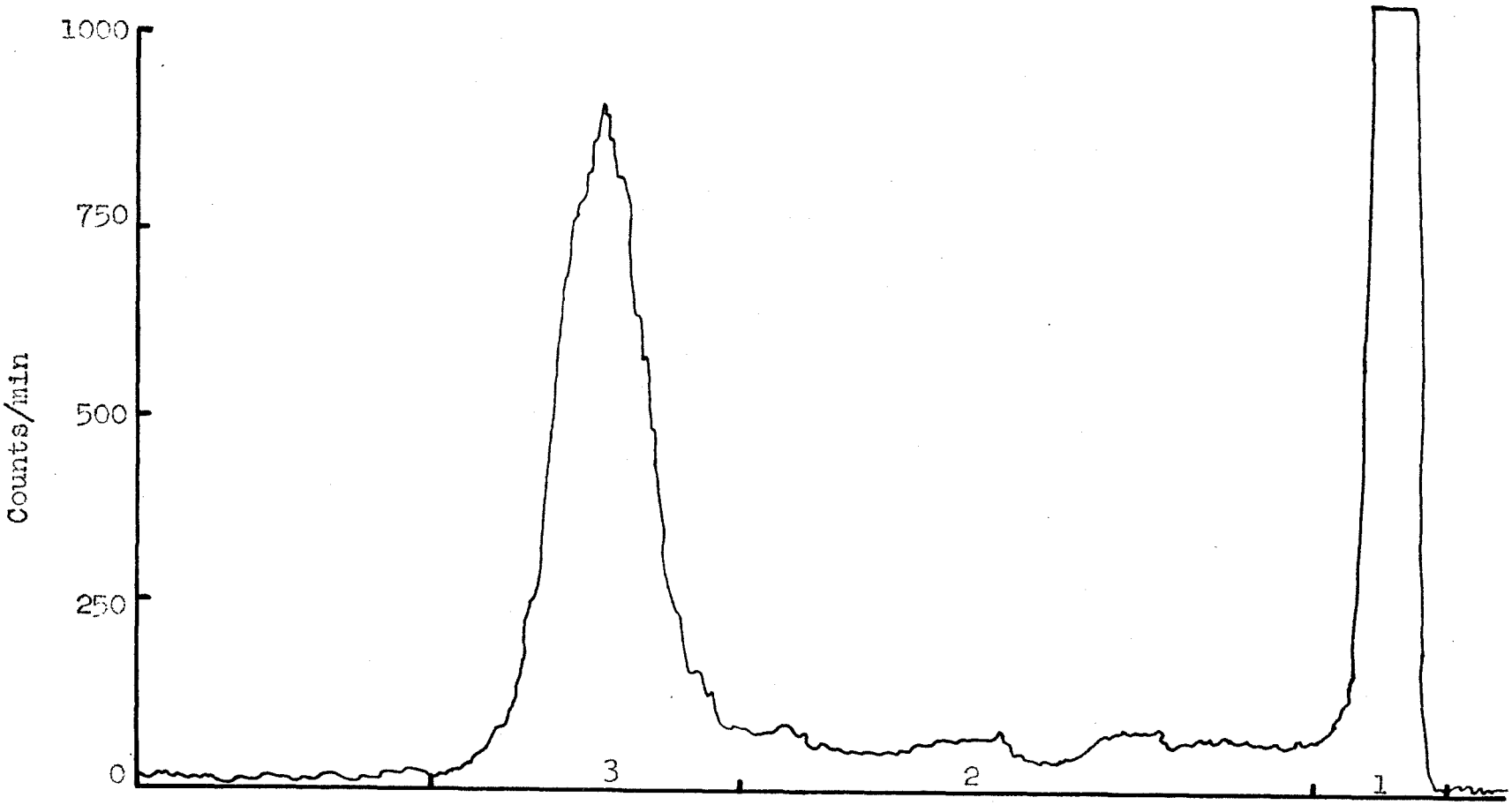

Figure 4

Distribution of radioactivity resulting from the paper chromatography of squashed whole animals injected with C^{14} valine (10 μC/20 λ). Numbers indicate areas measured by the planimeter with 1 being the chromatographic origin and 3 most closely matching the position of known valine.

Table 3

Eighty-four hour larvae were injected with a solution of 10 μC/20 λ of L-valine and 3 larvae were squashed on Whatman #1 paper at the time from injection indicated. After chromatography in the propanol-NH_3 solvent the chromatograms were scanned with the strip counter and the areas determined by planimetry. Radioactivity is reported as the per cent of total radioactivity present. Areas refer to those designated in Figure 4.

Time min.	Total Area	% of Total Radioactivity at Area 1	2	3
1.5	2.554	1.6	5.3	93.3
3	1.874	10.5	10.6	78.8
5	1.873	16.5	10.1	73.3
10	2.159	14.5	9.3	76.4
15	1.70	30.4	7.6	62.0
30	1.797	35.4	10.4	54.1
60	1.221	54.1	14.7	31.0

the origin and in the chromatographic position of valine. There was, however, a continuous zone of radioactivity between these two points. Though the results of the particular experiment recorded in Table 3 would seem to indicate considerable loss of radioactivity at the end of 60 minutes, it is felt that this is not the case. In other experiments with valine there was nearly as much total radioactivity remaining at 60 minutes as was found at earlier times in the series. It is most likely that the difference is attributable to variation in the amount initially injected.

