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An investigation was made into certain aspects of protein synthesis at different developmental stages of the flesh fly Sarcophaga bullata. This included measurements of the incorporation of labeled amino acids into protein by intact cells from a single organ, the larval fat body, and a study of amino acid activating enzymes in the fat body and in the developing pupa.

Fat bodies were dissected from Sarcophaga larvae and incubated for three hours with ^{14}C-labeled amino acids. The incorporation of amino acids into protein was greater with fat bodies from two or three day larvae than with those from six day larvae. In vitro protein synthesis by this tissue seemed to be correlated with the overall growth rate of the larvae. Incorporation of radioactivity into the lipid fraction of larval fat bodies followed the same pattern as that described for protein. It was also evident from these experiments that Sarcophaga fat bodies were capable of extensive degradation of amino acids to

carbon dioxide. The conversion of amino acids to carbon dioxide was most extensive with fat bodies from two day larvae, suggesting a greater energy requirement at this stage of rapid growth.

Amino acid activating enzymes were studied by the pyrophosphate exchange method in which the exchange of radioactivity between ^{32}P-labeled inorganic pyrophosphate and adenosine triphosphate is a measure of the enzymatic activity. Larval fat bodies were homogenized, fractionated by differential centrifugation, and the different fractions assayed for the presence of amino acid activating enzymes. The supernatant from one hour's centrifugation at 110,000 g. contained enzymes exhibiting activity toward nineteen amino acids, although there was considerable variation in the activation observed with different amino acids. It was further observed with this soluble fraction that the endogenous activity was always significant, probably as a result of the high level of free amino acids found in insect tissue; that the relative rates of activation did not agree with the relative rates of incorporation into fat body protein for those amino acids studied; that the sum of the activities observed with single amino acids was greater than the activity in the presence of a mixture of these amino acids; that preparations from different batches of larvae differed in their content of activating enzymes; and that the activity in the presence of D-valine was only slightly above the endogenous, although the greatest activity with a single amino acid was observed with L-valine. Activating enzymes

could be precipitated from the soluble fraction by adjusting the pH from 7.6 to 5.0 or lower. Although some separation of activity toward individual amino acids was attained, the specific activities of these fractions toward a mixture of amino acids were either less than or only slightly greater than the original soluble fraction.

The amino acid activating enzymes of Sarcophaga pupae were studied at different stages of pupal development. Activity decreased for the first few days, then rose to a maximum shortly before emergence of the adult flies. Determinations were also made on the concentrations of free amino acids and soluble protein. The patterns of amino acid activating enzymes, free amino acids, and soluble protein were consistent with the morphogenetic changes occurring during pupation, i.e. initial breakdown of larval tissue followed by synthesis of adult protein, utilizing amino acids produced by degeneration of the larval proteins.

PROTEIN SYNTHESIS IN *SARCOPHAGA BULLATA*

by

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PROTEIN SYNTHESIS IN SARCOPHAGA BULLATA

INTRODUCTION

One of the most important and fundamental problems of biochemistry has been that of the synthesis of proteins. At one time it was felt that the formation of these important constituents of living matter involved some mysterious "vital force", thus placing it beyond the realm of physical and chemical investigation. The crystallization of enzymes, however, revealed that proteins, although very large and complex, were well defined chemical substances. Research on protein synthesis remained very general nevertheless, simply because there were no tools available for more detailed studies.

Within the last twenty years this situation has changed completely. The development of new techniques, particularly the use of radioactive tracers, has permitted a very intensive investigation of the synthesis of proteins. The results of these studies have given us a fairly good understanding of the cellular components involved in protein synthesis, the mechanisms by which amino acids are brought together and united in peptide linkage, and even the manner in which the genetic material of a cell determines the sequential arrangement of amino acids.

In compiling this information, investigations of protein synthesis have been carried out with tissues from a wide

variety of organisms, including numerous mammalian and other vertebrate systems, plants, and all types of microorganisms. Aside from studies on the formation of silk, however, very little work has been done on protein synthesis in insects. Consequently an investigation was initiated into certain aspects of protein synthesis in the flesh fly Sarcophaga bullata, since the formation of protein during development of this insect is subject to a number of distinctive factors which may influence it. For example: insect hemolymph and tissue contain high levels of free amino acids, including the D-form in some species; the rate of protein synthesis is extremely high during the very rapid growth of the larva; the larval diet is composed primarily of protein with no carbohydrate and very little fat, so its amino acid metabolism may differ from that of organisms studied previously; the rate of protein synthesis undergoes extensive changes as the insect passes through different developmental stages; and the adult proteins are formed during pupation from the degradation products of larval proteins.

The majority of the work was with a single type of tissue isolated from the larval stage of the insect. This organ, known as the fat body, is composed of sheets of large cells loosely surrounding the digestive tract. While primarily a storage organ, the fat body contains many enzyme systems and actively synthesizes not only fat, but glycogen and protein as well. It has been likened in some ways to the mammalian liver (69, p 231).

The initial efforts were directed toward the *in vitro* incorporation of labeled amino acids into protein by intact fat bodies from larvae of different ages. Incorporation of amino acids into lipid and degradation to CO_2 were measured concurrently.

A more intensive study of a single step involved in the synthesis of protein was made by investigating the amino acid activating enzymes found in the larval fat bodies. This involved the determination of the cellular fraction containing the enzymes, an assay for specific amino acid activating enzymes, and a study of numerous factors affecting the activity of these enzymes.

Since one of the reasons for investigating protein synthesis in *Sarcophaga* was to try to correlate this activity with the extensive morphological changes taking place during development, the content of amino acid activating enzymes was determined at 24 hour intervals from the time of pupation to emergence of the adult fly.

HISTORICAL REVIEW

The classic work by Schoenheimer and his associates (78, p 333-344) provided a tremendous stimulus for the study of protein synthesis by introducing a powerful new tool, the use of radioactive tracers, while at the same time pointing out the complexity of protein metabolism in the whole animal. Early studies, therefore, were directed toward finding simpler systems which could incorporate amino acids into protein. This was first accomplished by Melchior and Tarver (67, p 309-315), who reported the incorporation of ^{35}S-containing methionine into the protein of rat liver slices. Similar findings were soon made with many amino acids and a variety of tissues, including rat intestinal mucosa (96, p 160-161), rat liver tumor (98, p 299-314), silkgland (100, p 624-626), and bone marrow cells (10, p 297-307). It was immediately found that energy yielding processes were required for the incorporation of amino acids, since the addition of respiratory poisons (96, p 160-161) or the uncoupling agent dinitrophenol (32, p 773-774) inhibited amino acid incorporation. Unfortunately, the demonstration of an energy requirement for uptake of amino acids by a system as complex as tissue slices did not provide any specific information about the mechanism of protein synthesis. It merely substantiated the endergonic nature of this process, a concept which had been generally accepted for some time (13, p 307-324).

Although it had been demonstrated repeatedly that tissue slices could incorporate amino acids, it was important to show that these results could be related to uptake of amino acids in the whole animal. One approach to this was to compare the initial rates of incorporation in the excised tissue with those observed *in vivo*. Although there are numerous factors making such a comparison quite difficult, substantial evidence was collected indicating that the initial rates of protein synthesis in tissue slices or whole cell suspensions were comparable to the rates for the same tissues in the intact animal (12, p 669-694).

Further substantiation that tissue slices were performing their *in vivo* function was provided by the findings of Peters and Anfinsen (75, p 171-179), who demonstrated that incorporation of labeled amino acids into chick liver slices occurred preferentially into serum albumin. Some examples of pure, identifiable proteins labeled by *in vitro* systems include serum albumin from chicken liver slices (74, p 461-470), ovalbumin from hen oviduct minces (4, p 739-744), and insulin and ribonuclease from calf pancreas slices (91, p 367-374).

While it is axiomatic that proteins must be derived ultimately from free amino acids, it was not established that free amino acids were the immediate precursors of a given protein molecule. The protein could conceivably be formed by combining peptide fragments or by the partial breakdown and reassembly of another protein molecule. However, experiments involving induction of enzymes in *Escherichia coli* (77, p 419-429),

in vivo incorporation of ^{14}C-labeled amino acids into rabbit muscle (85, p 61-71), *in vivo* labeling of casein in goat milk (5, p 105-115), antibody formation in rabbits (90, p 88-98) and *in vivo* synthesis of ferritin by rat liver (59, p 151-159) all indicated that protein synthesis proceeds from free amino acids with no significant participation of peptides or protein residues.

A logical prerequisite to the development of a satisfactory cell free system was the determination of the site of protein synthesis within the cell. One approach to this problem was employed by Borsook *et al* (11, p 839-848), who fractionated guinea pig liver excised 30 minutes after injection of labeled amino acids and determined the specific activities of different subcellular fractions. Although different amino acids were incorporated to different extents, the microsomes always showed the highest specific activity. More definitive were experiments in which the cells were fractionated after being labeled *in vivo* for various periods of time, thus permitting a study of the flow of amino acids within the cell. Utilizing this technique, Allfrey *et al* (3, p 157-175) found that the peak of activity in the microsome pellet was reached earlier than that of the supernatant fraction, but the radioactivity of the latter eventually became much greater. This implicated the microsomes as a site of synthesis for soluble proteins.

Even before the intracellular site of protein synthesis had been determined by *in vivo* experiments, attempts were made to

demonstrate the incorporation of labeled amino acids by cell free systems. Friedberg et al (34, p 441-442) were the first to obtain small amounts of incorporation into homogenates of several rat tissues. Siekevitz (84, p 549-565) reported that mitochondria and those substances necessary for oxidative phosphorylation were required in an incorporation system from rat liver, although the majority of the amino acid entered microsomal protein. It was found later that intact mitochondria could be omitted if hexose diphosphate, phosphocreatine, or phosphoenolpyruvate were added as a source of energy (99, p 337-354). If the soluble fraction were dialyzed, the addition of both adenosine triphosphate (ATP) and a high energy phosphate compound was required (51, p 45-59). Keller and Zamecnik (51, p 45-59) reported that the active component of the soluble fraction could be obtained by isoelectric precipitation at pH 5. This fraction was referred to as the "pH 5 enzyme". If the pH 5 enzyme were used in place of the dialyzed soluble fraction, which still contained nucleotides, it was necessary to add one more substance, either GDP or GTP. Incorporation of radioactivity into microsomal protein could now be demonstrated by incubating together a ^{14}C-amino acid, microsomes, pH 5 enzymes, guanosine diphosphate (GDP) or guanosine triphosphate (GTP), ATP and an ATP generating system. This was further refined by employing only the ribonucleoprotein particles (ribosomes) isolated from the microsome fraction by treatment with deoxycholate (58, p 111-123).

The requirement for ATP provided further substantiation that the condensation of amino acids into peptide linkage is an energy consuming process. In 1941, Lipmann suggested that compounds analogous to acetyl phosphate may serve as the activated form of amino acids (56, p 99-162). However, there was no progress toward identifying the activated precursors of protein until Hoagland (42, p 288-289) in 1955 demonstrated amino acid dependent exchange of radioactive pyrophosphate with the terminal phosphates of ATP in the presence of the soluble liver fraction required for amino acid incorporation into microsomes. A similar system in bacterial extracts was reported by DeMoss and Novelli (26, p 592-593). Exchange of ^{18}O has been observed between the carboxyl group of an amino acid and the adenosine monophosphate (AMP) moiety of ATP (45, p 215-217), suggesting that activation of the amino acid takes place through phosphorylation of the carboxyl group by the ribose bound phosphate of ATP, resulting in a mixed acid anhydride. Amino acid activating enzymes have been sought and found in all kinds of animal and plant tissues and in microorganisms (88, p 404). The activating enzymes are isoelectrically precipitated from the soluble fraction at pH 5, indicating that these enzymes are the constituents of the above mentioned pH 5 enzymes which are required for amino acid incorporation (43, p 345-358). DeMoss and Novelli (26, p 592-593) found that in the presence of their bacterial extracts eight different L-amino acids catalyzed the exchange of pyrophosphate into ATP.

By using proper precautions in preparing the enzymes, it was later possible to demonstrate activating enzymes for all common amino acids in preparations from E. coli (71, p 639-640); pigeon pancreas (57, p 67-73); pig liver, yeast, and pea seeds (94, p 125-134); and guinea pig liver (2, p 1061-1067). In 1956 Davie et al (22, p 21-38) were able to isolate a nearly pure enzyme from beef pancreas that was specific for L-tryptophan. Numerous other enzymes specific for single amino acids have since been prepared in highly purified form (76, p 287).

Since the aminoacyl adenylates are strongly bound to the activating enzymes (25, p 325-332), it was necessary to determine how the activated amino acids are transferred to the microsomal particles. Hoagland et al (46, p 215-216) in 1957 demonstrated that the pH 5 enzymes from rat liver contained approximately five percent ribonucleic acid (RNA), and that this RNA became labeled when the pH 5 enzymes were incubated with ATP and labeled leucine. The leucine-labeled RNA could be isolated, and when added to a microsomal suspension in the presence of GTP, leucine left the RNA and was transferred to microsomal protein material. This low molecular weight RNA has been studied in many systems (76, p 295-308), and is referred to as "soluble RNA" or "transfer RNA". Crude soluble RNA could bind several different amino acids and there was no competition, suggesting independent specific sites for each amino acid (44, p 241-257). Separation of soluble RNA into fractions binding specific amino acids indicates that

different molecular species act as specific acceptors of individual amino acids (15, p 103-104). In studying the transfer of activated amino acid from the enzyme to soluble RNA, Schweet et al (79, p 173-177) found that no cell fractions other than pure activating enzyme and soluble RNA were required. The activating enzymes are apparently responsible for both the activation of amino acids with the formation of enzyme bound aminoacyl adenylates, and the transfer of the aminoacyl residues to a specific soluble RNA molecule.

The activated amino acids bound to soluble RNA are then transferred to the ribosomes, where they are linked together by peptide bonds to form proteins. Little is known about this transfer, except that it seems to require GTP and to be stimulated by an enzyme factor from the soluble fraction after precipitation of pH 5 enzymes (44, p 241-257).

The process by which activated amino acids are condensed on the ribosomes in a genetically controlled sequence is perhaps the most important step in protein synthesis. Current thoughts on this matter are closely related to the hypothetical mechanisms proposed by Crick (20, p 138-163) in 1958 and by Hoagland et al (47, p 105-115) in 1959. They suggested that amino acids are bound to specific molecules of soluble RNA which act as adaptors to carry the amino acids to a template on the ribosome and locate the amino acids in the correct sequence. The template controlling the structure of the protein

being synthesized was supposed to be ribosomal RNA. In 1961 it was proposed that the template is actually an RNA molecule synthesized in the nucleus and with a base sequence related to the deoxyribonucleic acid (DNA), this RNA then becoming associated in some manner with the ribosomes (14, p 576-581, 38, p 581-585, 49, p 318-356). The order of amino acids is thought to be controlled by the sequence of nucleotides in this "messenger RNA", with a triplet of three nucleotides coding for a single amino acid (97, p 210-252). Apparently several ribosomes may be attached to a single strand of messenger RNA (93, p 1399-1403). The ribosomes presumably move along the RNA and the peptides grow on the ribosomes by sequential addition of amino acids as the amino acid code is read from the messenger RNA (36, p 148-157, 92, p 122-129).

There have been many studies of *in vitro* protein synthesis with excised silk glands, but very little work with tissues from other insects. Zamecnik *et al* (100, p 624-626) demonstrated in 1949 that silk glands from *Hyalophora cecropia* would incorporate radioactive glycine and alanine into silk protein *in vitro*. Under optimal conditions Shimura *et al* (83, p 285-294) observed a net synthesis of about 500 μg. of silk protein per hour in silk glands whose wet weight was approximately ten mg. *In vitro* fibroin synthesis by minces of the posterior silk gland was reported in 1958 by Takeyama *et al* (89, 233-243), who measured a rate of incorporation with this tissue that was nearly 200

times that of rat liver slices. These workers found that pre-incubation of the silk gland preparation with ribonuclease led to an impairment of its ability to incorporate glycine. When the mince used in these experiments was homogenized, its capacity for incorporation of ^{14}C-glycine into protein was almost completely lost, although there was some activity with a preparation containing only microsomes and the soluble fraction. Faulkner (30, p 71-78) observed in 1960 that the incorporation of labeled glycine into protein minces of silk gland from Bombyx mori was an aerobic process depending on the presence of divalent cations. Incubation under anaerobic conditions, or aerobically in the presence of either cyanide or ribonuclease, produced essentially no incorporation of radioactivity. The requirement for aerobic conditions for glycine incorporation into fibroin by excised silk gland was also reported by Shigematsu (82, p 295-320).

In 1959, Clements (17, p 665-675) incubated locust fat bodies for four hours in a saline solution containing approximately 0.5 μC. of glycine-U-^{14}C and observed incorporation of radioactivity into both the fat and protein fractions. Better than half of the ^{14}C taken up by whole fat bodies incubated with glycine-U-^{14}C or leucine-U-^{14}C was converted to carbon dioxide, while much less $^{14}CO_2$ was produced from acetate-2-^{14}C or glucose-U-^{14}C. This suggests that amino acids may be important substrates for respiration by fat body tissue. The incorporation of labeled amino acids into protein of isolated silkworm fat bodies has been reported by Shigematsu (80, p 880-882,

81, p 141-170). In contrast to findings with excised silk glands, a homogenate of the fat bodies was more active than the intact cells.

The investigation of amino acid activating enzymes in insects has received considerably less attention than studies of amino acid incorporation. Heller (40, p 397) examined the posterior silk gland from Bombyx mori for the presence of activating enzymes specific toward individual amino acids. Although glycine is a major constituent of silk fibroin, very low levels of glycine activation were observed. Maximum activity was for tryptophan, an amino acid found in only trace quantities in silk. The levels of amino acid activating enzymes during pupal development of the blowfly Lucilia cuprina were determined by Finch and Birt (31, p 59-64). The activities of these enzymes seemed to be correlated with the morphogenetic changes occurring during pupation. Howells and Birt (48, p 61-83) have recently extended these studies to include the complete life cycle of the blowfly.

MATERIALS

Unlabeled amino acids were obtained from California Corporation for Biochemical Research; ^{14}C-labeled amino acids and benzoic acid-1-^{14}C from New England Nuclear Corporation; ^{32}P-labeled orthophosphate from Nuclear Consultants Corporation; ^{32}P-labeled pyrophosphate from Nuclear-Chicago Corporation; ATP and liver concentrate from Sigma Chemical Company; p-terphenyl from Arapahoe Chemicals, Incorporated; 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP), 2,5-diphenyloxazole (PPO) and thixotropic gel from Packard Instrument Company; activated carbon, Tween 80, and Span 80 from Atlas Powder Company; and Cab-O-Sil from Cabot Corporation.

PART I

INCORPORATION OF LABELED AMINO ACIDS INTO PROTEIN

METHODS

Maintenance of Insect Colony and Rearing of Larvae

The adult Sarcophaga bullata were maintained in 11" x 12" x 15" cages in the laboratory, and were thus subject to a certain amount of temperature fluctuation. Each cage contained from two to three hundred flies, which were nourished solely by an aqueous sucrose solution.

The ovarian system of the female Sarcophaga does not develop until she has been exposed to meat. Therefore, small pieces of beef heart were placed in the cage for three or four days following emergence of the adult flies. In an attempt to keep the meat from drying out, it was usually supported by a pad of wet cotton in a petri dish. Since the meat still tended to become dry and hard, it was generally replaced daily. After about a week the females would deposit young, live larvae on the meat. Successive generations of larvae could be produced by exposure of the mature adults to meat for a short period of time. Addition of fresh meat a few days later would result in the deposition of larvae.