On elution and hydrolysis of the material from the area between the origin and valine, radioactive valine was recovered as were other radioactive components. The ninhydrin pattern of the hydrolyzed material was complex and it is certain that amino acids in bound form were present prior to hydrolysis. As was the case with leucine, radioactive components other than the origin peak increase very rapidly initially and then show a much reduced rate of increase during the rest of the series.

Distribution of Glutamic Acid and Valine in Chromatographic Fractions

The information contained in Table 4 and Figure 5 was obtained during an early attempt to prepare material for reinjection. Enough animals (300) were injected with a mixture of glutamic acid and valine to cover the origin of a large

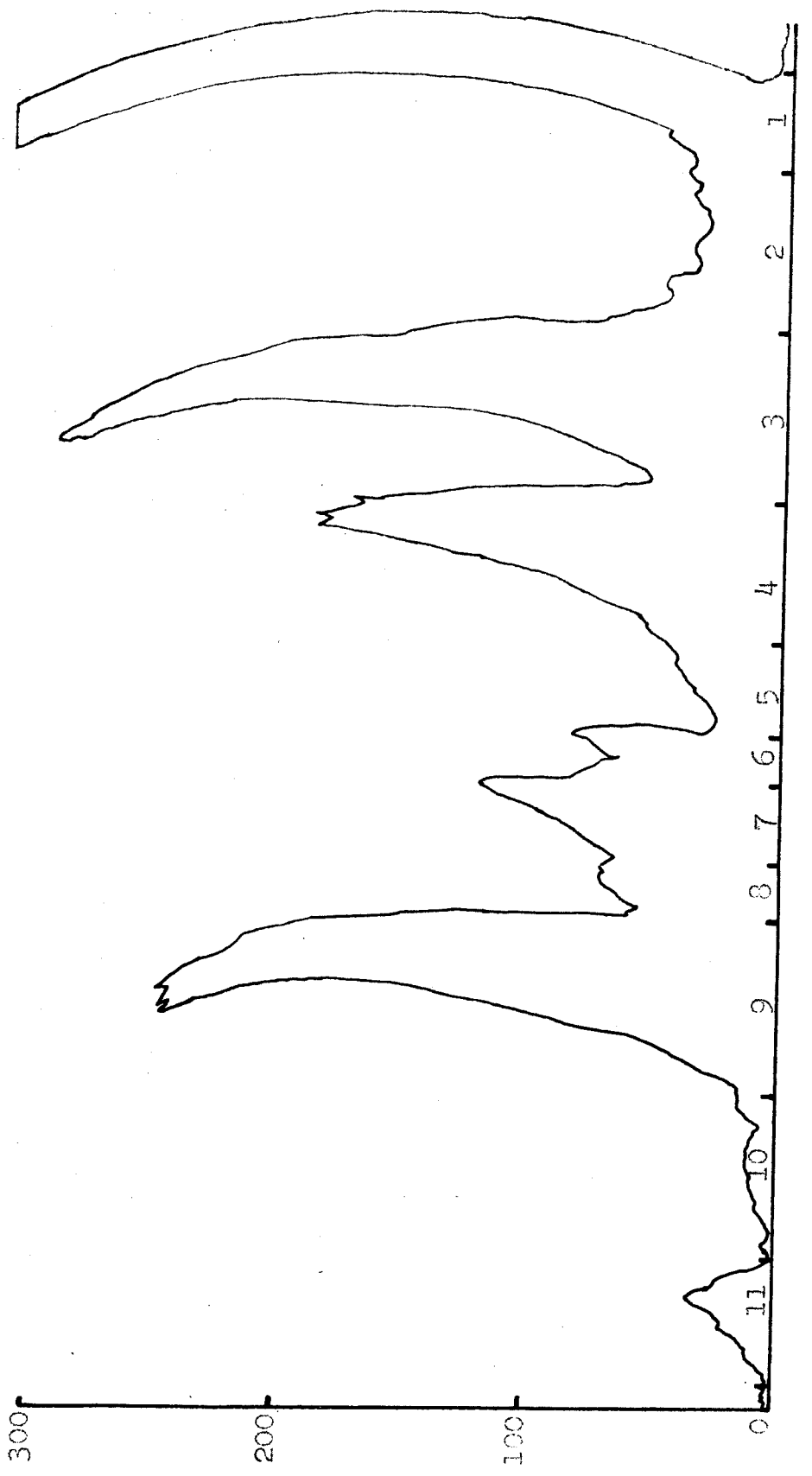

Figure 5

Distribution of radioactivity resulting from the paper chromatography of squashed whole animals injected with a mixture of radioactive glutamic acid and valine. The numbers indicate the areas eluted for hydrolysis with 1 being the chromatographic origin and 3 and 9 corresponding most closely to the positions of known glutamic acid and valine respectively.

Table 4

Distribution of Radioactivity in Chromatographic Fractions Following Hydrolysis

Aliquots were taken from the eluted fractions arising from the chromatography of squashed animals which had been injected with a mixture of radioactive glutamic acid and valine. Samples were hydrolyzed and chromatographed, then scanned with the strip counter. The estimated amount of radioactivity present is indicated by the number of + signs. Fractions refer to the areas indicated on Figure 5.

Fraction	Radioactivity at Position of Glutamic	Valine	Other
1	+	+++	0
2	+	+	±
3	+++++	+	±
4	+++++	+	±
5	+	+	++++
6	0	+	0
7	±	++++	++++
8	0	++++	0
9	0	++++++	±
10	+	+	0
11	+	±	0

sheet of washed chromatography paper. Thirty minutes after injection the animals were squashed. After drying, development in propanol-NH_3 was carried out. Following chromatography, a strip was cut from the sheet and scanned for radioactivity. Using this strip as a pattern the large sheet was cut into fractions for elution as is indicated in Figure 5. The eluted fractions were checked for chromatographic homogeneity, and after removing a small aliquot for hydrolysis attempts were made to reinject the remainder of the samples.

It was found that it was impossible to dissolve the eluted and dried fractions in a small enough volume to be suitable for reinjection. This was due to the large amount of material, other than the radioactive compounds, coming from the bodies of the whole animals. However, the results of the hydrolysis of the aliquots taken from the eluates are instructive in demonstrating the distribution of radioactivity in the chromatographically distinct fractions.

As Table 4 indicates the injected amino acids were found as peptides or other bound forms in a number of radioactive components. Radioactive material other than the injected amino acids was also found in several of the fractions following hydrolysis. In the case of Fractions 2, 3, and 4 this radioactivity remained at the chromatographic origin. In Fractions 5 and 7 the activity corresponded in chromatographic behavior to neither the injected amino acids nor the component

which had been hydrolyzed. No attempt was made to identify the radioactivity which was not present as either glutamic acid or valine.

Attempts to Alter the Incorporation Pattern

An extensive series of attempts were made to alter the incorporation pattern for radioactive amino acids. It was particularly hoped that compounds which have been found to act as inhibitors on protein synthesis in other systems (76,77) would prove effective in Drosophila, and that if this were the case intermediates in the synthesis would accumulate. Because Potter (78) in studying glutamic acid metabolism in mammalian brain tissue had observed that Metrazol has some effect on the incorporation pattern, this compound too was tested, along with other compounds also affecting the central nervous system. In addition to the chemicals used, the effect of starvation and of the simultaneous injection of a complete amino acid mixture was also tested.

Using valine as the injected C^{14} amino acid the data given in Table 5 were obtained. The values given are each taken from a time series and the values found at earlier times in the series were consistent with those reported in the table. The exact nature of the material found at the origin is not known. It is thought that a substantial part of it is protein. It is not possible to remove this material with either water or 10% TCA. Part of it can be eluted with

Table 5

Effect of Various Treatments on Incorporation of C^{14} Valine into Origin Material

Eighty-four hour larvae were injected with a solution of radioactive valine (10 μC/20λ) with the larvae being treated as indicated. The larvae were squashed on Whatman #1 paper and chromatographed. After scanning with the strip counter radioactive areas were measured and the percentage of the total radioactivity present was computed for each area. The values given are those for 60 minutes from the time of injection, with each being taken from a time series.

Treatment	% of Total Radioactivity at Origin
1. The injected larvae were placed on moist filter paper prior to being squashed on the chromatogram	54.1
2. As in 1 but with a complete mixture of amino acids from Drosophila protein injected simultaneously	62.9
3. Animals starved for 24 hours then injected with valine and placed on moist filter paper till squashed	50.7
4. As in 3 except that following injection the animals were allowed to feed on a moistened mixture of casein and brewers yeast prior to squashing	63.7
5. As in 4 except that the valine was dissolved in a solution of 10 mg/ml of p-fluorophenylalanine	65.0
6. As in 4 except that the valine was dissolved in a saturated solution (appr 0.04 mg/ml) of azaguanine	65.0
7. As in 4 except that the valine was dissolved in a saturated solution (2.5 mg/ml) of chloramphenicol	74.7

dilute NaOH. On hydrolysis this eluted material yielded valine as the only radioactive component.

None of the treatments listed in Table 5 was effective in altering the amount of radioactivity not found at the origin, or in the valine position. As the table indicates some variation in radioactivity incorporated at the origin was noted. Increase or decrease here was reflected in radioactivity present at the valine position. As the data in Figure 1 also demonstrated, added amino acids enhance incorporation when injected, and feeding larvae after injection has the same effect. Starvation prior to injection causes some reduction, but the effect is small.

The effect of chloramphenicol and p-fluorophenylalanine on protein synthesis was tested more directly by determining the specific activity of a protein extract. The specific activity of the protein preparation from treated larvae did not differ from the control values during a 60 minute time series in which activity was determined at 5 points. The increase found with chloramphenicol in the direct squash experiment has not been explained.

Using glycine as the labeled amino acid the accumulation of intermediates was searched for following the injection of the compounds listed: Desoxycholic acid (saturated, appx 0.24 mg/ml), linoleic acid (10 mg/ml), digitonin (10 mg/ml), arsenocholine (10 mg/ml), chloramphenicol (saturated, appx 2.5 mg/ml), p-fluorophenylalanine (10 mg/ml), streptomycin

(10 mg/ml) and 5 bromouracil (appx 3 mg/ml). In separate experiments the possible inhibitors were injected prior to, and simultaneously with the injection of the labeled amino acid. None of the compounds altered the pattern of distribution of radioactivity except desoxycholic acid, and this substance had caused extensive lysis in the body cavity.