If a large number of larvae were desired for stock purposes to maintain the colony, beef heart was added to the cage when the females were about ready to deposit their larvae. The meat

was left in the cage for approximately 24 hours. During this period, several hundred larvae were usually deposited on the beef heart. Since the larvae begin to eat immediately, and grow rapidly, they were of various sizes and ages when removed from the cage. This was of little importance, however, because these larvae were simply allowed to develop into adult flies for maintenance of the colony.

When rearing larvae for experiments involving amino acid metabolism by larval fat bodies or amino acid activating enzymes in larvae and pupae, however, it was desirable to have them as uniform as possible. Therefore, larvae were taken directly from the female. This can be accomplished fairly easily with Sarcophaga, since the larvae can be forced from the female by gently squeezing its abdomen. It was usually possible to find several females containg larvae, provided, of course, that they had been exposed to meat a few days earlier.

Larvae were reared on beef heart, which was placed on top of from one to two inches of wet sawdust in a 600 or 800 ml. beaker. Depending upon the size of the beaker, from 60 to 100 larvae were added. The number of larvae placed in any beaker was only a rough estimate, since the young larvae were small and difficult to handle. A paper towel was fastened over the top of the beaker, and it was placed in a constant temperature room at 30°C. The larvae to be used in the experiments described above were maintained at this temperature for two, three,

or six days.

When larvae were being reared for stock purposes, they were left on the meat at 30°C. for five or six days. Larvae were then removed from the meat, washed with tap water, blotted dry on a paper towel, and placed in a large beaker containing dry sawdust. Following pupation, the pupae were transferred from the sawdust to a large dish, which was placed in a clean cage. The pupae were allowed to develop at room temperature. When the first adults began to emerge, the sugar solution was added to the cage.

Fat Body Isolation

Sarcophaga bullata larvae of the desired age were removed from the meat on which they had been growing, washed well with tap water, and blotted dry on a paper towel. The removal of the larval fat bodies was done in insect Ringer's solution in a petri dish resting on crushed ice. The Ringer's solution was that of Ephrussi and Beadle (29, p 218-225) consisting of a pH 7 solution at 128 mM NaCl, 4.7 mM KCl, and 1.9 mM $CaCl_2$.

Extirpation of the fat body simply involved snipping off the anterior tip of the larva with watchmakers forceps and squeezing out the gut and fat body. The fat body was then freed from the digestive tract and any parts of the trachea which might have been removed with the fat body. It was desirable to avoid bursting the gut, because this was highly contaminated with bacteria. These procedures for obtaining larval fat

bodies, which are white in color, were facilitated by dissecting under a low power magnifying glass in a petri dish which had been painted black.

Fat bodies from ten larvae were pooled for each incubation with a radioactive amino acid. Prior to addition to the reaction flask, they were washed by suspending the fat bodies in cold Ringer's solution, allowing them to settle to the bottom, and decanting off the solution. This was repeated several times.

Incubation of Fat Bodies with ^{14}C-Amino Acids

Incubations were carried out in 50 ml. Erlenmeyer flasks which were fitted with a center well. Each flask contained a mixture (Table I) of eighteen L-amino acids (0.0725 mM each), a ^{14}C-amino acid (0.25 μC.), liver concentrate (1.45 mg./ml.), and the fat bodies from ten larvae in a total volume of 3.45 ml. of insect Ringer's solution. The center well contained one ml. of KOH to absorb $^{14}CO_2$. The flasks were sealed with a rubber serum bottle stopper and gently shaken in a Dubnoff shaker at 30°C. for the desired period of incubation.

The flasks were removed from the shaker at the end of the incubation, opened, and the KOH in the center well removed. The well was rinsed several times with distilled water to insure a quantitative transfer of the KOH. The radioactivity of the $^{14}CO_2$ trapped by the base was then measured.

In some of the experiments the fat bodies and reaction media were transferred directly to a Dounce homogenizer and

Table 1

Amino acid mixture used in studies of amino acid metabolism by larval fat bodies and amino acid activating enzymes of larvae and pupae[a]

L-alanine
L-arginine
L-aspartic acid
L-cysteine
L-glutamic acid
glycine
L-histidine
L-hydroxyproline
L-isoleucine
L-leucine
L-lysine
L-methionine
L-phenylalanine
L-proline
L-threonine
L-tryptophan
L-tyrosine
L-valine

[a]Equimolar quantities; occasionally, double the amount of DL-amino acid, was substituted for the L-form.

homogenized together. In other experiments, however, the fat bodies and media were removed from the reaction flask and the media decanted off. The fat bodies were washed four times with Ringer's solution to free them from external radioactivity, i.e. from the ^{14}C-amino acid in the reaction media. The washed fat bodies were then homogenized in glass distilled water.

The fat body homogenate obtained by either of the above methods was transferred to a 15 ml. glass centrifuge tube, mixed with an equal volume of $CHCl_3$-CH_3OH (2:1), stirred and centrifuged at low speed. Two liquid layers were formed, with solid material compacted at the interface. Both liquid layers were poured off, the solid adhering to the sides of the centrifuge tube. The $CHCl_3$-CH_3OH fraction was separated from the aqueous layer. The solid material in the tube was resuspended in the aqueous fraction and mixed again with an equal volume of $CHCl_3$-CH_3OH (2:1). Following centrifugation, the two liquid layers were poured off, separated, and the $CHCl_3$-CH_3OH was combined with that obtained previously. In some experiments, the second $CHCl_3$-CH_3OH extraction was carried out on the solid material alone, rather than on a suspension of the solid in the aqueous fraction. The combined $CHCl_3$-CH_3OH extracts, obtained by either method, were taken to dryness under a stream of air and the radioactivity of the residue was determined.

The aqueous fraction was made to a final concentration of five percent trichloroacetic acid (TCA) and centrifuged. The very small amount of precipitate thus obtained was added to

the solid material from the homogenate remaining after $CHCl_3$-CH_3OH extraction. In those experiments in which the fat bodies were washed free of the reaction media prior to homogenization, the TCA supernatant was filtered through glass wool and its radioactivity measured.

The solid fraction, including both the material from the homogenate after $CHCl_3$-CH_3OH extraction and the TCA precipitate from the aqueous fraction, was transferred to a 13 x 100 mm. culture tube. It was washed twice with 95 percent ethanol to remove all $CHCl_3$ and five times with five percent TCA. This was followed by five washings with 95 percent ethanol to remove all traces of TCA. The white or light tan precipitate was air dried, ground to a fine powder, and its radioactivity determined. This material was referred to as the protein fraction.

Measurement of Radioactivity

The dried, finely ground protein was carefully weighed into glass counting vials. Fifteen ml. of scintillator gel was added to each vial, and the protein was suspended in the gel by shaking. The scintillation gel was prepared by mixing p-terphenyl (300 mg.), POPOP (3 mg.), Cab-O-Sil (4 g.), and toluene (100 ml.) in a Waring blendor for five minutes. The protein samples were counted in a Tracerlab two channel liquid scintillation counter at a high voltage setting of 1550 volts and a gain of 16. A known amount of benzoic acid-1-^{14}C in toluene was added as

an internal standard, and the samples were counted again to determine the efficiency of the counting system.

$$\frac{a-b}{c} \times 100 = \% \text{ efficiency}$$

a - observed counts per minute (CPM) due to unknown and internal standard
b - observed CPM due to unknown sample
c - known disintegrations per minute (DPM) of internal sample

The radioactivity of the protein was expressed as DPM per mg. of dry protein.

The same instrument settings and procedure for determination of efficiency were used for measuring the radioactivity of the CO_2, the $CHCl_3$-CH_3OH extract, and the aqueous fraction.

The CO_2 which was trapped by KOH during incubation was prepared for counting in two ways. In early experiments the CO_2 was precipitated from KOH solution as $BaCO_3$, plated out, and dried. The dry $BaCO_3$ was removed from the planchets and placed in glass counting vials. It was then suspended in the gel described above and its radioactivity measured in the liquid scintillation counter. In other experiments, the volume of the KOH solution containing the CO_2 was determined and a one-half ml. aliquot was added to a counting vial. To this was added 14.5 ml. of a scintillation gel containing a 1:9 mixture of Tween 80-Span 80 (2.4 ml.), glycerol (3 ml.), Thixotropic gel (7.5 g.), PPO (1.2 g.), and toluene (300 ml.). The gel was prepared by mixing in a Waring blendor for two minutes, chilling in the cold room for a few minutes, and mixing again briefly. The

one-half ml. of KOH and 14.5 ml. of scintillator gel were mixed thoroughly by shaking and counted as described earlier. Radioactivity was expressed as DPM in the total CO_2 evolved by the fat bodies from ten larvae. There was no discernible difference in the results obtained by the two methods.

The $CHCl_3$-CH_3OH extracts were taken to dryness. The residue was dissolved in ten ml. of toluene and added to a counting vial. To this was added five ml. of toluene containing 60 mg. PPO and 0.75 mg. POPOP, giving a final concentration of 4 mg./ml. PPO and 0.05 mg./ml. POPOP. The samples were counted in the liquid scintillation counter, and radioactivity was expressed as DPM in the total $CHCl_3$-CH_3OH extract from the fat bodies of ten larvae.

The volume of the aqueous sample was measured and a one-half ml. aliquot added to a counting vial. This aliquot was mixed with 14.5 ml. of the Thixotropic gel described above for determination of $^{14}CO_2$ and counted in the same manner. The results were expressed as DPM in the total aqueous fraction from the fat bodies of ten larvae.

RESULTS

Metabolism of Amino Acids by Intact Fat Bodies

The primary purpose of experiments carried out with intact larval fat bodies was to study the incorporation of labeled amino acids into protein. In conjunction with this it was of interest to observe the evolution of $^{14}CO_2$ and the incorporation of radioactivity into the $CHCl_3$-CH_3OH fraction. This latter fraction was of interest since in these insects much of the carbon in lipids is derived from amino acids. The results of experiments in which ^{14}C-labeled amino acids were incubated with intact larval fat bodies are shown in Table II. The fat bodies were dissected from larvae which were two, three, or six days old and incubated with 0.25 μC. of DL-glutamic acid-3,4-^{14}C, L-histidine-2-ring-^{14}C, or L-valine-U-^{14}C for three hours at 30°C. All of the incubations were carried out in the presence of a mixture of eighteen unlabeled amino acids (Table I), which were 0.0725 mM for the L-form. The radioactivity of the protein is given as DPM per mg. of dry protein. Values for evolved CO_2 and the $CHCl_3$-CH_3OH fraction both represent the total activity of the fat bodies from ten larvae.

Time Course Studies

In order to observe the rate at which L-valine-U-^{14}C was incorporated into protein, incorporated into the aqueous fraction, and degraded to $^{14}CO_2$, time course studies were made.

Table II

Radioactivity of different fractions after incubation of ^{14}C-labeled amino acids with intact fat bodies[a,b]

^{14}C-Amino Acid	Larval Age (days)	Number of Experiments	Protein (DPM/mg. protein)	CO_2 (DPM)	$CHCl_3$-CH_3OH Fraction (DPM)
DL-glutamic acid-3,4-^{14}C	3	2[b]	663	34,762	11,165
DL-glutamic acid-3,4-^{14}C	6	1[b]	149	6,792	6,971
L-histidine-2-ring-^{14}C	3	2[b]	1,140	174	897
L-histidine-2-ring-^{14}C	6	1[b]	328	451	775
L-valine-U-^{14}C	2	1[b]	3,762	286,875	3,428
L-valine-U-^{14}C	3	9	2,608	36,758	2,007
L-valine-U-^{14}C	6	1[b]	1,429	1,188	1,834

[a]All incubations for three hours and in the presence of a complete mixture of amino acids

[b]Duplicate incubations carried out in each experiment

In these experiments, L-valine-U-^{14}C was incubated with intact fat bodies from three day larvae for various periods of time, from 20 minutes to four hours. The radioactivity of the protein, aqueous fraction, and total evolved CO_2 were measured at the end of all incubations. These results, expressed as percent of maximum activity attained in each individual experiment, are given in Table III.

The comparative activities after different periods of incubation are plotted in Figure 1 for the protein, aqueous fraction, and evolved CO_2.

Effect of Omitting Unlabeled Amino Acids

Since the fate of an amino acid within a cell may be strongly influenced by its availability and concentration, as well as that of all other amino acids, the effect of omitting unlabeled amino acids on the metabolism of L-valine-U-^{14}C by intact fat bodies from three day larvae was studied. Incubations were carried out in the presence of the complete amino acid mixture, in the presence of the complete mixture less valine, and in the absence of all added amino acids. All incubations were run in duplicate. Results for the incorporation of ^{14}C-valine into the protein and $CHCl_3$-CH_3OH fractions and for the evolution of $^{14}CO_2$ are in Table IV.

Variability of Amino Acid Incorporation into Protein

Fairly large variations were observed in the activities of fat bodies obtained from one set of larvae, as compared with

Table III

Radioactivity of the protein, aqueous fraction, and evolved CO_2 from intact fat bodies incubated with L-valine-U-^{14}C for various periods of time

Incubation Time (hours)	Protein				Aqueous Fraction				CO_2			
	Experiment				Experiment				Experiment			
	1	2	3	Ave.	1	2	3	Ave.	1	2	3	Ave.
1/3	61	49	22	44	82	100	100	94	3	5	4	4
2/3	76	57	37	57	100	74	69	81	9	14	16	13
1	90	67	44	67	66	87	60	71	18	33	45	32
2	87	100	60	82	64	59	66	63	74	90	81	82
3	97	98	100	98	47	47	36	43	100	100	88	96
4	100	--	91	96	42	--	56	49	96	--	100	98

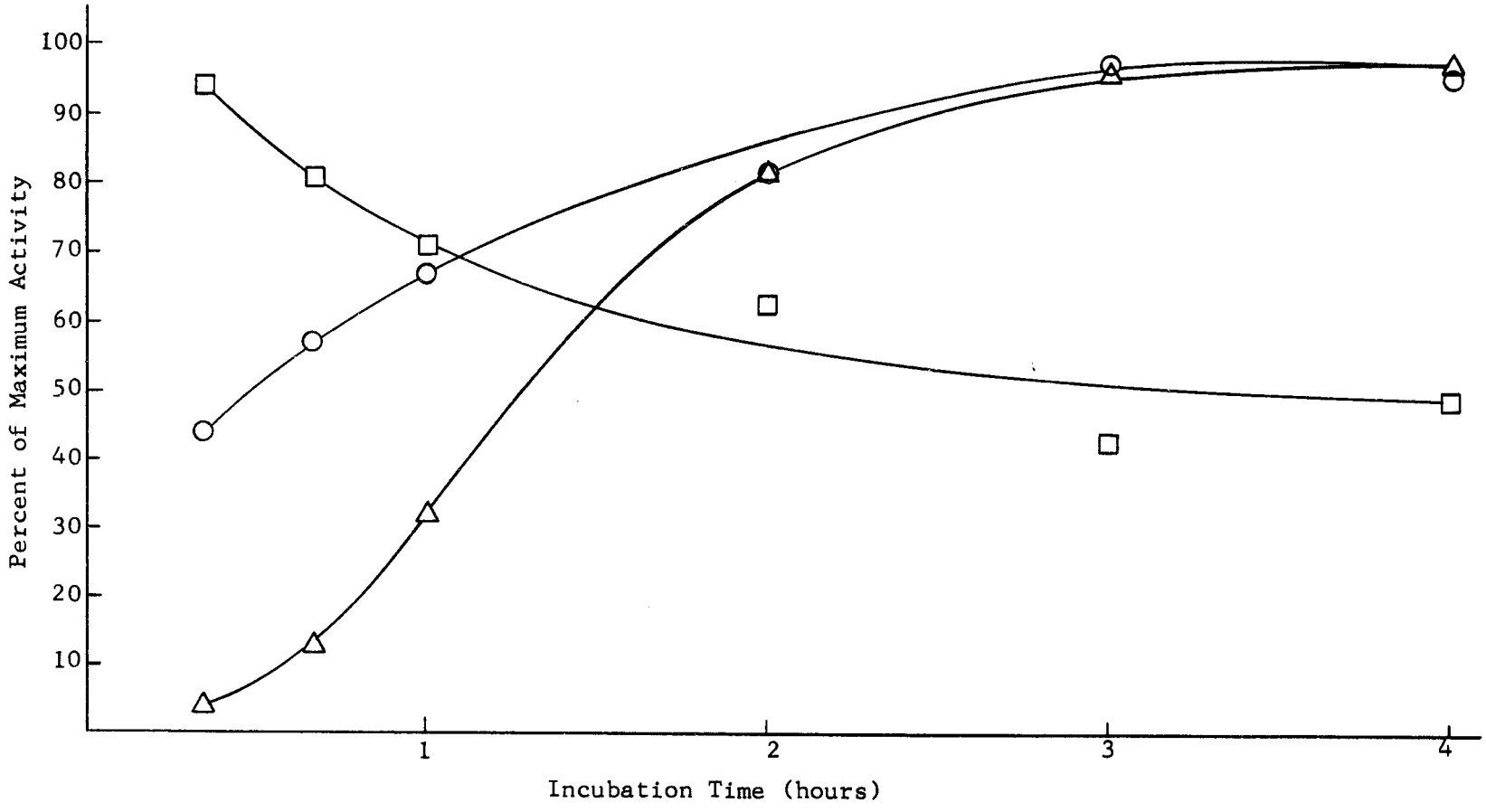

Figure 1. Time course of L-valine-U-^{14}C incorporation into protein ○—○, incorporation into aqueous fraction □—□ , and degradation to CO_2 △—△ by intact fat bodies.

Table IV

Effect of the omission of unlabeled amino acids from the incubation media on the radioactivity of different fractions[a,b]

Unlabeled Amino Acids	Protein (DPM/mg. protein)	CO_2 (DPM)	$CHCl_3$-CH_3OH Fraction (DPM)
Amino acid mixture	2203	55,400	672
Mixture less valine	3679	72,350	795
Less all amino acids	3239	101,375	444

[a] All incubations for three hours and in the presence of L-valine-U-^{14}C

[b] All incubations carried out in duplicate

fat bodies from a different batch which was presumably of the same age. This is illustrated in Table V, which gives the radioactivity of the protein obtained from nine different experiments. Duplicate incubations, utilizing fat bodies from larvae that were reared together, agreed to within plus or minus three per cent.

Table V

Radioactivity of protein of intact fat bodies incubated for three hours with L-valine-U-^{14}C

Experiment	Radioactivity of Protein (DPM/mg. protein)
I-10[a]	4473
I-12[a]	4758
I-14	2785
I-15	3347
I-16	1407
I-17	1552
I-19[a]	1219
I-20	1724
I-22[a]	2203
Average	2608
Standard Error of Mean	443

[a]Duplicate incubations, agreed to within plus or minus three percent.

DISCUSSION

One of the problems encountered in working with an experimental animal having a very rapid growth rate is to obtain individuals at a uniform stage of development. For example, freshly deposited Sarcophaga bullata larvae, which weigh less than one mg. and are approximately one mm. long, can attain a weight of nearly 200 mg. and a length of 15 mm. in forty-eight hours. The difficulties in selecting larvae of comparable size and maturity for a series of experiments are intensified, moreover, by the fact that the growth rate depends upon the number of larvae in the rearing flask, the texture of the meat on which they feed, the amount of moisture present, and undoubtedly many other unknown environmental factors. Conditions were controlled as closely as possible, but nevertheless there was considerable variation from one experiment to the next in the size of larvae of supposedly identical age. Consequently, the amount of fat body obtained from ten larvae, and perhaps its physiological condition, varied somewhat. Although fat bodies form a sticky mass which tends to dry out rapidly when they are removed from Ringer's solution, the differences in weight could have been overcome by weighing out known amounts of tissue for each experiment. This was not done, however, because it would not have eliminated differences due to the physiological state of the larvae, the additional handling of the fat bodies would produce cell damage

and perhaps loss of enzymatic activity, the fat bodies were uniform in size within each experiment, and the errors inherent in the experimental methods used were probably greater than any that could have been avoided by utilizing weighed amounts of tissue.

Before adding fat bodies to the reaction flask, they were always washed several times with cold Ringer's solution. The sheets of fat body, which settle rapidly to the bottom in insect Ringer's, were thus freed from the contents of broken cells and any bacterial contamination which might have occured during dissection. The later is especially important, since presence of bacteria in the reaction vessel could seriously alter the results.

In early experiments, the reaction vessel was simply unstoppered following incubation and the base containing evolved CO_2 was removed from the center well. The question then arose as to whether all of the CO_2 produced by the fat bodies was absorbed by the KOH from the media. In order to check this, the usual procedure was modified in one experiment. At the end of the incubation, one ml. of ten percent TCA was added to the reaction vessel through the rubber stopper with a hypodermic syringe. This would not only stop the reaction, but would also insure the release of all CO_2 from the media. The flask was left in the Dubnoff shaker for approximately five minutes after addition of the TCA. The stopper was then removed and the KOH pipetted

from the center well in the usual manner. There was no increase in the radioactivity of the CO_2 obtained in this experiment over previous values, so it was concluded that it was not necessary to acidify the reaction media to insure complete release of all CO_2. Therefore, this procedure was omitted in subsequent experiments.

The radioactivity of the CO_2 evolved by intact fat bodies incubated in the presence of ^{14}C-labeled amino acids is given in Table II. With fat bodies from three day larvae, DL-glutamic acid-3,4-^{14}C and L-valine-U-^{14}C yielded approximately the same amount to $^{14}CO_2$. The degradation of these amino acids was considerably reduced when older larvae were used as a source of fat body tissue. In an experiment with fat bodies from two day larvae, the $^{14}CO_2$ released was equivalent to over 50 percent of the radioactivity added as L-valine-U-^{14}C. As expected, considering the position of the label, the L-hisitidine-2-ring-^{14}C produced very little $^{14}CO_2$ upon incubation with larval fat bodies. Although the values are subject to differences of several magnitudes, it is interesting to note that the release of $^{14}CO_2$ from histidine was greater with fat bodies from the older larvae, contrary to the results with glutamic-3,4-^{14}C and valine-U-^{14}C.

Oxidation of glutamic acid-3,4-^{14}C probably proceeds via conversion of the glutamic acid to α-ketoglutarate, which could then enter the citric acid cycle. Kilby and Neville (52, p. 276-289) have reported the occurrence of a glutamic dehydrogenase, as well

as transaminases, in the fat body of the desert locust, Schistocerca gregaria. Conversion of pyruvic acid to alanine by transamination with glutamic acid has been observed in the silkgland and fat bodies of the silkworm larvae by Fukuda (35, p 505-510). McAllan and Chefurka (64, p 290-299) found a high level of glutamate-aspartate transaminase activity in housefly larvae.

Formation of $^{14}CO_2$ from histidine-2-ring- ^{14}C presumably involves formation of a formyl derivative of folic acid. Such a one-carbon compound might be ultimately converted, at least in part, into acetyl-coenzyme A. It has been reported (1, p 153-161) that the addition of folic acid increased formate oxidation by enzyme purified from larvae of the blowfly, Phormia regina (meig.). Since Sarcophaga larvae feed exclusively on meat and Phormia larvae can be reared on a diet containing amino acids as the sole carbon source, their metabolic pathways may be similar.

The first step in the degradation of valine is most likely removal of the amino group. This could take place most readily by transamination (35, p 505-510; 52, p 276-289). The α-keto-isovaleric acid thus formed might be converted to succinyl-coenzyme A and thus enter the citric acid cycle. It is apparent from Table II that degradation of valine is very extensive, at least in the fat bodies from two day larvae.

From the time course experiments with L-valine-U-^{14}C and fat bodies from three day larvae, it appears that after an

an initial lag the evolution of $^{14}CO_2$ proceeds rapidly for the first two hours. Activity levels off after this, so that by three hours essentially no $^{14}CO_2$ is being produced. This is no doubt due to loss of enzymatic activity, since the aqueous fraction of the fat bodies still contains a considerable amount of ^{14}C-labeled material, much of which is presumably free valine.

At the end of the incubation period, the fat bodies and media were homogenized together if the only fractions to be studied were the protein, $CHCl_3$-CH_3OH extract, and CO_2. The procedures used in purifying these fractions eliminated the possibility of significant contamination with ^{14}C-labeled amino acid from the incubation media.

In the time course studies that were made with L-valine-U-^{14}C, however, it was of interest to follow the level of radioactivity of the water soluble fraction within the fat bodies. Therefore, the fat bodies were removed from the incubation media and washed four times with Ringer's solution to free them from external radioactivity. They were then homogenized in distilled water. Following extraction of the homogenate with $CHCl_3$-CH_3OH and TCA precipitation of the protein, the radioactivity of the aqueous, or TCA soluble fraction was measured. This fraction contained free ^{14}C-valine that had entered the cells but had not yet reacted, as well as any of the intermediates in the breakdown of valine to CO_2. It can be seen from Table III and Figure 1 that the radioactivity of the acid soluble fraction is greatest at the earliest observed reaction time and that after

four hours it appears to be leveling off at roughly one-half its original activity. This is in agreement with the data for incorporation into protein and CO_2 evolution, i.e. the radioactivity of the soluble fraction decreases while the amino acid is being incorporated into protein or converted to CO_2, but becomes constant when amino acid incorporation and CO_2 evolution stop. After twenty minutes incubation, the TCA soluble fraction contained approximately one percent of the total radioactivity added to the incubation media.

The $CHCl_3$-CH_3OH extracts from the fat bodies incubated with DL-glutamic acid-3,4-^{14}C and L-valine-U-^{14}C contain significant amounts of radioactivity, as can be seen from Table II. Although fat bodies from younger larvae incorporate more of the label into $CHCl_3$-CH_3OH soluble products, the decrease in activity with age is not as great as it was for $^{14}CO_2$ evolution. With the six day larvae, in fact, the residue from the $CHCl_3$-CH_3OH extraction is more radioactive than the CO_2, The L-histidine-2-ring-^{14}C imparted a lesser degree of radioactivity to the $CHCl_3$-CH_3OH extract. The fat bodies from three day larvae were slightly more active than those from six day larvae.

As pointed out earlier in the discussion of the oxidation of amino acids to CO_2, both glutamic acid and valine may be converted to citric acid cycle intermediates. Formation of lipids could then proceed via acetyl-coenzyme A. The position of the labeled carbon atoms within the molecules of glutamic

acid and valine could conceivably account for the differences in radioactivity of the $CHCl_3$-CH_3OH soluble compounds produced.

Although degradation of histidine yields glutamic acid, which can be incorporated into lipids as described above, it must be remembered that the histidine used in these experiments was labeled in the two position of the imidazole ring. Since this carbon is lost as a one-carbon unit upon formation of glutamic acid from histidine, the incorporation of labeled carbon into the $CHCl_3$-CH_3OH fraction must proceed by a more circuitous route.

In discussing possible pathways for degradation and interconversion of amino acids by the fat bodies of Sarcophaga larvae, it is necessary to consider the diet on which these organisms are grown. Since the larvae feed exclusively on meat, essentially all of their carbohydrate, and probably much of their lipid, must be synthesized from amino acids. Therefore, the rates of the reactions, and possibly even the reactions themselves, may be quite different from those encountered in higher animals.

During purification of the protein fraction, it was washed several times with a five percent solution of TCA, primarily to rid the protein of contamination with free amino acids. In some of the early experiments, nucleic acids were extracted from the TCA precipitate by carrying out the final TCA wash at 90°C. for a period of fifteen minutes. Since this step had no observable effect on the radioactivity of the protein fraction, in the majority of the experiments all TCA washings were carried out at room temperature.

As described earlier, the protein was freed of all traces of TCA by repeated washing with 95 percent ethanol. This was necessary since TCA acts as a quencher in the liquid scintillation counting system used to measure the radioactivity of the protein.

The values given in Table II for the radioactivity of fat body protein indicate that valine was incorporated much better than either glutamic acid or histidine. If it is assumed that only the L-form of the glutamic acid was utilized, then glutamic acid and histidine were incorporated to an almost identical degree. This was true whether the fat bodies were obtained from three or six day larvae.

The radioactivity of the fat body protein was expressed as specific activity based on the dry weight of the protein, and thus differs from values given for the CO_2 or $CHCl_3$-CH_3OH extract. This was necessary since the numerous steps involved in purifying and preparing the protein for counting resulted in loss of material, making it impossible to determine the total amount of ^{14}C-amino acid incorporated. A rough estimate might be made by assuming a yield of 20 mg. protein from the fat bodies of ten larvae. Based on such an assumption, it can be estimated that the fat bodies from ten three day larvae incorporated approximately ten percent of the added L-valine-U-^{14}C into protein.

When unlabeled valine was omitted from the incubation

media (Table IV), there was an approximately two-thirds increase in the amount of valine-U-^{14}C incorporated into the protein. Evolution of $^{14}CO_2$ was accelerated to a somewhat lesser extent, and incorporation into the $CHCl_3$-CH_3OH fraction may have increased slightly. Since omission of the unlabeled amino acid would have the effect of increasing the specific activity of the valine available to the cell, these results were to be expected.

If all unlabeled amino acids were omitted, the incorporation of radioactive valine into protein was nearly 50 percent greater than in the presence of the complete amino acid mixture. This high level of incorporation once again reflects the increased specific activity of the valine. Although a complete complement of amino acids is considered necessary for de novo protein synthesis, incorporation of valine-U-^{14}C into protein by intact fat bodies in the absence of added amino acids does not imply that such incorporation is due to mechanisms other than true synthesis. Insect hemolymph and tissue, including the fat body, are known to contain high levels of free amino acids (37, p 235-238), so the endogenous amino acids present in the fat bodies may well provide an adequate amino acid pool for de novo protein synthesis.

Degradation of the valine-U-^{14}C to $^{14}CO_2$ was nearly doubled by the omission of the amino acid mixture from the incubation media. By reducing the concentration of free amino acids, the concentration of intermediates common to the catabolic metabolism

of many amino acids, including valine, may have also been lowered. This could quite conceivably increase the rate at which valine was degraded to CO_2. For some reason, incorporation of valine into the $CHCl_3$-CH_3OH fraction was decreased in the absence of the complete amino acid mixture.

The results of individual experiments on the incorporation of valine into fat body protein (Table V) illustrates the wide variation in the activity of tissue obtained from different batches of larvae. As mentioned earlier, the size and physiological state of the fat bodies used from one experiment to the next could be controlled to only a limited degree. Consequently the specific activities can be seen to vary by as much as four-fold. That these differences are due to the nature of the fat bodies used, and not to the procedure and techniques involved in measuring the enzymatic activity, is apparent from the fact that duplicate incubations utilizing fat bodies from Sarcophaga larvae reared at the same time show less than plus or minus three percent variation in the specific activity of the protein.

Similar difficulties were encountered by Stevenson and Wyatt (87, p 65-71) in studying the incorporation of leucine-1-^{14}C into fat body tissue of Hyalophora cecropia larvae. They reported that duplicate incubations with fat body tissue from a single larva gave results which differed by less than five percent. However, in three experiments utilizing fat body tissue from different larvae at the same stage of development, Stevenson and Wyatt obtained protein with an average specific activity of

29,200 CPM per mg. protein and a standard error of mean of 10,700. Therefore, the standard error of mean for amino acid incorporation into larval fat body protein of the flesh fly and the silkmoth amounts to from 17 to 30 percent of the average specific activity.

The general relationship between larval age and enzymatic activity of the fat body was the same for incorporation of amino acids into protein, evolution of $^{14}CO_2$, and incorporation into the $CHCl_3$-CH_3OH fraction, i.e. the younger larvae were more active. This variation with age was most significant in the degradation of the amino acids to CO_2, and least evident in the formation of the $CHCl_3$-CH_3OH fraction. It will be recalled that the oxidation of the labeled carbon of histidine-2-ring-^{14}C to $^{14}CO_2$ was the single possible exception to this general pattern.

The differences with age in incorporation of amino acids into protein probably reflect a general differential in the overall rate of protein synthesis, since in the younger larvae the fat body cells are rapidly growing in size and consequently are synthesizing more protein than in the mature larvae. However, it is not clear why this effect is less noticeable with valine than with glutamic acid and histidine. The greater degradation of glutamic acid and valine to CO_2 in the younger larvae suggests a greater energy requirement by the two and three day larvae. This can be correlated with the rate of larval growth, which is greatest at two days and least at six days. The fact that fat bodies from six day larvae degrade glutamic acid more

extensively than valine may indicate that when the energy requirements of the larvae are lower, those amino acids which can enter the citric acid cycle most readily are preferentially converted to CO_2. Since the pathway by which $^{14}CO_2$ is produced from L-histidine-2-ring-^{14}C is unclear, it is not possible to offer an explanation for the greater activity of fat bodies from older larvae. The incorporation of radioactivity into the $CHCl_3$-CH_3OH fraction is more nearly constant for fat bodies from larvae of different ages. This would suggest that the rate of lipid synthesis decreases slowly with larval age.

PART II

AMINO ACID ACTIVATION

METHODS

Rearing of Adult Flies and Larvae

During the early work on amino acid activating enzymes in Sarcophaga bullata, the adults and larvae were reared exactly as described in the Methods section of Part I. The food provided for the adult flies was later changed from an aqueous sucrose solution to a diet composed of a dry mixture of sugar, powdered milk, and powdered egg (6:6:1 by volume). This change was made because it was felt that the flies would live longer on the latter diet. Larvae were reared on either beef or pork liver for a few experiments, when beef heart was unavailable. Neither of these dietary changes had any noticeable effect on the amino acid activating enzymes of the fractions investigated.

If fat bodies were to be removed from the larvae for studies of amino acid activating enzymes, the average larval weight was determined prior to dissection.

Fractionation of Larval Fat Bodies

The isolation of larval fat bodies was described in the Methods section of Part I. For the investigation of amino acid activating enzymes, the fat bodies from approximately 100 two day old larvae were pooled and washed four times with cold

insect Ringer's solution. The tissue was then suspended in as small a volume of this solution as possible (approximately four ml.) and transferred to a Dounce homogenizer. An equal volume of a cold solution of 0.5 M sucrose and 0.2 M tris(hydroxymethyl) amino methane (Tris) buffer (pH 7.6) was added, and the fat bodies were homogenized with ten strokes of the tight pestle.

The homogenate was separated into various subcellular fractions by differential centrifugation. Unbroken cells and cell debris were sedimented by centrifuging for 15 minutes at 480 g. in a Servall Model RC-2 centrifuge. A thick layer of lipid and material associated with it remained on top of the solution. This was removed with either a spatula or a cotton swab. The milky supernatant was then centrifuged in the Servall at 10,000 g. for 15 minutes to sediment the mitochondrial fraction. The surface of the supernatant was again covered with a layer of lipoidal material, which was removed as before. The latter supernatant, which had a somewhat opalescent appearance, was centrifuged in a Spinco Model L preparative ultracentrifuge at 110,000 g. for one hour. This final centrifugation resulted in the sedimentation of the microsomal fraction. When desired for use as a source of amino acid activating enzymes, the microsomes were resuspended in a 0.25 M sucrose and 0.1 M Tris solution (pH 7.6) and centrifuged again for one hour at 110,000 g. The microsomes were then taken up and suspended in a small volume of the buffered sucrose solution. The supernatant from the initial 110,000 g. centrifugation

(designated the "soluble" fraction) was often used directly as enzyme. In some experiments, one normal acetic acid was carefully added to the soluble fraction until the pH was reduced to four. The resulting precipitate was removed by centrifugation at 10,000 g. for ten minutes and dissolved in a small amount of the 0.25 M sucrose and 0.1 M Tris solution (pH 7.6). This solution was again centrifuged for ten minutes at 10,000 g., any residue was discarded, and the supernatant taken as the "pH 4 enzyme" fraction. The pH of the supernatant from the acid precipitation was adjusted from four to 7.6 with one normal NaOH. This fraction was designated the "pH 4 supernatant". All procedures in the preparation of enzyme fractions from larval fat bodies were performed at 0-4°C. The fractionation procedure is summarized in Figure 2.

Selection of Pupae at Different Developmental Stages

To study amino acid activating enzymes in developing pupae, it was necessary to rear large numbers of larvae to obtain sufficient material at the desired stage of development. Freshly hatched larvae were placed on meat, either beef heart or beef liver, and grown at 30°C. as usual. Larvae were removed from the meat after three days growth, washed, and transferred to large beakers of sawdust. They were then maintained at room temperature and allowed to pupate. All newly formed pupae were removed from the sawdust at 12 hour intervals. Groups of ten pupae were weighed, placed in test tubes, and permitted to develop further at 30°C. for varying lengths of time. Amino acid activating

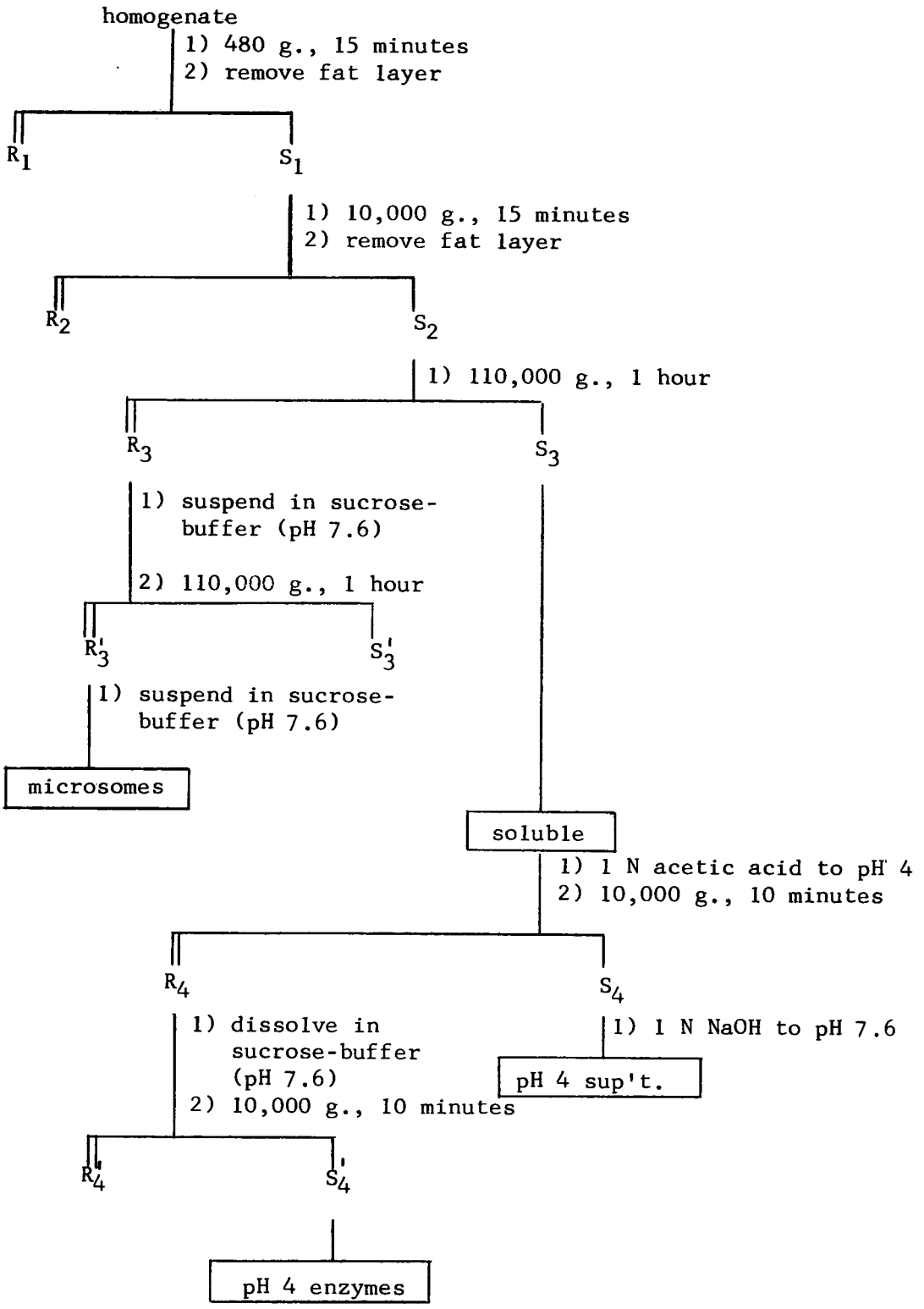

Figure 2. Fractionation of larval fat body homogenate.

enzymes from pupae at different stages of development were then studied, utilizing ten pupae of a particular age for each enzyme preparation. It was thus possible to observe the amino acid activating enzymes from pupae representing a complete range of pupal development. No ten day pupae were used, because in every available group of ten pupae at this age, some adults had emerged. All of the flies had emerged by 11 days, so these adults were treated like the pupae and included in the experiments.