The only compounds tested which affected the whole pattern of incorporation were Metrazol and chlorpromazine. When a 3% solution of Metrazol was injected, a concentration which allowed the animals to complete development to the adult stage, the specific radioactivity of the 30 minute protein extract was halved. This was true using either leucine or glutamic acid as the tracer compound. Injecting a 10% solution of Metrazol, which allowed the animals to survive for at least 24 hours prior to death, resulted in a 4-fold decrease in protein specific activity at 30 minutes as compared to the control value. The scanning charts of injected larvae treated with Metrazol showed a depression of all radioactive peaks usually seen, with the exception of the one attributable to the injected amino acid.

Chlorpromazine injected as a 4% solution was similar in effect to Metrazol. The same depression of chromatographic radioactive areas was observed and protein specific activity was reduced 4-fold. An attempt was also made to study the effect of the proprietary preparations Marsilid, Miltown and Reserpine. With these substances no effect was observed, but

since each was but slightly soluble in the volume required for injection the result was inconclusive.

Column Separation and Additional Purification of Radioactive Fractions

The work with injected larvae that were squashed directly on paper and then chromatographed made it clear that the system being studied was very complex. It was hoped that greater resolution could be obtained through the use of ion exchange chromatography. After preparing the extract as previously described the fractionation plotted in Figure 6 was carried out. A total of seven column fractionations of larval extracts have been made (71). The distribution of ninhydrin positive material, before and after hydrolysis, was similar in each fractionation.

As the elution diagram indicates, the extract was resolved into a large number of components. However, the elution pattern does not depict the true complexity of the mixture that is present in the larvae. As Table 6 indicates, very few of the fractions contain only one ninhydrin positive component. The validity of these few must be doubted since the experiments of Mitchell and Simmons (71) showed that to find more ninhydrin positive compounds it was only necessary to extract more larvae, and thus bring compounds at low concentration into the range of the analytical procedure. The measure of the heterogeneity of the system is not exhausted by

μ MOLES
COUNTS / MIN.
FRACTION NO.
cys.a
asp
thr
ser
pro
glu
gly
ala
val
iso
leu
cys
phe
tyr
lys
his
try
arg

Figure 6

Figure 6 (cont)

Elution diagram resulting from the ion exchange chromatography of a larval extract. The before hydrolysis concentration of ninhydrin positive material is indicated by the height of the open bars and the after hydrolysis concentration by that of the black bars. Radioactivity is shown by the continuous black line. The fractions in which known amino acids were eluted are indicated. This information was obtained by separating a known amino acid mixture under the same conditions used in this experiment. Introduction of eluting solvents was started with ammonium formate buffer (pH 2.49, 0.05M with respect to ammonium) after the collection of Fraction 11. Elution was continued to Fraction 75 with this buffer. At this point a pH and concentration gradient was started by slowly introducing ammonium formate buffer at pH 2.9 and 0.1M into the solvent reservoir (100 ml) containing the first buffer. Rapid mixing was assured by mechanical stirring. The addition of the pH 2.9 buffer continued until the introduction of the next buffer started. The other buffers, and the fraction at which their introduction into the mixing reservoir was started, were as follows:

Buffer	pH	Molarity	Fraction
Ammonium Formate	3.3	0.15	142
Ammonium Formate	3.65	0.2	191
Ammonium Acetate	5.5	0.4	259
Ammonium Acetate	6.8	0.6	294
Ammonium Acetate	8.0	1.0	359

Fractions eluted from the column were analyzed by the methods previously given.

Table 6

Homogeneity of Fractions from Dowex Column

Using the methods listed in the Methods and Materials Section an aliquot from each column fraction was analyzed. Information on radioactivity pertains only to those fractions up to, and including, the tubes containing free glutamic acid.

Criteria	# of Fractions
Total number of fractions analyzed	400
Number of fractions giving only one ninhydrin positive spot prior to hydrolysis	52
Number of fractions giving only one ninhydrin positive spot after hydrolysis	16
Number of fractions scanned for chromatographic position of radioactivity	131
Number of fractions having only one radioactive peak prior to hydrolysis	33
Number of fractions having only one radioactive peak after hydrolysis	83
Number of fractions containing C^{14} glutamic acid after hydrolysis	127
Number of fractions containing only C^{14} glutamic acid as a radioactive component after hydrolysis	78

ion exchange chromatography followed by chromatography of fractions with the propanol-NH_3 solvent. Paper electrophoresis and paper chromatography using other solvents showed that many of the spots seen following the first separation by paper chromatography were, in fact, multicomponent. The same complexity that was revealed by the ninhydrin reaction was also seen with the distribution of radioactive material.

In one series of attempts to purify radioactive components for reinjection, 26 glutamic acid containing compounds were separated from 5 fractions which had been formed by combining apparently similar fractions from the column fractionation. In addition to the 26 finally isolated, a number of compounds having low radioactivity were discarded during the purification procedure. In the same series 7 compounds were found which were radioactive, but ninhydrin negative, and did give a positive reaction for -NH- on application of the chlorination test. On hydrolysis these 7 components gave a ninhydrin spot corresponding to glutamic acid plus other ninhydrin positive areas, presumably amino acids.

The results of this column fractionation, taken with similar ones of Mitchell and Simmons (71), make it clear that much of the amino acid material in Drosophila is present in bound form as peptides. Comparing total ninhydrin positive material after hydrolysis to the total before, the column fractionated material gave a consistent total increase of

just less than 3-fold on hydrolysis. However, there is strong evidence to indicate that not all of the bound amino acids are in peptide linkage. An example is shown in Figure 7. Before hydrolysis both fractions were homogeneous in two two-dimensional chromatographic systems. Following hydrolysis both showed more than one ninhydrin positive spot, but while A showed a substantial increase in total ninhydrin positive material B showed none. Dr. H. K. Mitchell, who is currently investigating compounds of this type, has shown that many of the B type compounds give a positive periodate reaction.

With respect to radioactivity, Table 6 and Figure 6 indicate that the injected C^{14} glutamic acid was incorporated into a large number of fractions which were separated from the free amino acid. Many of these behave as orthodox peptides, others show the behavior of the B type compound illustrated in Figure 7. No fractions were recovered which contained enough radioactive leucine, which could be recognized as being bound, to detect by means of the strip counter. Since a primary purpose of the ion exchange fractionation was to recover peptides having enough radioactivity to allow reinjection, more sensitive methods for detecting bound C^{14} leucine were not used. Mitchell (65) has demonstrated that leucine is present in the larvae in a number of complex peptide fractions.

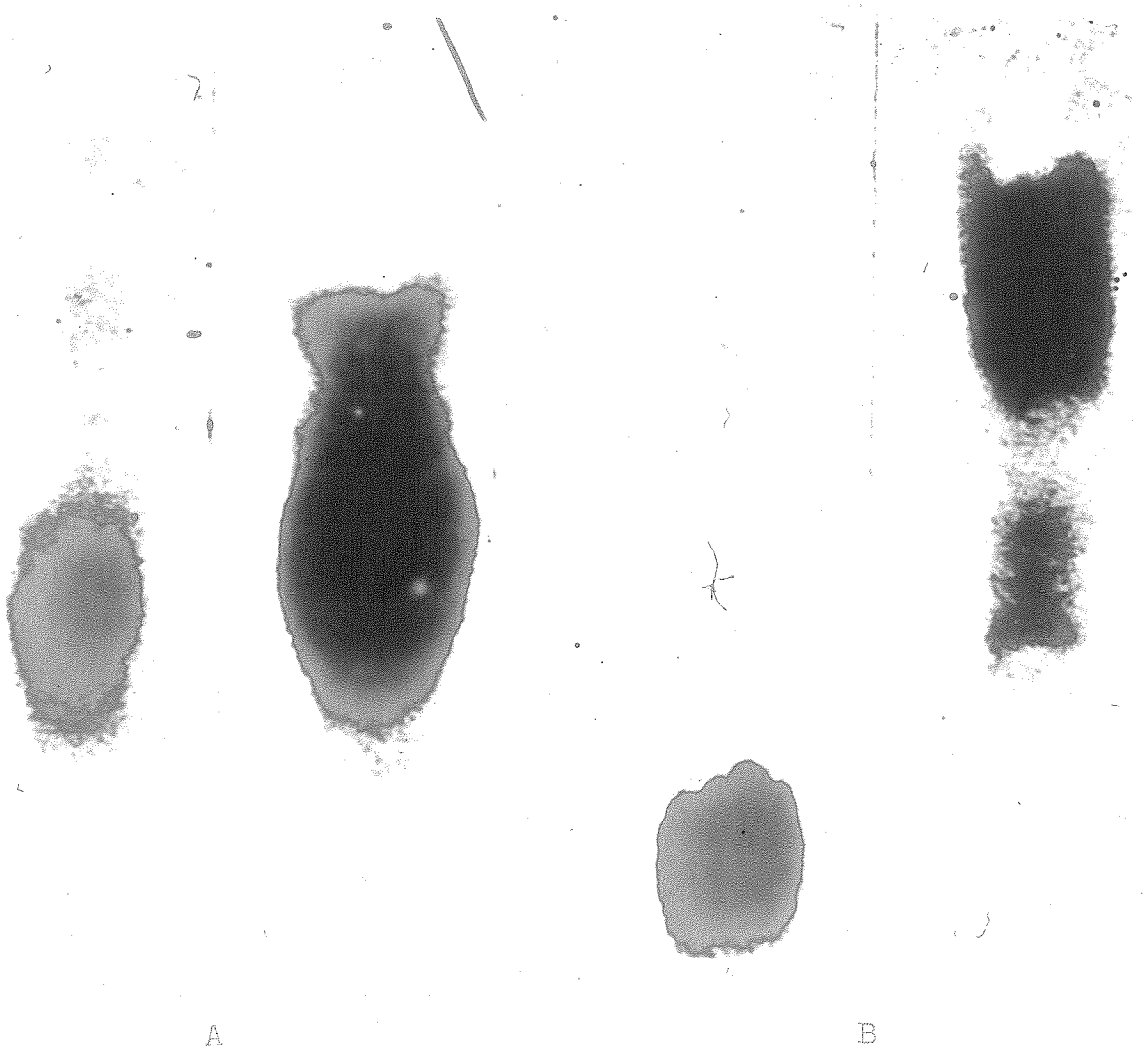

Figure 7

Picture of chromatograms which compare two types of compounds containing bound amino acids. In each pair the ninhydrin positive material found before hydrolysis is shown on the left and the after hydrolysis material on the right.