Fractionation of Pupal Tissue

In preliminary experiments, the enzyme fraction from pupae was prepared by cutting open the pupal case and transferring the contents directly to a Dounce homogenizer. With the younger pupae, however, it was found that the tissue was too liquid to be removed without considerable loss. In the procedure finally adopted, ten pupae were gently ground in four ml. of a cold 0.25 M sucrose and 0.1 M Tris solution (pH 7.6) with a mortar and pestle. This mixture was then transferred to a Dounce homogenizer. The mortar was rinsed with three ml. of sucrose-buffer, which was added to the Dounce. This was further homogenized with three strokes of the loose fitting pestle. Fragments of the pupal case were not broken up by this treatment, but were merely compressed at the bottom of the Dounce homogenizer and later discarded. The homogenate thus obtained was separated into various cellular fractions by differential centrifugation as described for the larval fat body. The soluble fraction was the only one studied, however,

and was not submitted to further fractionation. Since many enzymatic activities and analytical measurements were expressed on the basis of the total activity or quantity of a substance with respect to the initial weight of the ten pupae, the soluble fraction was always made to a known volume with the cold sucrose and Tris solution described above. All steps in the preparation of the soluble fraction from pupae were carried out at 0-4°C.

Assay Procedure for Amino Acid Activating Enzymes

The assay for amino acid activating enzymes was based on the observation of Hoagland (42, p 288-289) that the activation of amino acids is a reversible process, resulting in an exchange of radioactivity between added ^{32}P-labeled inorganic pyrophosphate (^{32}PP) and ATP. The reaction involved may be written as follows:

$$\text{Amino acid} + \text{ATP} + \text{enzyme} \rightleftharpoons \text{amino acid-AMP-enzyme} + \text{PP}$$

The activity of an enzyme preparation was determined by measuring the ^{32}PP-ATP exchange occurring in the presence of the enzyme, ^{32}PP, ATP, and an amino acid or mixture of amino acids. Table VI gives the composition and concentrations of the complete reaction mixture used in these assays. Magnesium was required for the exchange to occur, as will be pointed out later. Potassium fluoride was added to inhibit the action of pyrophosphatases, which could alter the concentrations of PP and ATP and thereby influence the apparent activity of the enzyme. In one experiment, for example, the fat body soluble fraction was incubated with the amino acid mixture in the presence and in the absence of KF.

Table VI

Composition of reaction mixture for ^{32}PP-ATP exchange assay of amino acid activating enzymes[a]

Component	Concentration
Tris buffer	100 mM
$MgCl_2 \cdot 6H_2O$	10 mM
KF	5 mM
^{32}PP (2-5 x 10^5 cpm)	2 mM
ATP	2 mM
Amino acid mixture[b] (if added)	1 mM(for L-form of each amino acid)
Individual amino acids (if added)	2 mM(for L-form of each amino acid)
Enzyme	12-86 μg. protein nitrogen

[a]Total volume one ml.

[b]See Table I

The calculated enzymatic activity in the absence of KF was only 43 percent of that obtained for the same enzyme incubated in the presence of five mM KF.

The exchange reaction was performed in open 13 x 100 mm. culture tubes, which were gently shaken in a Dubnoff shaker at 30° C. for 15 minutes. At the end of the incubation period, the exchange reaction was halted and protein precipitated by the addition of two ml. of cold 7.5 percent TCA. The protein was sedimented by centrifugation of the reaction tubes for 30 minutes at 1800 revolutions per minute in a Phillips-Drucker model 7011 centrifuge. Two ml. of the clear supernatant were transferred to a 15 ml. conical glass centrifuge tube.

The inorganic pyrophosphate and ATP in this fraction were separated by the method of Crane and Lipmann (19, p 235-243). One hundred mg. of activated carbon were added to the solution and thoroughly mixed by swirling. The ATP was adsorbed on the surface of the carbon, while the free pyrophosphate remained in solution. Two ml. of 0.1 M non-radioactive sodium pyrophosphate solution (pH 7.6) were added and the contents of the centrifuge tube again mixed by swirling. This mixture was centrifuged for 30 minutes, as above, and the supernatant carefully decanted off. The charcoal was washed two more times with 2.5 ml. portions of the pyrophosphate solution. The supernatants from the initial charcoal wash and the two subsequent washings were combined and made to ten ml. with distilled water.

The activated carbon, with the ATP still adsorbed to its surface

was suspended in four ml. of one normal HCl and heated for 20 minutes in a boiling water bath. This resulted in the hydrolysis of the two terminal phosphates of the ATP (23, p 299-308). The charcoal solution was centrifuged as described above, the supernatant carefully decanted off, and the charcoal washed twice more with 2.5 ml. portions of distilled water. The washings were combined with the HCl hydrolysate and made to ten ml. with distilled water.

Prior to all centrifugations, 0.2 ml. of 95 percent ethanol were used to wash down the carbon from the sides of the tube.

Two ten ml. fractions were thus obtained from each reaction tube. One fraction contained the free pyrophosphate and the other the terminal phosphates of the ATP from two-thirds of the reaction media. The radioactivity of each of these was determined by placing 0.5 ml. aliquots in glass planchets and counting using a thin window Geiger counter.

The observed exchange of radioactivity between labeled ^{32}PP and ATP was expressed as a percent of the total exchange which would be attained at equilibrium (22, p 21-38).

$$\text{Percent exchange} = \frac{AT^{32}P}{\left[\frac{(ATP)}{(ATP)+(PP)}\right]\left[AT^{32}P + {}^{32}PP\right]} \times 100$$

(ATP) and (PP) --- total concentrations in μmoles per ml.

$AT^{32}P$ and ^{32}PP --- total radioactivity in isolated ATP and PP

Using equal amounts of ^{32}PP and ATP, one-half of the radioactivity would be in the PP and one-half in the ATP at equilibrium. The percent exchange would increase with time from zero to a theoretical value of 100 at equilibrium. This increase would become slower and slower as the process went toward equilibrium, due to the back

exchange of radioactivity between newly formed $AT^{32}P$ and PP.

A more meaningful expression of the activity of the enzymes was obtained by use of an equation similar to that derived by Duffield and Calvin (28, p 557-561) for determining the rate of this type of reaction.

$$R = \frac{(ATP)\ (PP)}{(ATP)+(PP)} \times \frac{2.3}{t} \times \log \frac{100}{100 - \%\ \text{exchange}} \times 1000$$

R - rate of exchange in mμmoles per minute
(ATP) and (PP) - total concentrations in μmoles per ml.
t - length of incubation period in minutes
% exchange - as defined above

In this calculation, exchange between PP and the ever increasing amount of $AT^{32}P$ is taken into account, to give a true rate of exchange. If there were no loss of enzymatic activity with time, the rate of exchange determined by this equation would be constant, no matter when the radioactivity of the PP and ATP were determined, up to the point of equilibrium.

The observed exchange between ^{32}PP and ATP resulted not only from the action of amino acid activating enzymes, but also from other enzymes, such as those involved in acetate or fatty acid activation. In an attempt to determine the amino acid dependent ^{32}PP-ATP exchange, i.e. exchange related only to the activity of the amino acid activating enzymes, the endogenous rate of exchange was subtracted from that occurring in the presence of added amino acids. The value obtained in this way, designated by the term R_{aa}, was somewhat less than the true amino acid dependent exchange, however, because the endogenous exchange was at least partially due to amino acid activating enzymes reacting with free amino acids in the enzyme preparation. This error could be significant, especially if the enzyme

being assayed required a low level of its amino acid substrate to become saturated.

Protein and Nucleic Acid Isolation

Aliquots were taken from all enzyme fractions for the determination of protein nitrogen and RNA. For these determinations, usually carried out in triplicate, an enzyme sample of from 0.1 to 0.4 ml. was placed in a three ml. conical glass centrifuge tube and made to a final volume of one ml. with glass distilled water. The protein and nucleic acids were precipitated by the addition of one ml. of cold ten percent TCA. Nucleic acids were removed from the precipitate by extraction with 0.5 ml. of one normal $HClO_4$ at 70°C. for 20 minutes. A second, identical $HClO_4$ extraction was carried out to insure complete isolation of the nucleic acids. The isolation procedure is outlined in Figure 3.

In the above procedure for the isolation of protein and nucleic acids from the different enzyme preparations (Figure 3), there is no provision for the removal of lipids. Although the TCA precipitate was extracted with $CHCl_3$-CH_3OH (2:1) in some of the early experiments, it was found that deletion of this step had no effect on the results of protein nitrogen or RNA determinations. Since it was desirable to employ as few steps as possible, for the sake of both accuracy and convenience, the $CHCl_3$-CH_3OH extraction was routinely omitted.

Determination of Protein Nitrogen

Nitrogen was determined by a modification of the method described by Lang (54, p 1692-1694). A solution of DL-serine

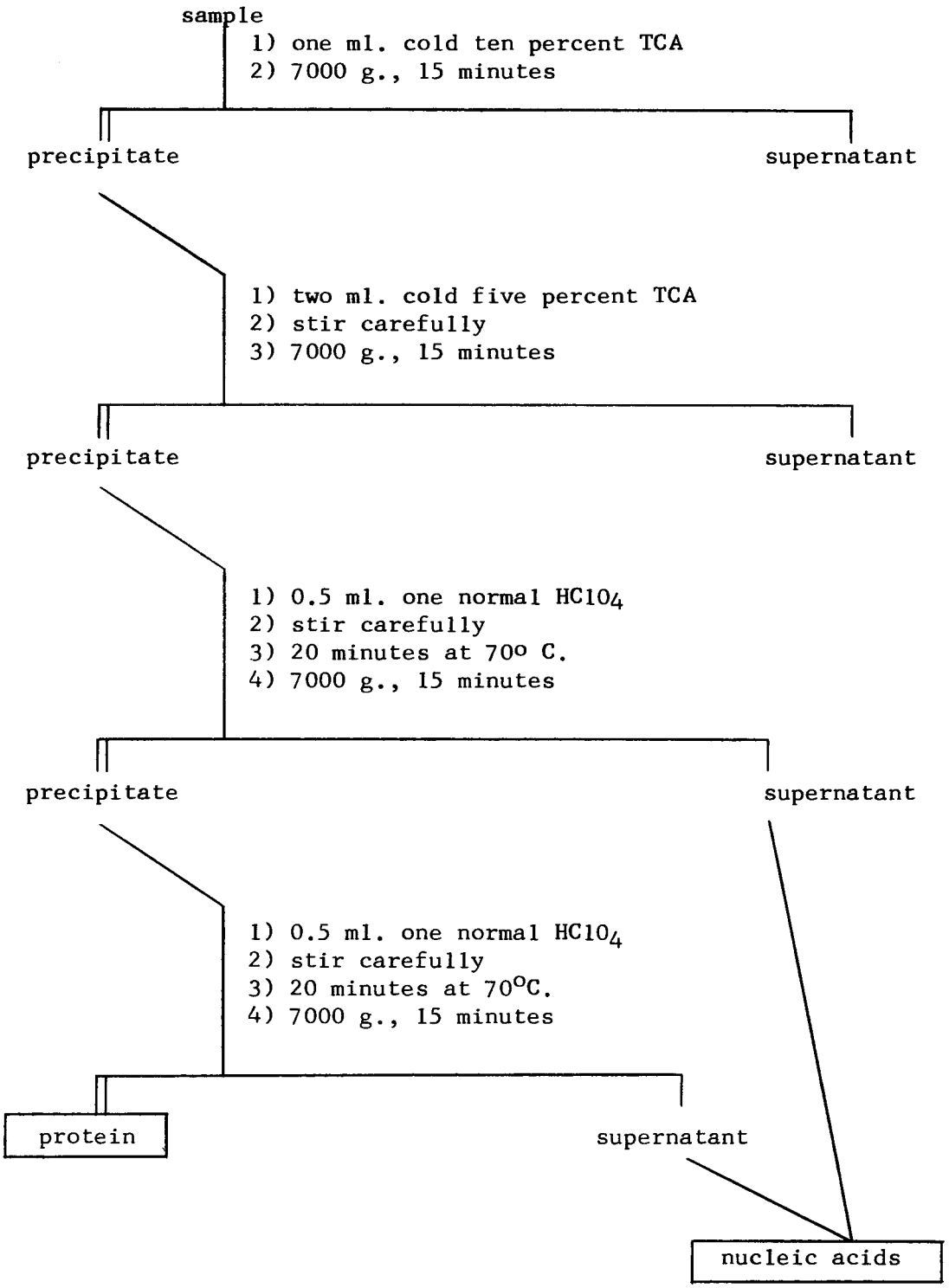

Figure 3. Procedure for isolation of protein and nucleic acids.

containing 140 μg. of nitrogen per ml. was used as a standard. Aliquots of the serine solution, containing from 14 to 140 μg. of nitrogen, were added to 13 x 100 mm. culture tubes and taken to dryness. Two-tenths ml. of digestion mixture were added to each standard. The same amount of digestion mixture was added to three ml. conical centrifuge tubes containing the protein being assayed. All tubes were placed in a sand bath and slowly heated to 210-250° C. They were maintained at this temperature until digestion was complete, usually from 16 to 24 hours. Complete digestion was indicated by the absence of any dark residue and by a colorless protein digest. Tubes were then removed from the sand bath and allowed to cool. Standards were made to eight ml. with glass distilled water. Unknowns were quantitatively transferred to 13 x 100 mm. culture tubes and made to eight ml. with glass distilled water. Both standards and unknowns were mixed thoroughly. Three ml. of this diluted digest were combined with one ml. of glass distilled water and two ml. of Nessler reagent in a clean culture tube. After mixing, the tubes were placed in the dark for approximately ten minutes and then read at 500 mμ in a colorimeter.

The digestion mixture was prepared by combining 40 g. of potassium sulfate, two ml. of selenium oxychloride, and distilled water to a final volume of 250 ml. To this mixture were added 250 ml. of concentrated sulfuric acid.

The Nessler reagent was prepared as described by Koch and McMeekin (53, p 2066-2069). Two hundred and twenty five g. of

iodine were dissolved in 200 ml. of water containing 300 g. of potassium iodide. When solution was complete, 300 g. of pure metallic mercury were added, and the mixture was stirred until the supernatant liquid had lost all of its yellow color due to iodine. During this time the mixture was cooled by intermittently immersing it in tap water. The aqueous supernatant solution was decanted off, and a small portion of it tested for iodine with starch solution. A negative result would indicate that the solution may contain mercurous compounds. If the test were negative, a few drops of iodine solution of the same concentration described above were added until a faint excess of free iodine was detected by the starch test. The solution was then diluted to two liters with distilled water and mixed well. The entire two liters were added to 9.75 liters of accurately prepared ten percent (w/v) NaOH. This final solution was mixed and allowed to clear by standing. The Nessler reagent was stored in a clear glass carboy in the laboratory, and single preparations were used for nitrogen determination for well over a year.

Determination of Ribonucleic Acid

Ribonucleic acid determinations were carried out by a modification of the method of Mejbaum (66, p 117-120). An orcinol reagent was used, with ribose standards. One μg. of ribose was considered to be equivalent to 3.76 μg. of RNA. Standards, containing ribose equivalent to from eight to 80 μg. of RNA per 0.5 ml., were prepared in one normal $HClO_4$. One-half ml. of the nucleic

acid solution being assayed, or 0.5 ml. of the standards were placed in clean 13 x 100 mm. culture tubes. To this was added one ml. of one normal $HClO_4$ and 1.5 ml. of orcinol reagent. The tubes were shaken and then heated for ten minutes at 100°C. After cooling, the optical density at 660 mμ was determined.

The orcinol reagent was prepared by dissolving 48 mg. of ferric sulfate in 100 ml. of concentrated HCl. Immediately prior to use, one g. of orcinol was added to the ferric sulfate solution.

Determination of Free Amino Acids

The free amino acids of an enzyme fraction were determined with ninhydrin. One ml. of enzyme was combined with one ml. of ten percent TCA. The protein was sedimented by centrifugation and the clear supernatant carefully removed. This supernatant, which was 0.125 M sucrose, 0.05 M Tris, and five percent TCA, was diluted one to ten with a sucrose, Tris, and TCA solution of the same concentrations. One-tenth and 0.5 ml. aliquots were added to clean 13 x 100 mm. culture tubes and made to one ml. with the sucrose, Tris, and TCA mixture. Standards containing from 0.04 to 0.20 μmoles of L-leucine per ml. were prepared, again utilizing the 0.125 M sucrose, 0.05 M Tris, and five percent TCA solution. One ml. of ninhydrin reagent was added to each unknown and standard. The tubes were heated for five minutes in a boiling water bath, cooled, and diluted with five ml. of a 1:1 water-n-propanol solution. After 15 minutes, the amount of amino acid present was determined by measuring the optical density at 570 mμ.

The ninhydrin reagent was prepared by first combining 300 ml. of methyl-Cellosolve and 100 ml. of four normal sodium acetate buffer (pH 4.5). Eight g. of ninhydrin and 160 mg. of $SnCl_2 \cdot 2H_2O$ were then dissolved in the above solution.

Preparation of ^{32}P-Labeled Pyrophosphate Solution

The majority of the ^{32}P-labeled pyrophosphate used in the study of amino acid activating enzymes was obtained from Nuclear-Chicago Corporation, Des Plaines, Illinois. However, some ^{32}PP was prepared by pyrolysis of labeled orthophosphate. Five ml. of sodium phosphate solution (10 mC.) and ten mg. of carrier K_2HPO_4 were placed in a crucible. The mixture was taken to dryness in an oven at approximately 95°C. and heated over a Meeker burner for 30 minutes. The residue was taken up in five ml. of distilled water and the entire procedure repeated twice. The phosphate and pyrophosphate were separated by ion exchange chromatography according to the method of Martonosi (61, p 12-14). A column of Dowex 1-X10 resin was converted to the bicarbonate form, and the solution containing the radioactive material added. The phosphate compounds were eluted by the successive addition of three, four and eight percent $KHCO_3$ to the 500 ml. mixing vessel of a gradient elution system which initially contained distilled water. The column was run at room temperature under a few pounds nitrogen pressure, ten ml. fractions being collected. The radioactivity of the eluate was followed by counting 0.1 ml. aliquots from every second

or third fraction with a thin window Geiger counter. The pyrophosphate peak was identified by its position, and the tubes within this peak were pooled. Mild heating was used to concentrate this fraction. Sixty percent $HClO_4$ was added dropwise with rapid stirring to remove the bicarbonate as CO_2 and to precipitate the potassium ions as $KClO_4$. The gentle heating and addition of $HClO_4$ were repeated several times, until the ^{32}PP was contained in ten ml. of essentially bicarbonate free solution.