Material Prepared by the Revised Extraction Procedure

Table 7 gives the results of the ninhydrin analysis of material prepared by the mild conditions of the revised extraction procedure. It will be noted that hydrolysis produces nearly a 4-fold increase in ninhydrin positive material compared to a 3-fold increase with the column separated material. The presence of compounds unstable to either mild acidic or basic conditions was indicated when samples from Fraction 1 were chromatographed in three different solvents: propanol:H_2O (2:1), propanol:1% aqueous NH_3 (2:1) and propanol:30% aqueous acetic acid (2:1). When some bands resolved in these systems were eluted and rechromatographed in the same solvent, it was found that both the acidic and basic solvent produced splitting of the originally homogenous bands. The propanol:H_2O solvent did not produce this effect. For this reason only the propanol:H_2O solvent was used in partial purification of radioactive samples prepared by the revised extraction procedure.

Reinjection of Material Prepared from Drosophila Larvae

As has been previously indicated, attempts at purification of fractions from the ion exchange column produced a large number of radioactive fractions. As a result of this division no one fraction contained very much radioactivity. There was also difficulty in dissolving the separated fractions in volumes small enough to be suitable for reinjection. Attempts were made to reinject purified fractions, and while

Table 7

Ninhydrin Positive Material from the Revised Extraction Procedure

Equal aliquots from the fractions prepared by the revised extraction procedure were analyzed for ninhydrin positive material before and after hydrolysis. Concentration in μM was computed by comparison to a standard amino acid mixture chromatographed on the same paper.

Fraction	μ Moles Before Hyd	μ Moles After Hyd
1	330	610
2	0	0
3	18	108
4	18	125
5	70	660
6	10	200
Total	446	1703

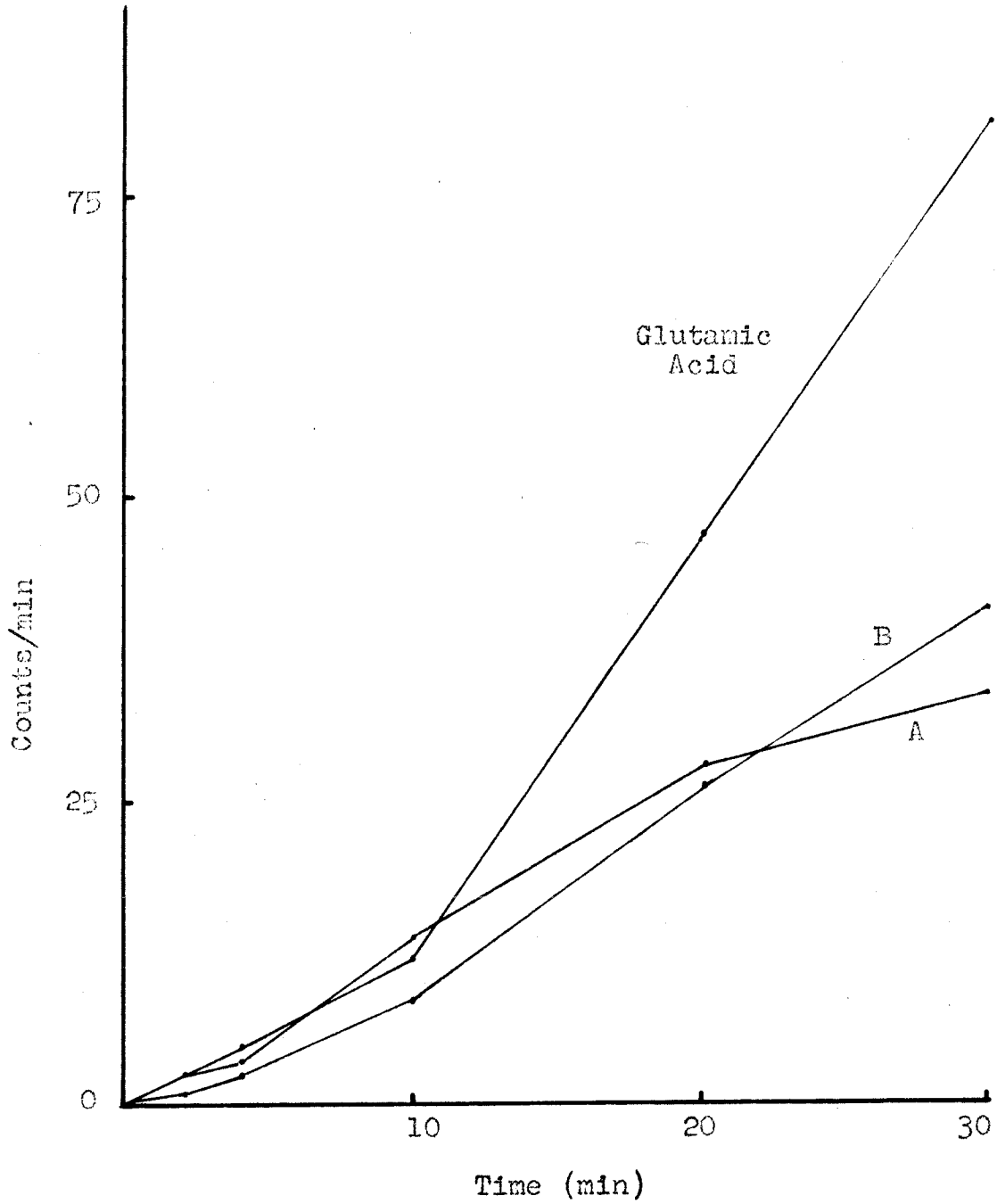

Figure 8

Larvae were injected with either chromatographic component A or B from Fraction 1 of the revised extraction procedure or with known glutamic acid. All three of the injected materials had an approximate specific activity of 70,000 counts/min/μM of glutamic acid present. Protein was prepared and its specific activity determined by the methods previously given.

the results indicated that radioactivity from the injected material was found in the protein extracted from the larvae, the amounts were so small that no critical analysis could be made.

Larvae were also injected with material from Fraction 1 of the revised extraction procedure. Using mild conditions chromatographic components A and B were obtained from Fraction 1. Component A moves to the same chromatographic R_F as glutamic acid does in the solvent used. On hydrolysis A gave three spots, probably glycine and lysine in addition to glutamic acid on the basis of chromatographic behavior. The glutamic acid concentration was approximately 3 times that of either of the other amino acids. The overall increase in ninhydrin reactivity following hydrolysis was about 2-fold. Component B separated cleanly from glutamic acid on chromatography in the propanol:H_2O solvent. Hydrolyses followed by two-dimensional chromatography revealed 4 ninhydrin positive spots, probably lysine, glycine and aspartic acid in addition to glutamic acid. The intensity of the ninhydrin color of the glutamic acid spot was about 4 times that of lysine and aspartic acid and about twice that of the glycine. The overall increase on hydrolysis was again about 2-fold.

The results obtained by injecting A and B and then determining protein specific activity are given in Figure 8, and are compared with the result of a control experiment.

The results leave little doubt that both A and B are different than free glutamic acid with respect to incorporation into larval protein. The possible implications of this finding will be discussed in the following section.

DISCUSSION

Amino Acids in Drosophila

As has been previously stated, there are many reports concerning the large quantities of amino acids found in insects. In the quantitative sense the present work supports these reports. In the matter of the form in which the amino acids occur there is less agreement. Assuming that Drosophila does not present a special case, the experiments reported here, taken with the concurrent ones of Mitchell and Simmons (71), offer proof that the concept that the hemolymph amino acids occur largely in the free form is not correct. Rather there is a very complex mixture of non-protein amino acid containing compounds (Table 6, Figure 6). There is good evidence that many of these compounds are peptides. The most straightforward explanation of the increase in ninhydrin positive material found following the hydrolysis of larval extracts would indicate, in fact, that a major portion of the amino acids are bound in peptide linkage. However, it is also indicated by the experimental results that in addition to being in peptides, amino acids also occur in combination with other constituents whose nature is yet incompletely defined (Figure 7).

The difference in the results of the present work and those reported by other workers is a reflection of the techniques that were used. A large part of the data which have been

reported by other workers was obtained by identifying hemolymph constituents on the basis of their chromatographic behavior on paper. In this regard the report of Deane and Truter (79) is significant. These workers made an extract of sheep wool. On the basis of chromatographic behavior in two-dimensional systems they were able to identify eleven "amino acids." However, on isolation and hydrolysis of these supposed amino acids they found that each of them was a peptide containing from eight to eleven amino acids. That this was not an isolated case was indicated when the same workers tested a second biological material (human perspiration) and found a similar phenomenon. Present experience with the ninhydrin positive material from Drosophila has also indicated that identical chromatographic behavior of peptides and amino acids is common. No doubt mistakes in identification have been made in the past when two-dimensional paper chromatography has been used to identify the elements of the complex mixture found in insects. In writing of the identification of free amino acids by means of paper chromatography, Deane and Truter stated, "The minimum requirement is that the chromatograms before and after hydrolysis should be identical; preferably, the single components should be hydrolyzed and examined separately." This is sound advice. It could be added that a quantitative comparison of the amount of ninhydrin positive material present before and after hydrolysis can also indicate the presence of peptides in a supposed

mixture of free amino acids.

The present experiments support the finding of other workers (5) that generally the non-essential amino acids are in higher concentration in insects than are the essential ones. The fact that a greater amount of radioactivity from leucine than from glutamic acid was found in protein (Figure 1) when they were injected in similar amounts and specific activities is most likely a reflection of the difference in pool size (both bound and free) for the two amino acids. Also in the fractions taken from the column separation (Table 6, Figure 6) many different fractions were found containing a considerable amount of radioactive glutamic acid while, for the most part, the amount of radioactivity present as leucine in bound form was below the sensitivity of the analytical methods used. The ninhydrin analysis for amino acids likewise indicated the presence of much more free and bound glutamic acid than was the case for leucine.