The purity of the pyrophosphate solutions was checked by paper chromatography on Whatman 41 H paper using a CH_3OH, HCOOH, H_2O (80:14.5:5.5) solvent (7, p 405-410). The location of radioactive material was determined using a Forro Radiochromatograph strip counter. A modified Hanes-Isherwood spray (50, p 1091-1097) was used for the chemical location of phosphate and pyrophosphate. After drying, the color was developed by placing the chromatogram in a closed metal can and heating in an autoclave for two minutes. Both compounds were revealed as blue spots against a white or light brown background. All radioactivity was found to be confined to a single spot which corresponded chromatographically to inorganic pyrophosphate.

Determination of Phosphorus

Aliquots of the ^{32}PP solution were taken for determination of its phosphorus content. The method described by Bartlett (8, p 466-468) was used, with slight modification. One-half ml. of ten normal sulfuric acid was added to 0.1 ml. of the ^{32}PP

solution in a 13 x 100 mm. culture tube, and the mixture was heated at 150-160°C. for three hours. After cooling, two drops of 30 percent H_2O_2 (phosphorus free) were added, followed by another treatment of 1.5 hours at 150-160°C. Standards, containing from 0.02 to 0.10 μmole of inorganic orthophosphate, were treated in the same manner. After the sulfuric acid digests had cooled, 4.6 ml. of 0.22 percent ammonium molybdate and 0.2 ml. of Fiske-SubbaRow reagent were added and mixed thoroughly. The solutions were then heated in a boiling water bath for seven minutes, cooled, and their optical densities at 830 mμ measured in a spectrophotometer.

The Fiske-SubbaRow reagent was prepared by first dissolving 0.5 g. of 1-amino-2-naphthol-4-sulfonic acid in 200 ml. of freshly prepared 15 percent sodium bisulfite (anhydrous) with a mechanical stirrer, followed by the addition of one g. of anhydrous sodium sulfite. The final solution was filtered and stored in a brown glass bottle in the refrigerator.

RESULTS

Time Course for ^{32}PP-ATP Exchange

The results of an experiment to determine the optimum incubation time for the ^{32}PP-ATP exchange are shown in Figure 4. Both the percent exchange and the rate of exchange are plotted for this experiment, which was carried out with the soluble fraction from larval fat bodies and in the presence of the amino acid mixture. On the basis of this information, it was decided to use a 15 minute incubation period for the assay of amino acid activating enzymes. A shorter time would allow only a very small amount of exchange when individual amino acids were used, and any variations in incubation time would become more significant. Although the rate of exchange is not constant, indicating that the enzyme is somewhat unstable under assay conditions, this loss of activity is linear for at least the first 15 minutes.

pH Optimum for Amino Acid Activating Enzymes

The pH optimum was determined for the amino acid activating enzymes in the soluble fraction from fat bodies (Figure 5). Since the measurements were made in the presence of the amino acid mixture, the observed ^{32}PP-ATP exchange represents the sum of the activities of all individual activating enzymes. Figure 5 illustrates the variation in the rate of exchange with variation of the pH of the reaction media. Although specific enzymes from this fraction, or the mixture of enzymes from other fractions, may differ slightly

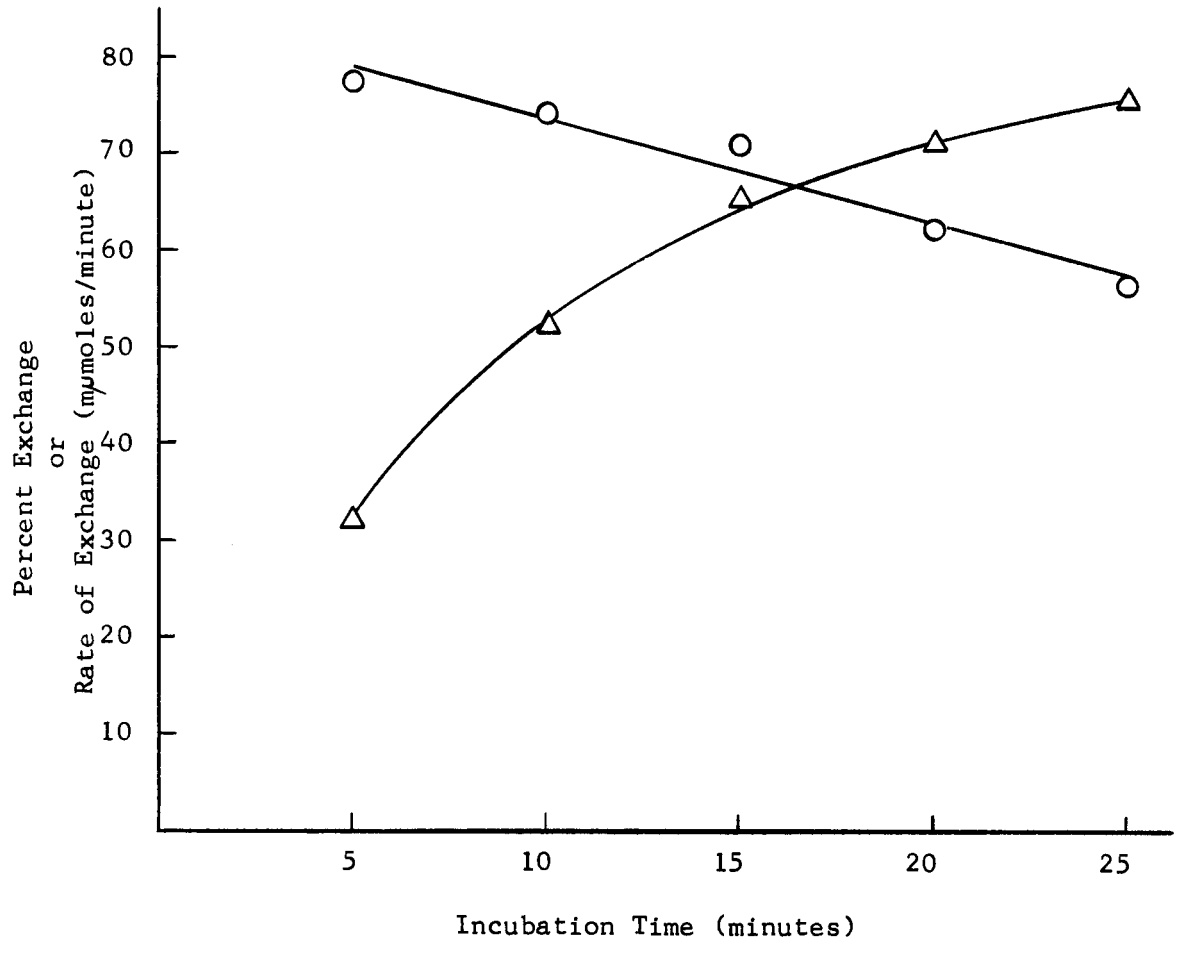

Figure 4. Time course of ^{32}PP-ATP exchange during incubation; percent exchange △—△, rate of exchange ○—○.

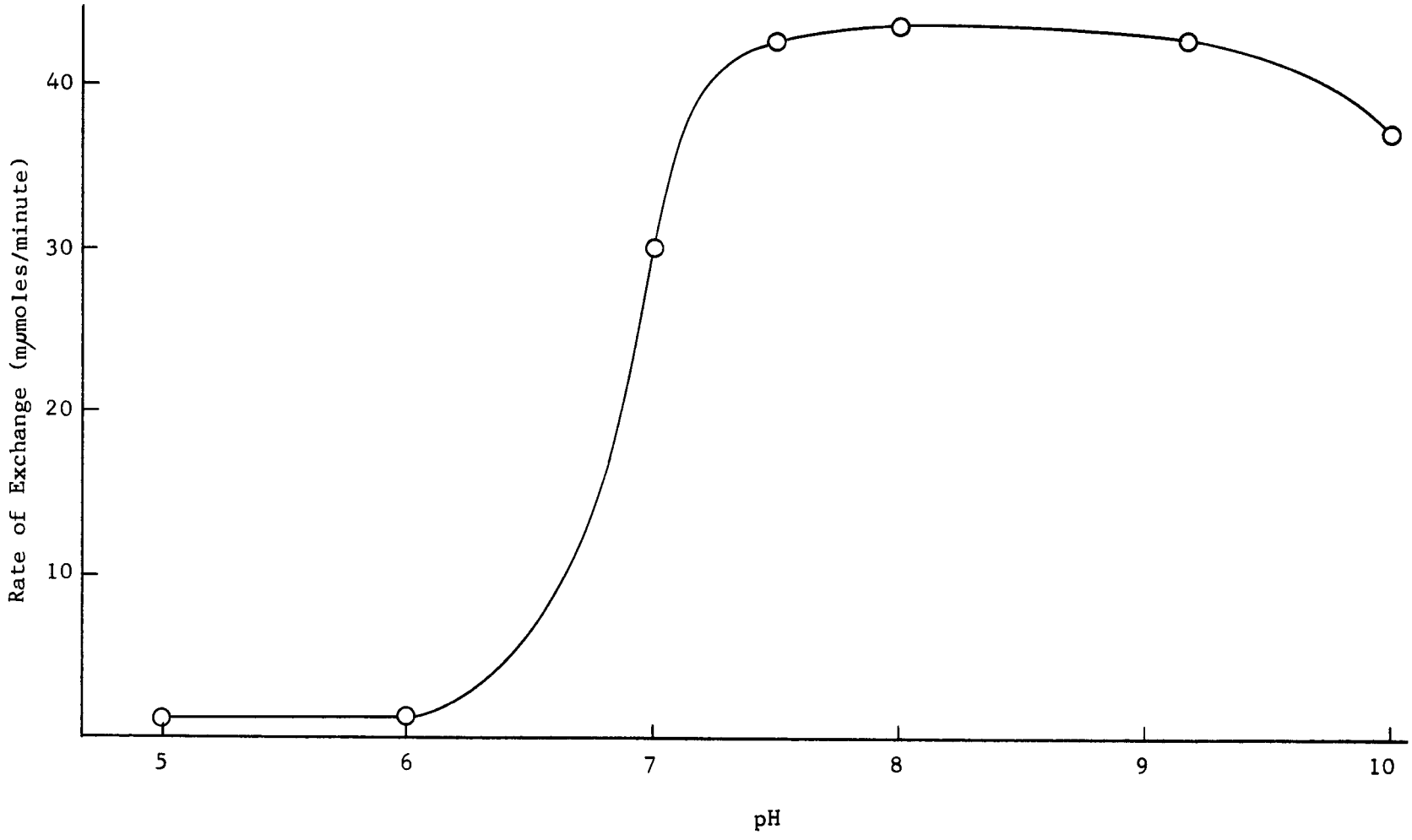

Figure 5. Effect of pH on rate of ^{32}PP-ATP exchange

in their optimum pH, all measurements of ^{32}PP-ATP exchange were performed at pH 7.6.

Effect of Enzyme Concentration on ^{32}PP-ATP Exchange

It is evident from Figure 6 that the rate of ^{32}PP-ATP exchange was proportional to the amount of fat body soluble fraction added, at least for the lower levels of enzyme concentration. Such a relationship had to be shown, if specific activities of amino acid activating enzymes, based on protein nitrogen, were to be compared between experiments in which the protein content of the added enzyme differed. Although datawere available suggesting that this linear relationship might extend to higher concentrations, no comparisons were made between results obtained with different enzyme preparations unless the protein nitrogen content of the added enzyme fell within or very near the limits of linearity indicated in Figure 6. It was stated in Table VI that the added enzyme sometimes contained as much as 86 μg. protein nitrogen. However, all experiments in which the enzyme contained more than 33 μg. protein nitrogen were complete within themselves and specific activities were not calculated. The pH 4 enzyme from larval fat bodies also showed a linear relationship between ^{32}PP-ATP exchange and enzyme concentration (Figure 7).

Effect of Amino Acid Concentration on ^{32}PP-ATP Exchange

In the assay employed for measuring the concentration of amino acid activating enzymes, the enzyme must be the rate limiting factor. Therefore, it had to be determined that the amounts of

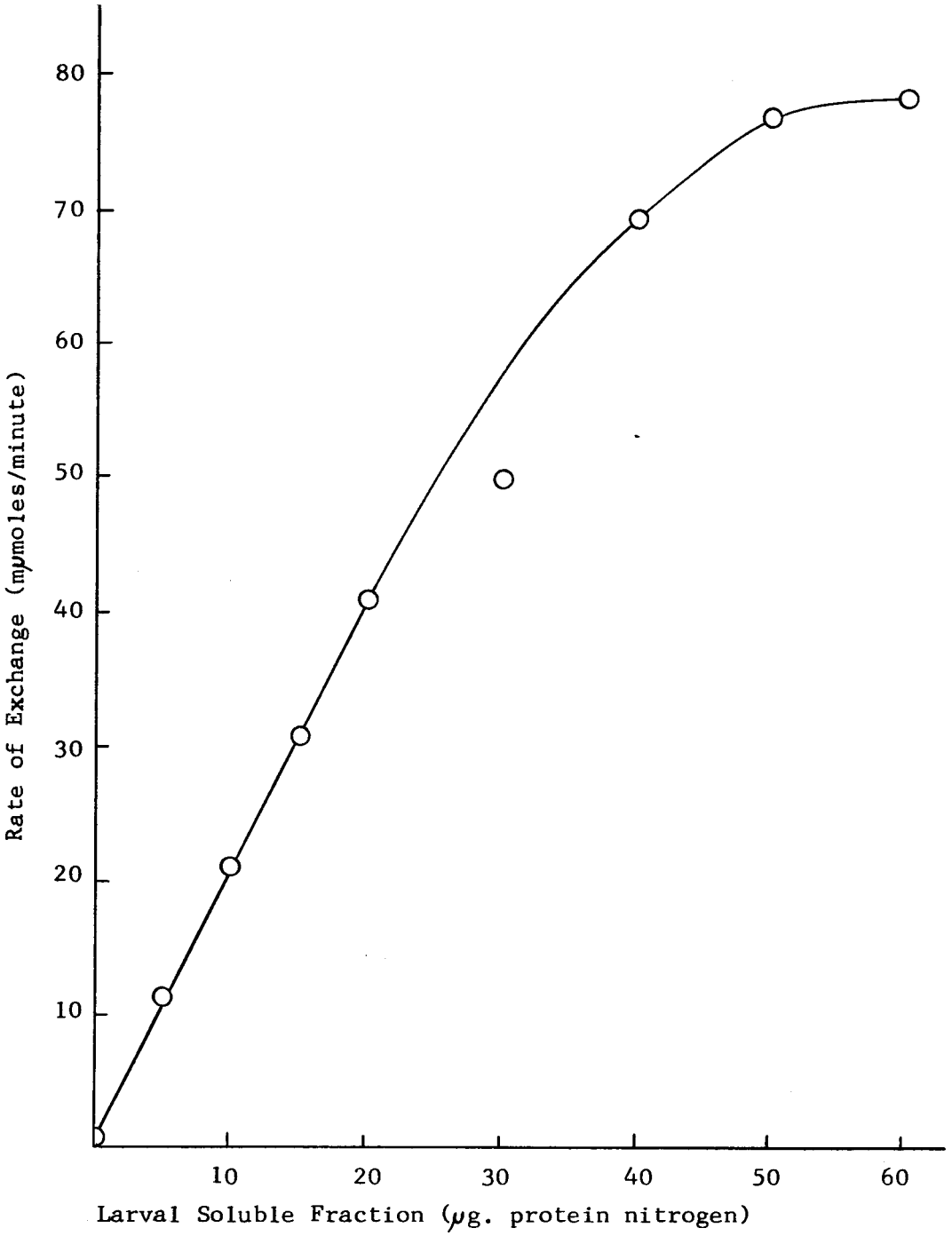

Figure 6. Effect of larval soluble fraction concentration on ^{32}PP-ATP exchange.

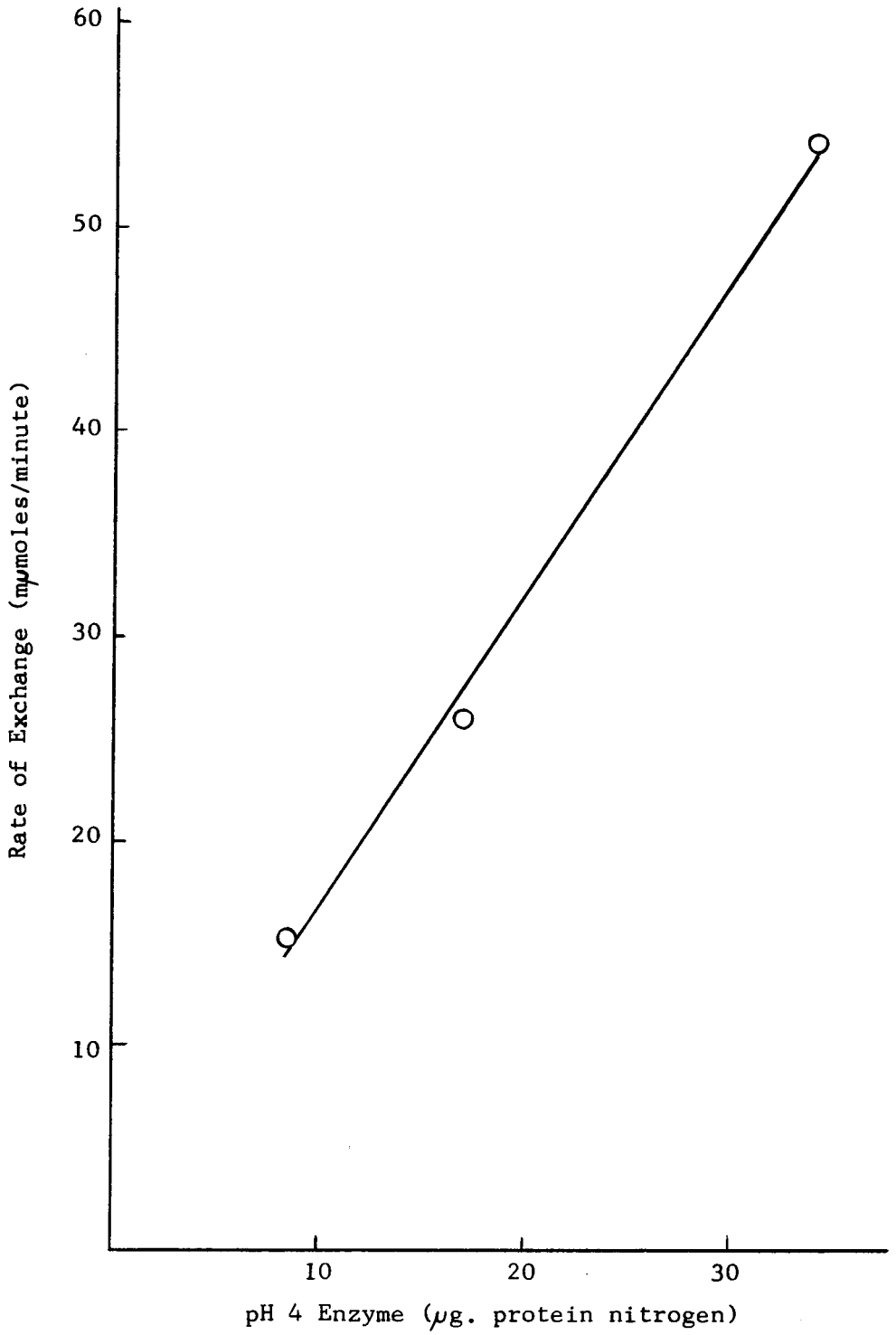

Figure 7. Effect of pH 4 enzyme concentration on ^{32}PP-ATP exchange.

amino acid being added, either in the amino acid mixture or as individual amino acids, were in excess.

Figure 8 illustrates the effect of amino acid concentration on the rate of ^{32}PP-ATP exchange induced by the soluble fraction from larval fat bodies. Three enzyme preparations were studied in the presence of various amounts of the amino acid mixture, alanine, cysteine, histidine, methionine, phenylalanine, or valine. The effect of valine concentration was determined twice. It may be noted that the endogenous rate of exchange varied from 4.5 to 12.6 mμmoles per minute for the three enzyme preparations. From these curves it was decided to use two μmoles of amino acid in all experiments involving ^{32}PP-ATP exchange in the presence of single amino acids. Because of some solubility difficulties, however, an amount of amino acid mixture equivalent to one μmole of each of the L-amino acids was utilized in all studies of ^{32}PP-ATP exchange. A similar effect of the concentration of added amino acid mixture on the rate of exchange was shown for the pH 4 enzymes (Figure 9).

Effect of Magnesium Ion Concentration on ^{32}PP-ATP Exchange

Hele (39, p 329-339) has reported on the pronounced effect of magnesium ion concentration on the activities of partially purified isoleucine, leucine, and lysine activating enzymes from rat liver. In the absence of magnesium, activity was negligible. As the magnesium ion concentration was increased, the rate of ^{32}PP-ATP exchange rose to a maximum and then fell off. Optimum

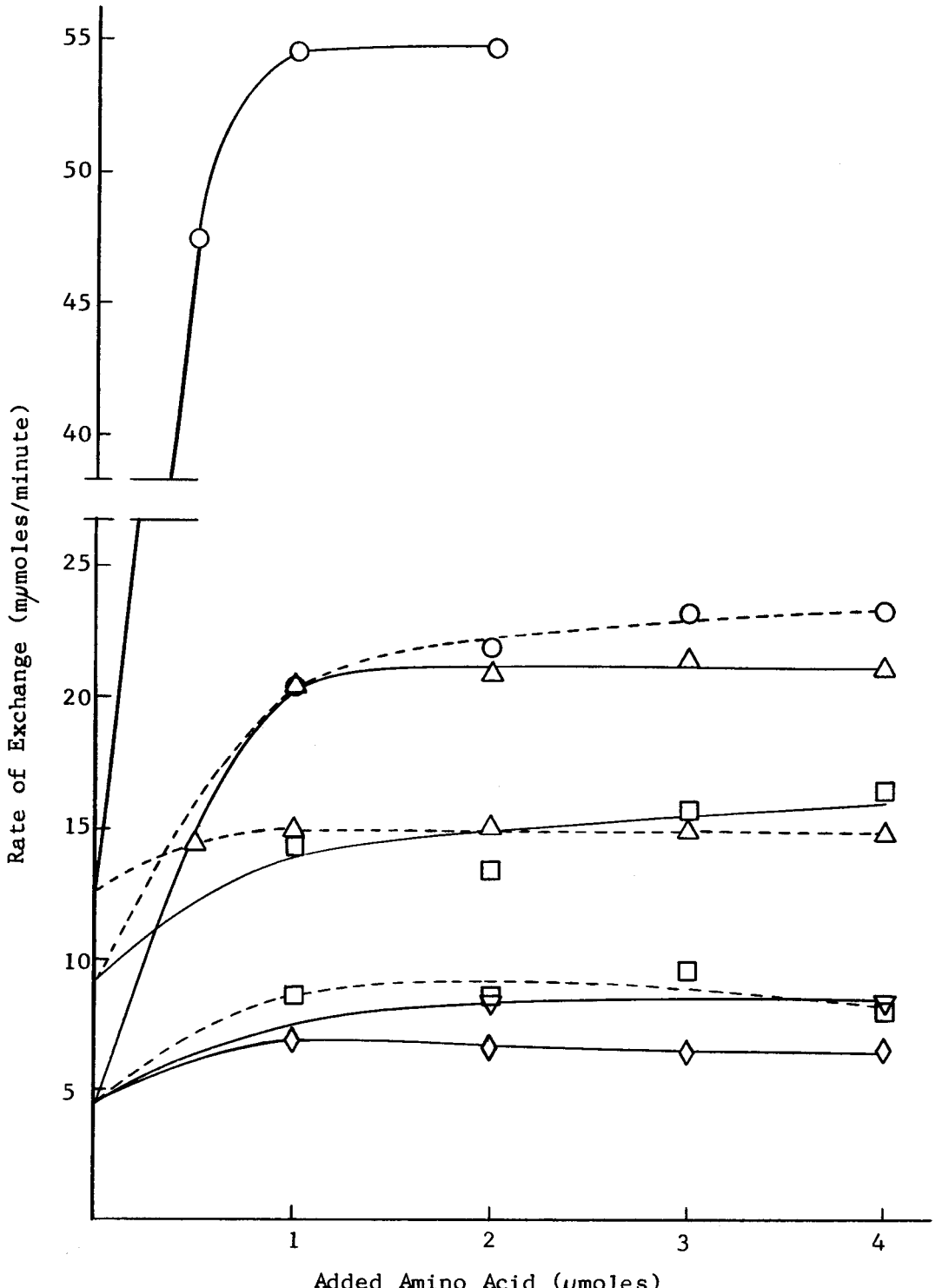

Figure 8. Effect of amino acid concentration on rate of ^{32}PP-ATP exchange in presence of three different preparations of the larval soluble fraction with the amino acid mixture ○——○, alanine △----△, cysteine ▽——▽, histidine □---□, methionine ◇——◇, phenylalanine □——□, or valine ○----○ and △——△.

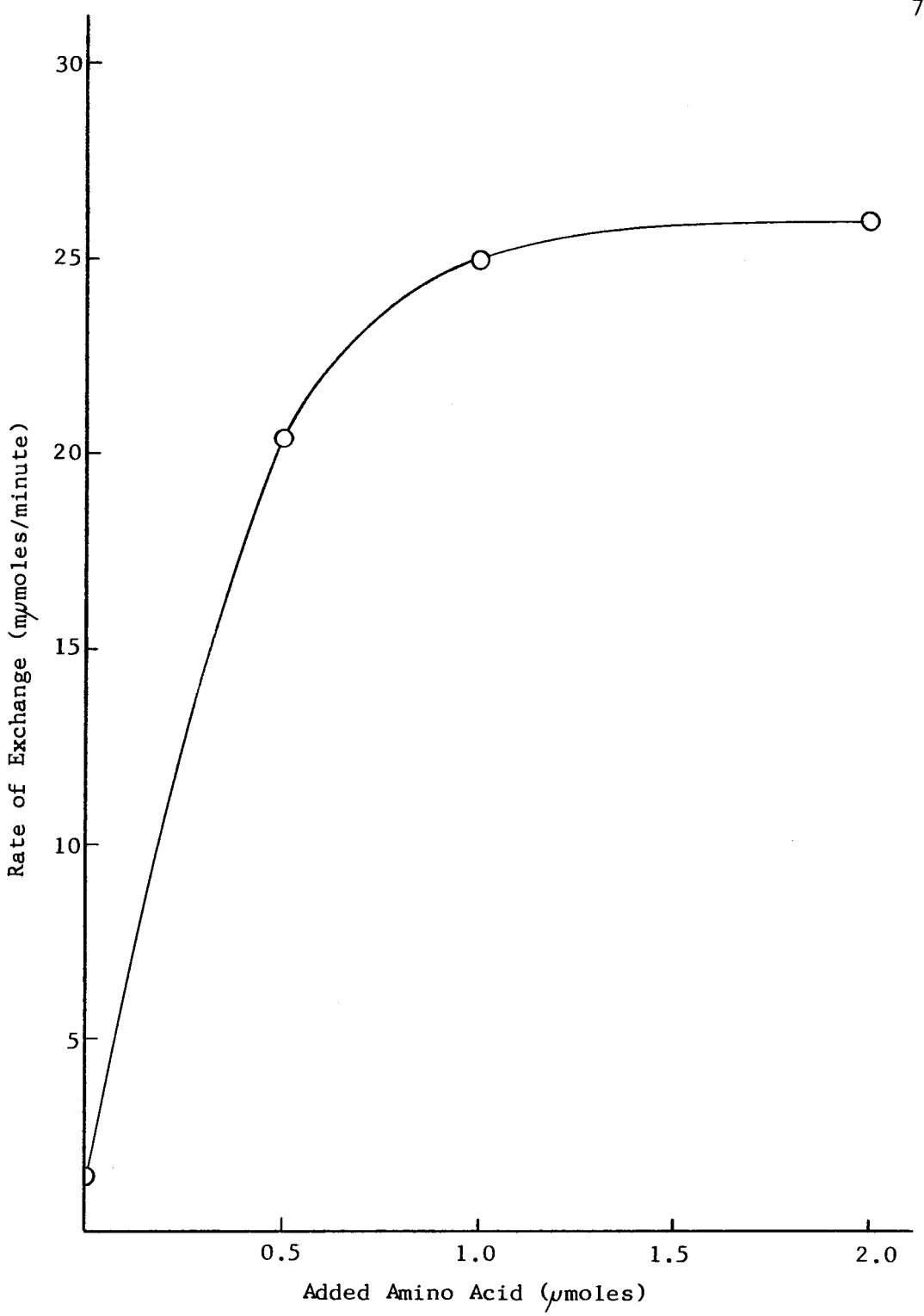

Figure 9. Effect of amino acid concentration on rate of ^{32}PP-ATP exchange in presence of pH 4 enzymes and the amino mixture.

concentration varied from five to 35 mM for the different enzymes.

Similar results were obtained when the fat body soluble fraction was incubated with histidine, phenylalanine, or valine at different magnesium ion concentrations (Figure 10). Rates of ^{32}PP-ATP exchange were insignificant in the absence of the metal ion, maximum at five or ten mM magnesium, and either unaffected or inhibited at higher concentrations. The inhibitory action of greater than optimum magnesium ion concentration was much less extensive than in the experiments reported by Hele. This may represent inherent differences in the amino acid activating enzymes specific for different amino acids, or it may be related to the fact that the enzymes studied by Hele were partially purified.

In the presence of the amino acid mixture, the activities of both the soluble fraction and pH 4 enzyme from fat bodies were maximum when the magnesium ion concentration was ten mM (Figures 11 and 12). The rate of ^{32}PP-ATP exchange decreased slightly at higher magnesium concentrations.

Although some individual amino acid activating enzymes may exhibit optimum activity at different concentrations, ten mM magnesium was used routinely in ^{32}PP-ATP exchange experiments. Howells and Birt (48, p 61-83) reported recently that ten mM magnesium was optimum for ^{32}PP-ATP exchange in the presence of the 105,000 g. supernatant fraction from two day larvae of the blowfly Lucilia cuprina.

A comparison of the maximum activities indicated in

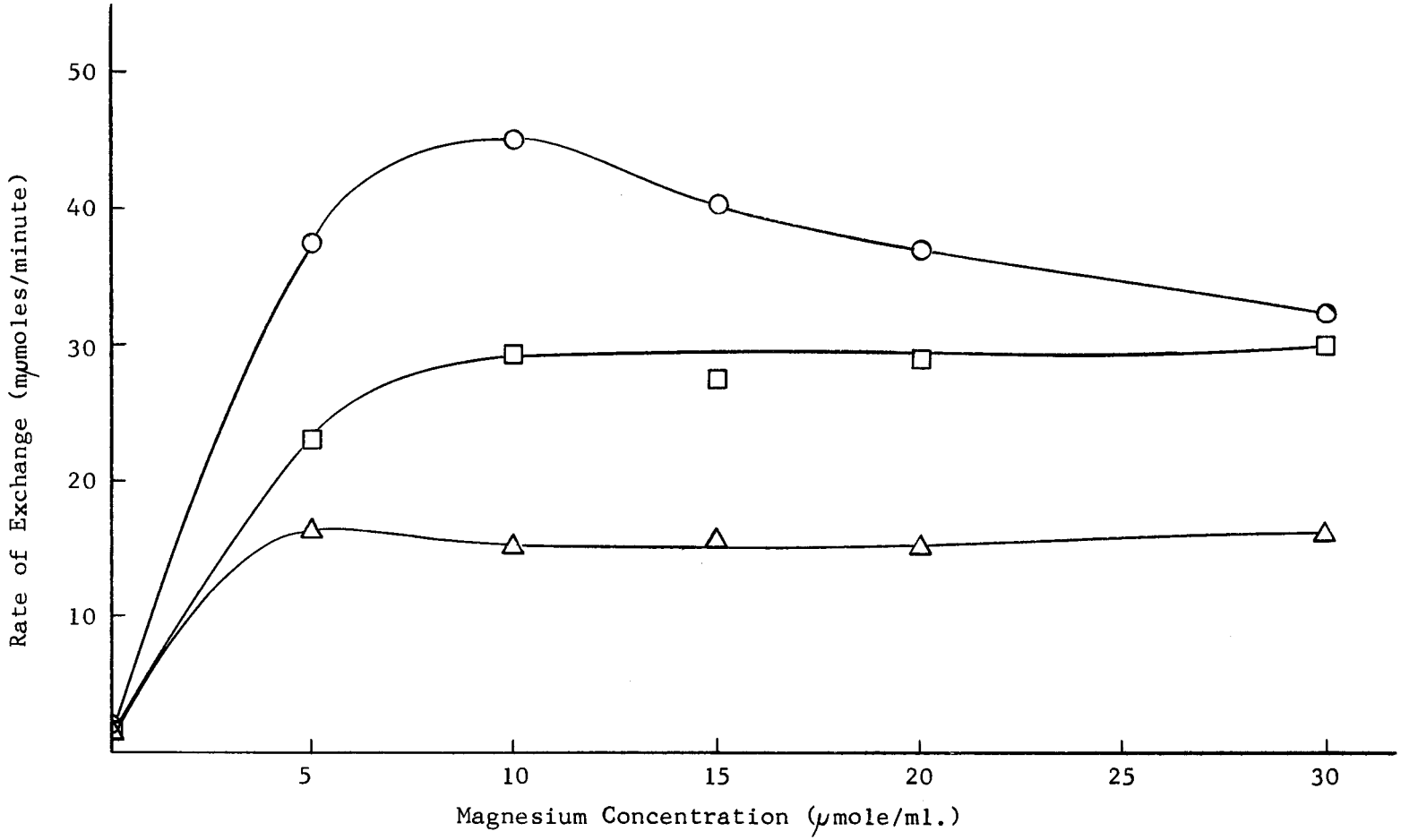

Figure 10. Effect of magnesium ion concentration on rate of ^{32}PP-ATP exchange in presence of larval soluble fraction and histidine △——△, phenylalanine □——□, or valine ○——○.

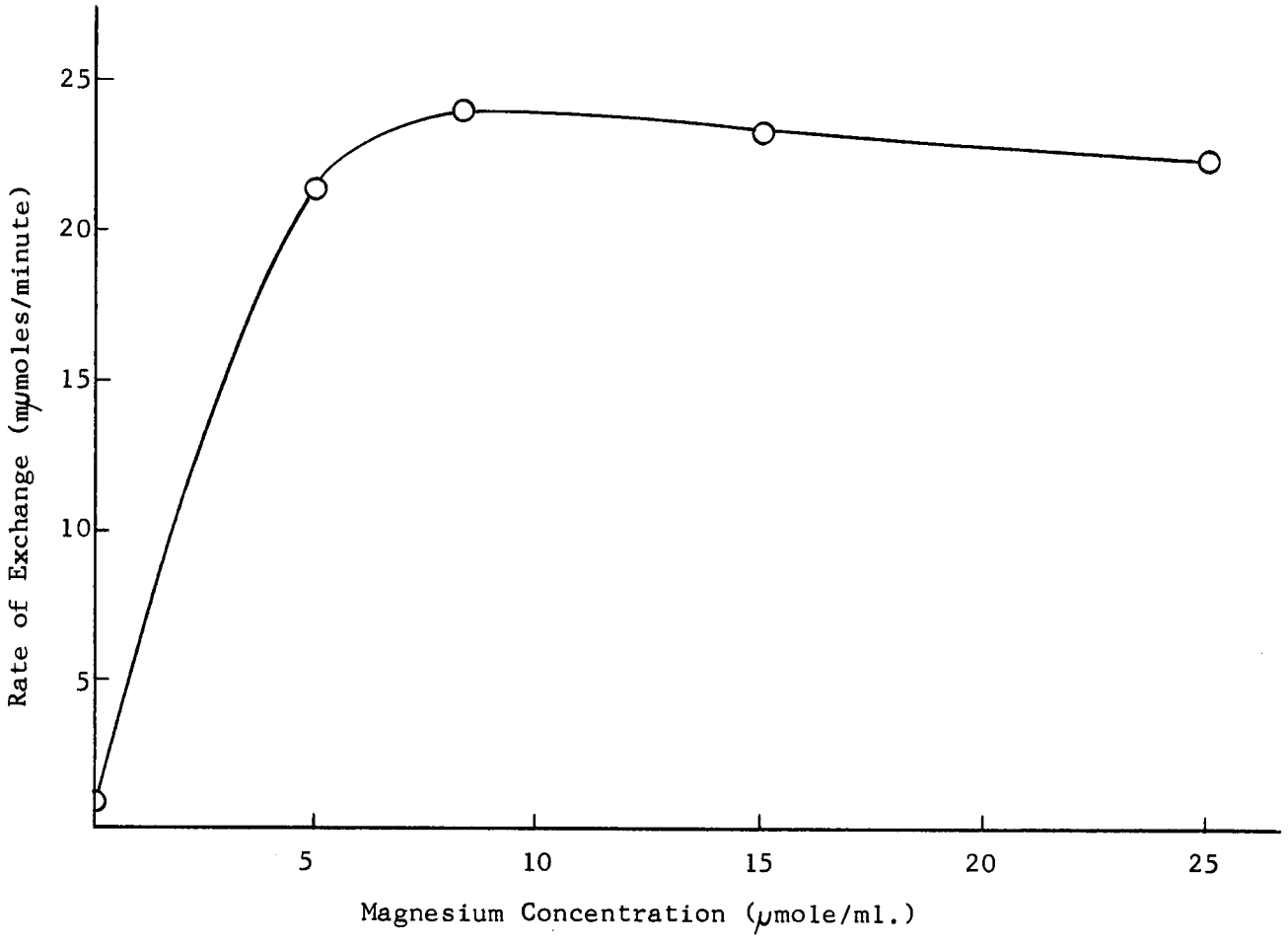

Figure 11. Effect of magnesium ion concentration on rate of ^{32}PP-ATP exchange in presence of larval soluble fraction and the amino acid mixture.