Amino Acid Incorporation into Bound Forms

The complexity of the mixture of amino acid containing compounds present in the larvae made it difficult to analyze the results of the experiments in which animals were injected and squashed directly on the chromatograms. It is clear that many components present in the hemolymph were labeled rapidly with the amino acids that were tested (Tables 1, 2, 3 and 4;

Figures 2, 3, 4 and 5). The time course of incorporation for a given component was confused, however, by the inability to be certain that the same component was always being measured on the counting chart. The compounds in the hemolymph which contain radioactive amino acids might arise from either the degradation of more complex material, such as protein, or by de novo synthesis. The experiments conducted did not critically distinguish between these two possibilities, but the rapid initial incorporation of the labeled amino acids into peptides and other bound forms, which exceeded the initial incorporation into protein, would seem to favor de novo synthesis. The failure to find large amounts of radioactive amino acids in bound form at the end of the time series when the initial rate of incorporation has been rapid would indicate that the amino acids contained in bound form are not inert, but are utilized further in the metabolic processes of the animal.

In the hope of accumulating greater quantities of compounds containing radioactive amino acids in bound forms, inhibitors such as chloramphenicol and p-fluorophenylalanine which are known to affect protein synthesis in some systems were used. In addition to these compounds twelve other possible inhibitors were tested as well as variations in feeding the larvae. With the exception noted, none of the treatments employed was effective in interfering with the utilization of the radioactive amino acid (Table 5). The failure

of starvation to reduce substantially incorporation of such amino acids is likely a result of the tendency for insects to maintain the hemolymph non-protein nitrogen pool at the expense of protein formed prior to the starvation period (16), thus making amino acids available for synthetic purposes.

Of the various compounds tested only Metrazol and chlorpromazine were effective in reducing protein synthesis. This was not accompanied by an accumulation of radioactivity into bound forms, rather incorporation into all components was much reduced. This depression of incorporation may result from a toxic effect on a general process such as energy supply, though it should be noted that levels of the drugs which produced a 2-fold reduction in incorporation during treatment did not interfere with normal development of the larvae to the adult stage. As contrasted to a general effect, the action of the compounds might be a more direct one on an early step in amino acid utilization, such as activation.

Functions of Bound Amino Acids

Considering that amino acids do occur as peptides, and in other bound forms, it may be asked, what is their origin and function? Turba and Esser (61) suggested these possibilities: a) peptides are intermediates in protein synthesis, b) they arise from a side reaction of unknown function, c) they are formed by non-enzymatic reaction of

activated amino acids. To these possibilities Connell and Watson (64) added a fourth, that the peptides arise from degradation processes as a result of experimental conditions. The present experiments were conducted in a manner designed to minimize the last possibility. The possibility of the bound amino acids being formed non-enzymatically is not ruled out directly, though neither the whole larval homogenate nor the material from extracts contains large quantities of activated amino acids as measured by hydroxamate formation (65). If the process is non-enzymatic it is not random. It was possible to isolate a limited number of distinct compounds rather than finding a complete spectrum of molecular types which might be expected from random combination of activated amino acids.

Of greatest interest in considering the function of compounds containing bound amino acids is the possibility that they are utilized in protein synthesis. In Drosophila, amino acids contained in bound form are incorporated (Figure 8), but how this is accomplished is not known. The combination of low specific activity in the radioactive materials isolated from the larvae, and the solubility problems introduced by the necessity of dissolving compounds in small volumes made the performance of critical test difficult. Central to the solution of these problems is the necessity to gain a detailed understanding of the chemical nature of the amino acid containing compounds found in Drosophila. With this accomplished,

peptides, or other amino acid containing compounds of interest, could be synthesized having the composition and specific activity desired.

With compounds of known constitution and specific activity it would be possible to examine the mechanism of incorporation more closely. Using labeled peptides in competition studies with unlabeled amino acids would seem to be a promising technique, particularly the use of peptides containing two C^{14} amino acids of known specific activity appears attractive. With such a compound the specific activity of amino acids incorporated into protein could be determined and the ratio of the specific activities in the protein compared to those in the peptide which was injected. From this it might be possible to decide whether the amino acids were incorporated intact in the bound form or were first degraded to the free amino acids.

From the present work it can be said that an amino acid contained in a bound form was incorporated into protein, but no mechanism is implied. The slower incorporation of the bound components A and B (Figure 8) as compared to free glutamic acid may indicate that the bound forms are being degraded prior to incorporation. The same data might be taken to indicate that the bound forms are limited in the kinds of protein into which they can be incorporated while the free glutamic acid is available for incorporation into all types of protein. Both components A and B and the free

glutamic acid injected at the same level of radioactivity showed a lag period prior to the phase of most rapid incorporation. Reactions prior to incorporation into protein thus seem to be necessary. This might involve the formation of stable intermediates, such as peptides, or reflect a period necessary for such a process as activation to occur. The existence of an organism which is in a very active metabolic state and which has considerable amounts, and many kinds of compounds containing amino acids in bound forms would seem to offer excellent opportunities for studies which will elucidate the role of such compounds in metabolic processes.

As was indicated, C^{14} amino acids were first injected into larvae in an attempt to study their association with lipid material. Since the initial experiments no attempts have been specifically made to further investigate the relation between amino acids and lipids. However, in the experiments where the specific activities of protein preparations were determined, it was found that the increase of radioactivity with time in the ether wash of the homogenized larvae paralleled the increase in specific activity found in the protein. This, perhaps, is of interest in view of the report of Hendler (80) which suggests a role for lipids in amino acid incorporation.

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