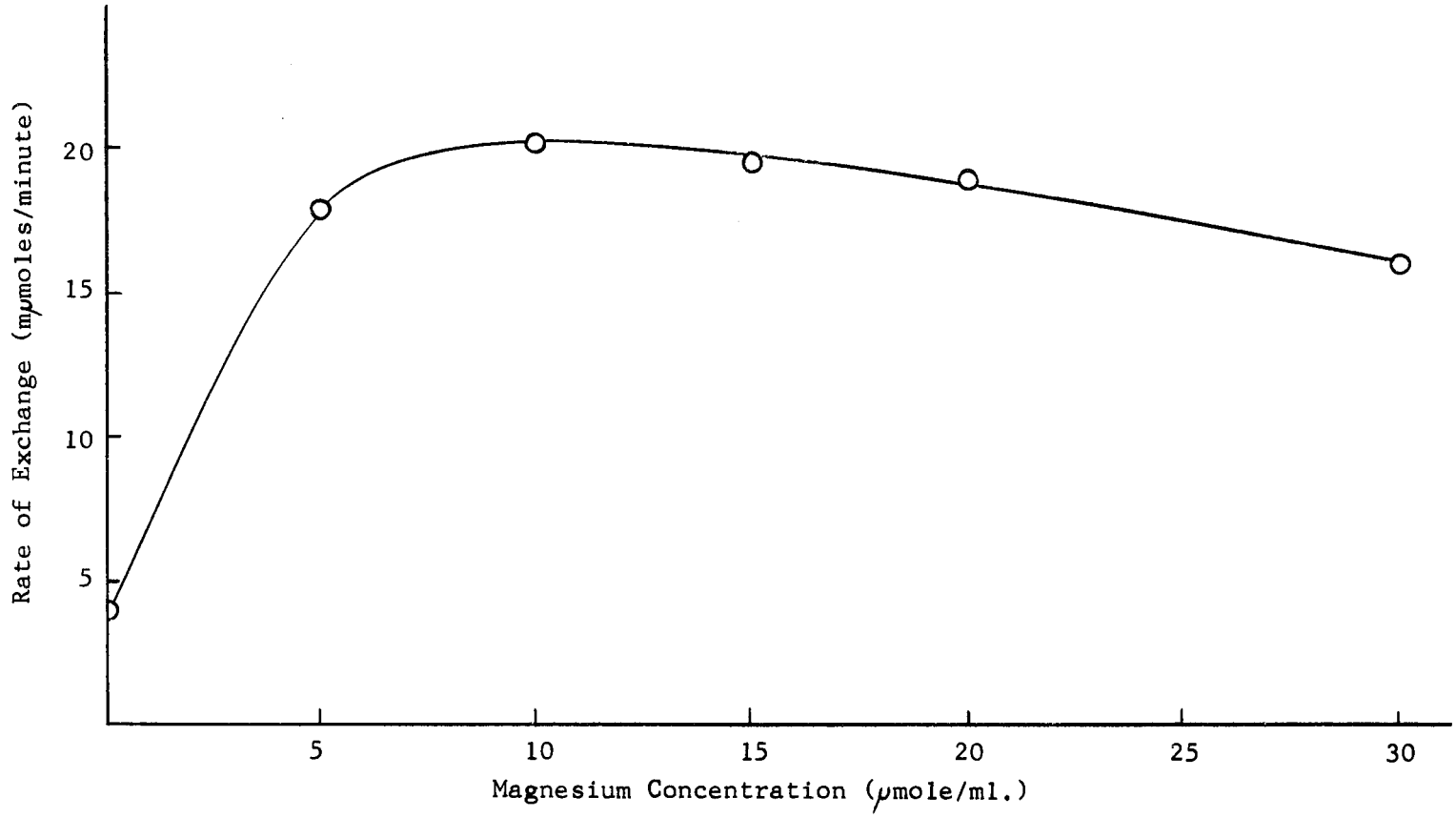

Figure 12. Effect of magnesium ion concentration on rate of ^{32}PP-ATP exchange in presence of pH 4 enzymes and the amino acid mixture.

Figures 10 and 11 would suggest that the rate of ^{32}PP-ATP was greater in the presence of either phenylalanine or valine than when the mixture of eighteen amino acids was added. This apparent discrepancy resulted from the fact that two different preparations of the fat body soluble fraction were used in obtaining the data. Differences in the specific activity and/or concentration of enzyme in the two preparations would account for the greater observed exchange with the single amino acids.

Although amino acid activating enzymes appear to have an absolute requirement for divalent metal ions, magnesium may be replaced in some systems. Webster (95, p 141-152) reported that ^{32}PP-ATP exchange took place with an alanine activating enzyme from pig liver in the presence of either magnesium or cobalt. No exchange was detected with manganese, calcium, barium, strontium, cadmium, or zinc. Manganese partially replaced magnesium with an extract from root nodules, but cobalt and molybdate were ineffective (68, p 93-97). Incorporation of glycine-^{14}C into soluble RNA, dependent upon a glycine activating enzyme from rat liver, required either magnesium or manganese. No enzyme dependent incorporation occurred with iron, cobalt, or nickel (33, p 1123-1133).

Effect of Dialysis on the Fat Body Soluble Fraction

The rate of exchange observed when the fat body soluble fraction was incubated in the usual manner, but in the absence of added amino acids, was always significant. This endogenous

activity occasionally resulted in as much as ten percent exchange. Since insect tissue contains high levels of free amino acids, it was reasonable to assume that the majority of this exchange might be due to the presence of these compounds. Dialysis of the soluble fraction, to remove the free amino acids, reduced the endogenous activity to less than one percent exchange. However, the ^{32}PP-ATP exchange in the presence of added amino acids was also decreased, in some instances to less than one-fifth that of the freshly prepared, undialyzed enzyme. It was decided, therefore, that dialysis of the fat body soluble fraction was impractical, and no further efforts were made to reduce the endogenous exchange activity.

Amino Acid Activating Enzymes in the Fat Body Soluble Fraction

The ^{32}PP-ATP exchange method was utilized to assay for individual amino acid activating enzymes in the soluble fraction from larval fat bodies by incubating this fraction with 20 different amino acids. Both the endogenous exchange and the exchange occurring in the presence of the amino acid mixture were determined for every enzyme preparation. The activity is expressed as the rate of exchange in the presence of added amino acid less the endogenous rate. Since the soluble fractions prepared at different times varied in their protein content, all values are expressed on the basis of protein nitrogen in the added enzyme. The results of these experiments are given in Table VII.

Table VII

Rate of amino acid dependent ^{32}PP-ATP exchange with the soluble fraction from larval fat bodies

Amino Acid	Number of Experiments	R_{aa}/mg. Protein nitrogen[a,b]
L-valine	7	695
L-phenylalanine	5	388
L-histidine	6	225
L-cysteine	5	188
L-lysine	4	160
L-threonine	4	125
L-tyrosine	1	98
L-alanine	4	82
L-methionine	3	78
L-leucine	3	51
L-aspartic acid	2	49
L-tryptophan	1	46
L-asparagine	1	25
L-serine	2	22
L-arginine	2	21
L-proline	2	21
L-isoleucine	3	17
L-glutamic acid	2	16
glycine	2	16
L-hydroxyproline	2	1
Amino acid mixture	9	1536
Sum of individual amino acids		2324

[a]See Methods, Part II, pp 52-53.

[b]Protein nitrogen of the added enzyme varied from 16 to 33 μg.

When it became apparent that the sum of the activities of individual enzymes exceeded the activity observed in the presence of the amino acid mixture, experiments were designed to test one of the possible explanations. The rate of ^{32}PP-ATP exchange occurring in the presence of a mixture of two or three amino acids was compared with the sum of rates of exchange obtained when the same amino acids were incubated separately (Table VIII). If the discrepancy noted above were due to competition for single activating enzymes by more than one amino acid, it was hoped that these experiments would detect it. Although the sensitivity of the enzyme assay was not sufficient to allow a positive conclusion there did not appear to be any extensive competition. The significance of these results will be discussed later.

Activation of a D-Amino Acid

A single experiment was carried out to determine if the soluble fraction from larval fat bodies would activate a D-amino acid. The rates of ^{32}PP-ATP exchange obtained from duplicate incubations with either no added amino acid or with D-valine are shown in Table IX.

Variability of ^{32}PP-ATP Exchange

Considerable variation was observed in the ^{32}PP-ATP exchange activity of fat body soluble fractions prepared at different times from larvae which were presumably of the same age. Table X gives the rates of exchange obtained with nine soluble

Table VIII

Rate of amino acid dependent ^{32}PP-ATP exchange with individual amino acids and with mixtures of the same amino acids

Amino Acid	Rates of Amino Acid Dependent Exchange[a]	
	Activity of Mixture	Sum of Individual Activities
cysteine, histidine	21.6	17.1
cysteine, valine	37.5	35.5
histidine, phenylalanine	24.4	24.3
histidine, valine	39.8	31.0
phenylalanine, valine	37.5	40.3
alanine, cysteine, lysine	11.0	10.1
alanine, cysteine, threonine	12.1	10.5
alanine, histidine, lysine	9.9	9.7
alanine, histidine, threonine	11.1	10.1
alanine, lysine, threonine	7.7	6.8
cysteine, histidine, lysine	12.0	14.8
cysteine, histidine, threonine	14.2	15.2
cysteine, lysine, threonine	14.9	11.9
histidine, lysine, threonine	12.9	11.5

[a]See Methods, Part II, pp 52-53

Table IX

Rate of ^{32}PP-ATP exchange with the soluble fraction from larval fat bodies and a D-amino acid

Amino Acid	R/0.1 ml. enzyme[a]
none	10.3
none	10.7
D-valine	12.6
D-valine	12.6

[a]See Methods, Part II, pp 52-53

Table X

Rate of amino acid dependent ^{32}PP-ATP exchange with the soluble fraction from larval fat bodies and the amino acid mixture

Experiment	R_{aa}/mg. protein nitrogen[a,b]
III-4	1635
III-6	558
III-7	2314
III-8	2192
IV-6	1243
IV-15	1377
V-10	1331
VI-1	1425
VI-5	1753
Average	1536
Standard error of mean	175

[a]See Methods, Part II, pp 52-53

[b]Protein nitrogen content of added enzyme varied from 16 to 33 μg.

fractions prepared from larval fat bodies. Duplicate assays with single enzyme preparations agreed to within plus or minus four percent.

Amino Acid Activating Enzymes in Different Larval Fat Body Fractions

The rates of ^{32}PP-ATP exchange in the presence of the amino acid mixture were measured for the microsomes, soluble, pH 4 enzyme, and pH 4 supernatant fractions from fat bodies of Sarcophaga larvae (Table XI). It will be recalled that the pH 4 enzyme and pH 4 supernatant were actually subfractions of the soluble. Since all of these activities were expressed on the basis of protein nitrogen, it was not possible to compare the total amounts of enzyme in the different fractions. It was apparent, however, that the non-particulate fraction had the highest specific activity and that this activity was precipitated at pH 4. The fact that the pH 4 enzyme was only slightly more active than the soluble and the very low activity of the pH 4 supernatant would suggest that a better fractionation of the soluble might have been attained at a less acid pH. However, the specific activity of the material precipitated at pH 5.0, 4.6, or 4.5 was always less than that of the soluble fraction, and the supernatant from such precipitates was not particularly active. Although the ^{32}PP-ATP exchange given for the microsomes may have been due to soluble enzymes adsorbed onto the microsomes, these enzymes must have been firmly bound, because washing did not lower the activity of the microsome fraction. All of these exchange values were obtained

Table XI

Rate of amino acid dependent ^{32}PP-ATP exchange with different fractions from larval fat bodies and the amino acid mixture[a]

Fraction	Number of Experiments	R_{aa}/mg. protein nitrogen[b]
microsomes	3	293
soluble	4	1482
pH 4 enzyme	3	1610
pH 4 supernatant	2	155

[a]The incubation media was only 5mM $MgCl_2 \cdot 6H_2O$ instead of 10 mM.

[b]See Methods, Part II, pp. 52-53

in the presence of five mM magnesium, since the experiments were run prior to the time when it was determined that ten mM was the optimum concentration.

In one experiment the soluble fraction from larval fat bodies was treated with one normal acetic acid to reduce the pH to 4.6. The precipitate and supernatant were treated as described in the Methods section for the preparation of the pH 4 enzyme and the pH 4 supernatant. The soluble fraction, pH 4.6 enzyme and pH 4.6 supernatant were each incubated with the amino acid mixture, histidine, phenylalanine, and valine. Table XII gives the ^{32}PP-ATP exchange obtained with these various combinations. Although each fraction was active toward all three individual amino acids, there was some concentration of activity (Figure 13). The pH 4.6 enzyme and pH 4.6 supernatant were about equally active with the amino acid mixture. The specific activity of the histidine activating enzyme was higher in the pH 4.6 enzyme, but the rates of phenylalanine and valine dependent ^{32}PP-ATP exchange were greater with the pH 4.6 supernatant.

Amino Acid Activating Enzymes in Developing Pupae

The soluble fractions from pupae of various ages were incubated both with the amino acid mixture and in the absence of added amino acids. The observed rates of ^{32}PP-ATP exchange were first corrected to give the total activity obtained from each group of ten pupae. In order to make the values for different developmental stages comparable, however, still another correction

Table XII

Rate of amino acid dependent ^{32}PP-ATP exchange with different fractions from larval fat bodies and individual amino acids

	R_{aa}/mg. protein nitrogen[a]		
Amino Acid	soluble	pH 4.6 enzyme	pH 4.6 supernatant
amino acid mixture	439	244	277
L-histidine	76	120	67
L-phenylalanine	79	29	53
L-valine	120	67	130

[a]See Methods, Part II, pp 52-53

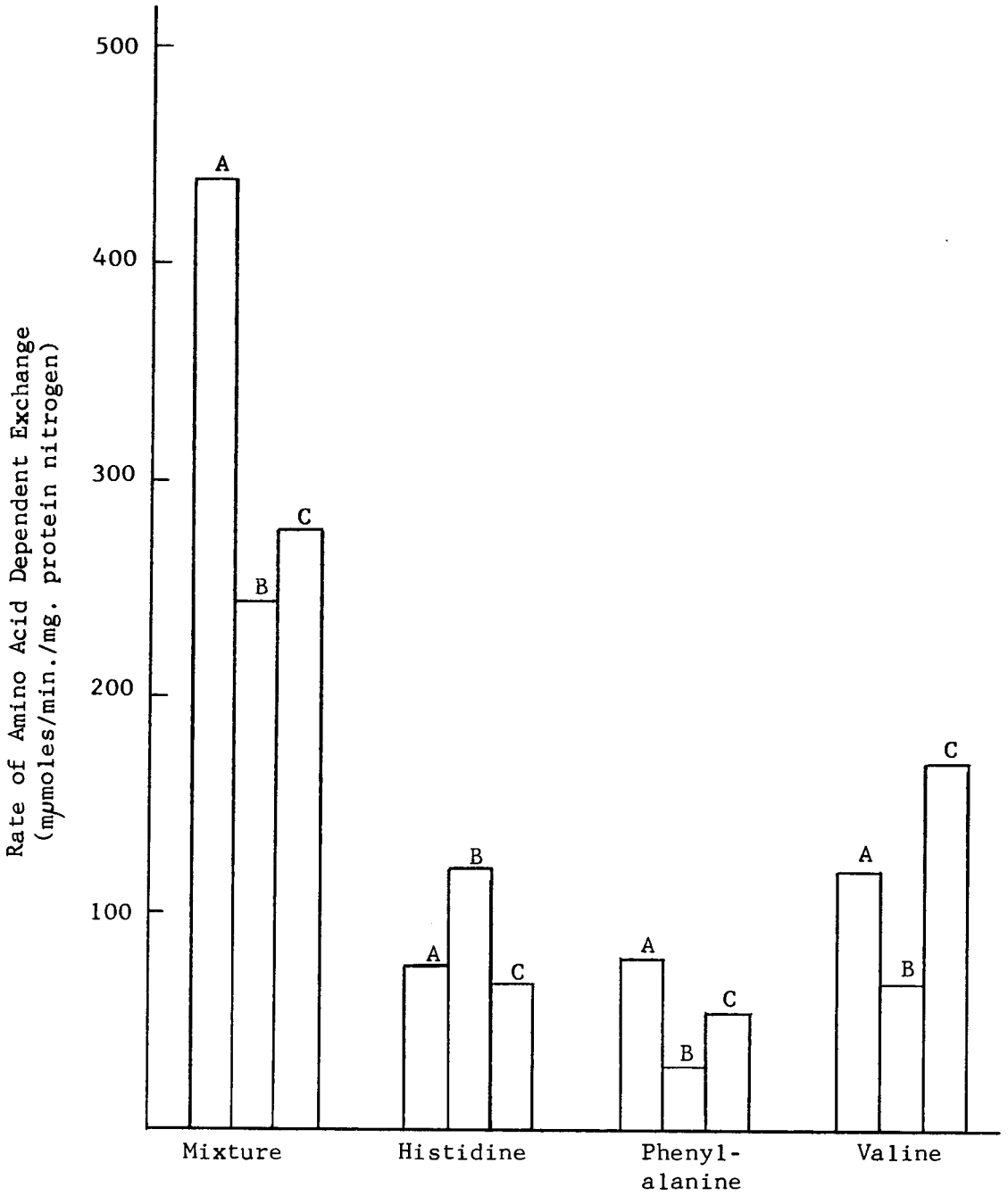

Figure 13. Distribution of specific amino acid activating enzymes among different enzyme fractions from larval fat bodies. A = soluble, B = pH 4.6 enzymes, C = pH 4.6 supernatant.

was necessary. Since the initial weights of the pupae used for preparation of the soluble fractions varied from 1514 to 1928 mg., all rates of exchange were expressed as the total activity in 1500 mg. pupae (Table XIII and Figure 14). It will be recalled that values reported for the eleventh day are for the soluble fraction from adult Sarcophaga. An experiment in which incubations were made in the presence of histidine, phenylalanine, or valine produced a pattern similar to that obtained with the amino acid mixture.

In order to calculate the specific activities of the amino acid activating enzymes in the soluble fractions from developing pupae, the protein nitrogen of these preparations was determined. From Figure 15 it can be seen that the soluble protein was fairly constant for the first seven days, then decreased sharply in the older pupae and adult flies. The rates of ^{32}PP-ATP exchange, based on protein nitrogen, are shown in Table XIII and Figure 16. The variations with age were similar to those obtained for the total enzyme content, although increases in activity with the older larvae were more pronounced and continued on into the adult stage.

Since the values for ^{32}PP-ATP exchange produced by soluble fractions from pupae at different stages of development may be influenced by the endogenous amino acids present, it was of interest to ascertain the free amino acid content of these preparations. The results of such determinations are shown in

Table XIII

Rate of ^{32}PP-ATP exchange with the soluble fraction from pupae at different stages of development

Pupal Age (days)	Number of Exp'ts.	R/1500 mg. pupae[a]			R/mg. Protein nitrogen[a]		
		amino acid mixture	no added amino acids	amino acid dependent	amino acid mixture	no added amino acids	amino acid dependent
1	2	693	351[b]	342	95	50[b]	45
2	3	636	297	339	90	42	48
3	3	577	291	286	84	42	42
4	3	563	245	318	83	36	47
5	5	536	284	252	79	41	38
6	3	471	211	261	71	31	40
7	3	529	220	309	77	33	44
8	3	773	272	501	129	45	84
9	3	737	293	444	159	64	95
11	2	632	199	433	249	78	171

[a]See Methods, Part II, pp 52-53.
[b]Only one experiment

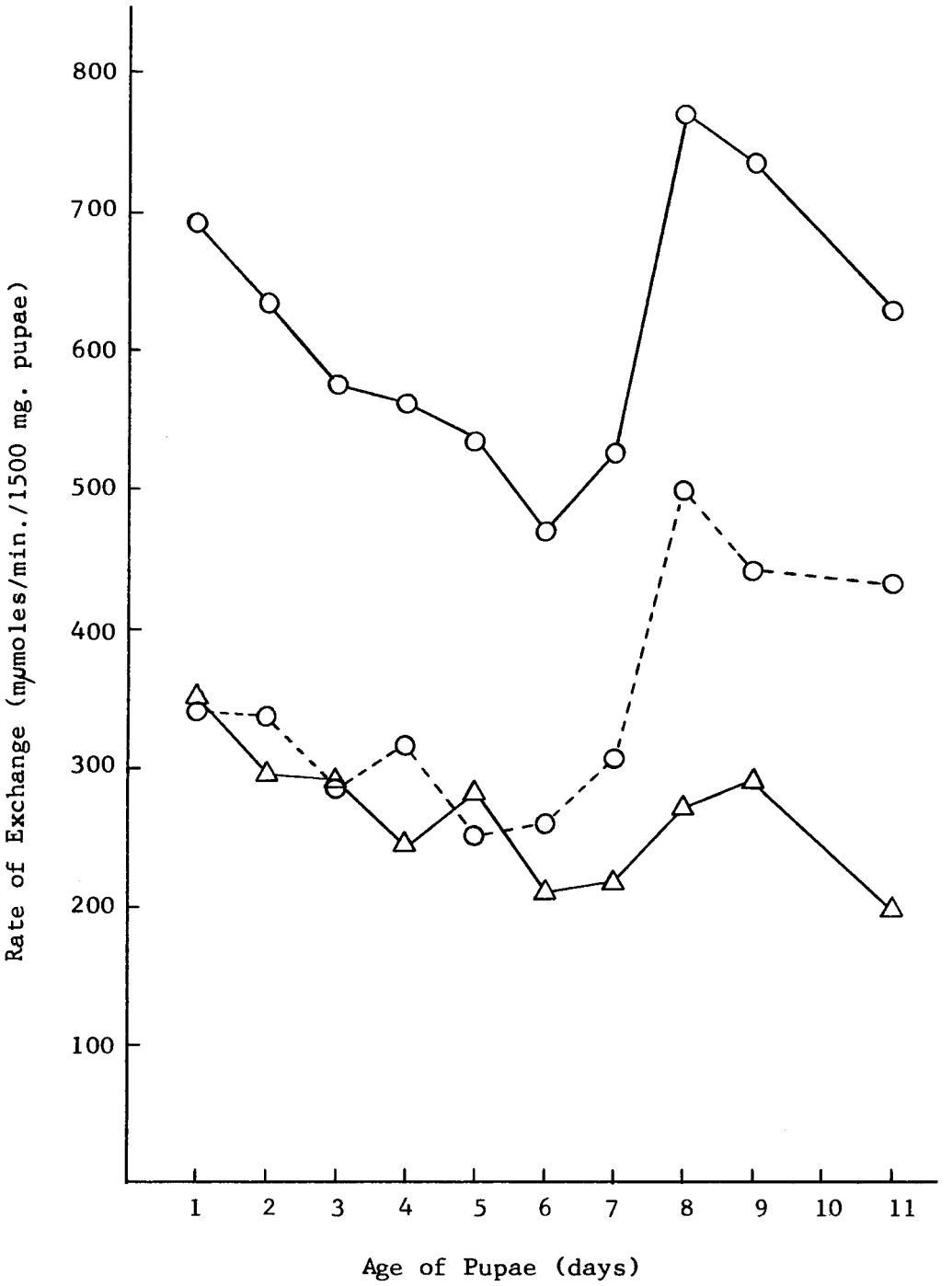

Figure 14. Total ^{32}PP-ATP exchange activity in the soluble fraction from developing pupae in presence of an amino acid mixture O——O or in absence of added amino acids △——△, and amino acid dependent exchange O----O .

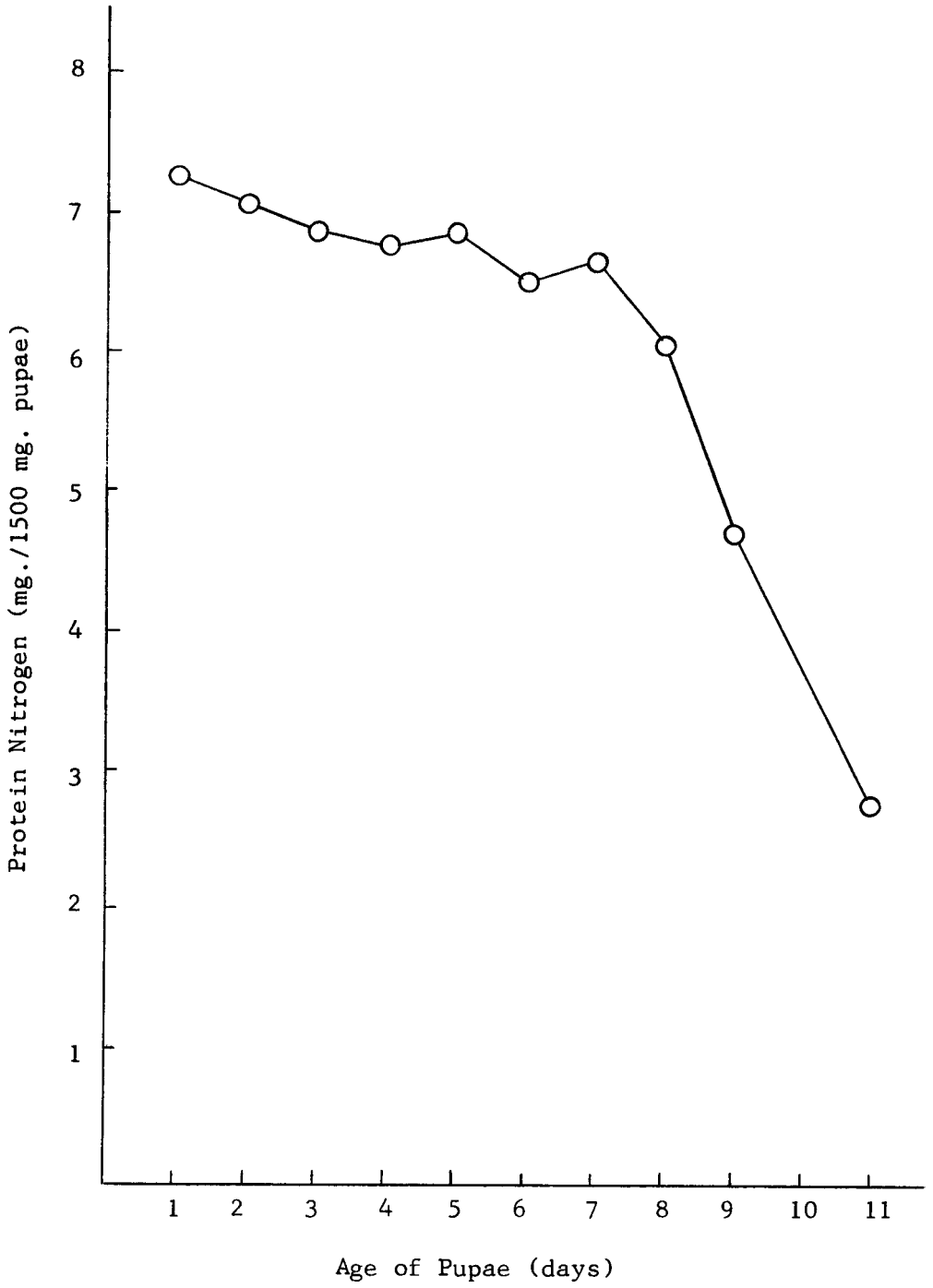

Figure 15. Protein nitrogen content of soluble fraction from developing pupae.

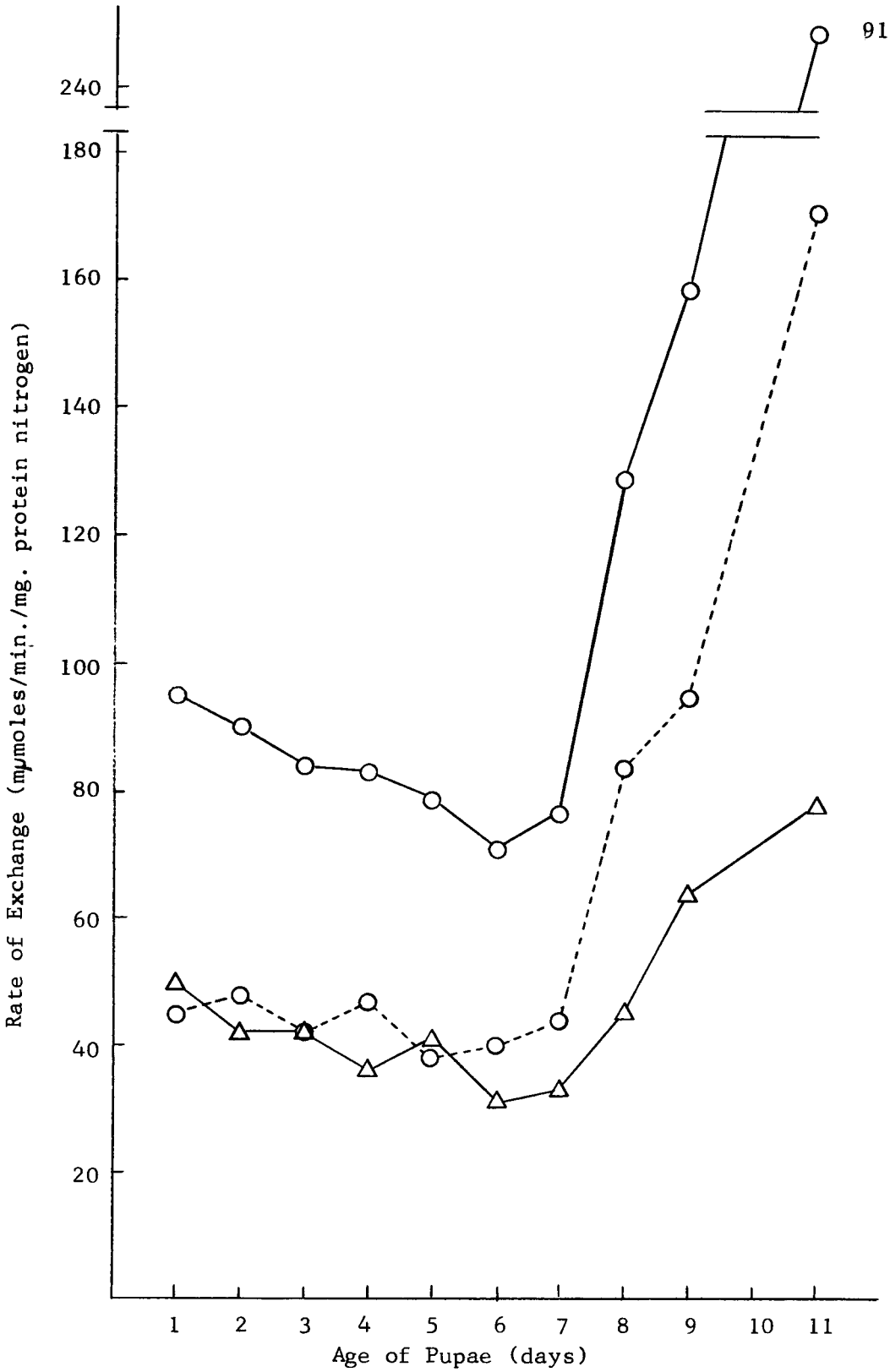

Figure 16. Specific activity of ^{32}PP-ATP exchange in the soluble fraction from developing pupae in presence of an amino acid mixture ○——○ or in absence of added amino acids △——△, and amino acid dependent exchange ○----○.

Figure 17. In general, free amino acids decreased to a minimum at eight days, then increased on the ninth day.

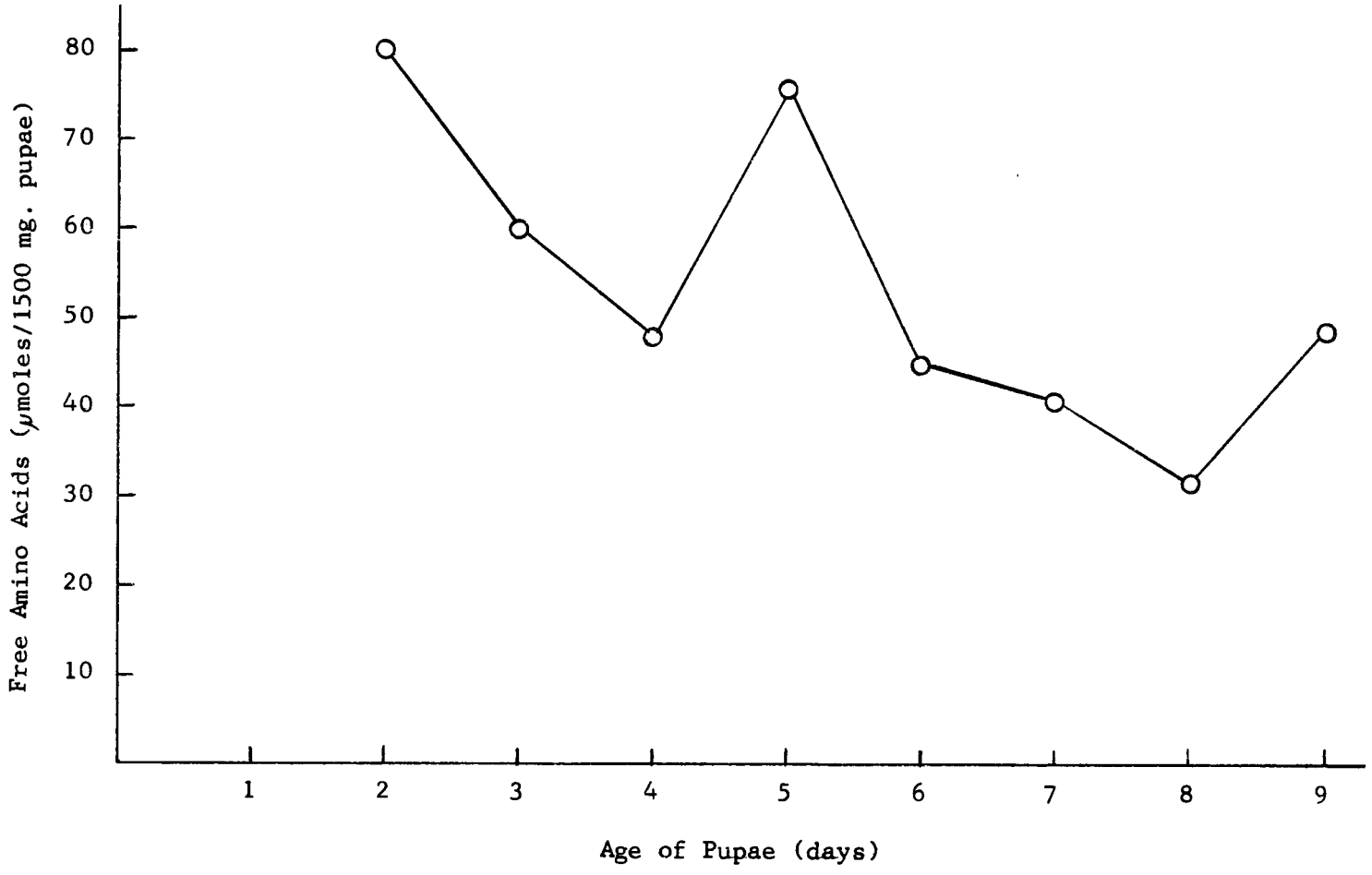

Figure 17. Free amino acid content of soluble fraction from developing pupae.

DISCUSSION

In the early investigations of amino acid activating enzymes, activity could usually be shown toward only a few amino acids (26, p 592-593; 42, p 288-289). However, it soon became possible to demonstrate a complete range of activating enzymes for all naturally occurring amino acids (2, p 1061-1067; 57, p 67-73; 71, p 639-640; 94, p 125-134). This was true for the soluble fraction from larval fat bodies, which induced ^{32}PP-ATP exchange in the presence of all amino acids tested, except hydroxyproline (Table VII). The failure to observe a hydroxyproline activating enzyme was to be expected, since this amino acid is apparently not incorporated into protein in its hydroxylated form (86, p 31-33). There is considerable evidence that the hydroxyproline of such proteins as collagen is formed from proline, this amino acid being hydroxylated at some point subsequent to its activation (18, p 75-81; 73, p 3966-3977).

Although activation was demonstrated for 19 amino acids, the variation in the activities toward different amino acids was extensive. The greatest rate of ^{32}PP-ATP exchange occurred in the presence of valine, with glycine and glutamic acid being the least active (Table VII). It is not known why the valine activating enzyme should appear to be more than 40 times as active as the enzymes for some other amino acids. These great differences in the activities of the enzymes for specific amino acids may well

represent differences in stability toward the fractionation and assay procedures used. It is also possible, however, that there is actually more of the valine activating enzyme present.

Several authors (40, p 397; 41, p 821-823; 76, p 283-326) have reported that there is no direct correlation between the relative rates of activation of amino acids and the extent to which they are incorporated into protein. A classic example of this is found in the posterior silkgland of the silkworm Bombyx mori, for which Heller (40, p 397) observed very low levels of glycine activation, although this amino acid constitutes 42 per cent of the fibroin being synthesized. The maximum activity was for tryptophan, which is found in only trace quantities in silk.

A similar lack of correlation is seen when activation of glutamic acid, histidine, and valine is compared with the incorporation of these amino acids into protein by fat bodies from Sarcophaga bullata larvae. The relative rates of ^{32}PP-ATP exchange were 1:14:44 for glutamic acid, histidine, and valine, respectively (Table VII), while these same amino acids were incorporated into protein at the ratio of 1:1.7:3.9 (Table II).

However, this does not prove that a correlation between the relative rates of activation of amino acids and their incorporation into protein does not exist in vivo. Although the above-mentioned ratio of 1:14:44 for different amino acid activating enzymes may correctly represent the relative amounts

of activation occurring under the conditions of the assay, this may not correspond to the situation in the intact animal. The concentration of the amino acid substrates, the pH, and the concentration of metal ions would all be different *in vivo*, and could result in an entirely different ratio for the activation of amino acids. In addition, the relative activities observed *in vitro* may be indicative of the stabilities of the different enzymes, rather than their original concentrations.

The sum of the activities for the individual amino acid activating enzymes was considerably larger than the rate of ^{32}PP-ATP exchange in the presence of the amino acid mixture (Table VII). Although the mixture of amino acids did not contain asparagine or serine, the contribution of these two amino acids could not account for the differences noted above. The same relationship between the sum of individual activities and the activity with a mixture of amino acids has been reported for the amino acid activating enzymes from *E. coli* (27, p 49-61), mouse and human skeletal muscle (72, p 205-208), frog and tadpole liver (24, p 595-604), root nodules of *Galega officinalis* (68, p 93-97), and larvae and pupae of the blowfly *Lucilia cuprina* (48, p 61-83).

An obvious explanation for this discrepancy is competition for activating enzymes by two or more amino acids. In an attempt to support or discredit this hypothesis, experiments were performed with mixtures of only two or three amino acids, hoping to detect

any competition which might exist between specific amino acids. Contrary to the findings with the complete amino acid mixture (Table VII), the rates of ^{32}PP-ATP exchange observed with mixtures of two or three amino acids were in most instances equal to or slightly greater than the sums of the activities of these amino acids incubated individually (Table VIII). It was concluded from this data that no extensive competition existed between any of the amino acids examined, supporting the concept that each amino acid has its own specific activating enzyme. These experiments agreed with those of Matsushita (62, p 21-28), who reported that the activities of four amino acid activating enzymes in a partially purified preparation from yeast were additive. Since the fat body soluble fraction was studied with only a few of the possible combinations of amino acids, however, it is still conceivable that the difference between the ^{32}PP-ATP exchange with the complete amino acid mixture and the sum of the individual activities might be due to competition for the same activating sites by different amino acids.

Another possible explanation could be that the ^{32}PP or ATP were limiting when incubated with the amino acid mixture. However, Howells and Birt (48, p 61-83) measured the activating enzymes from blowfly pupae under optimum concentrations of ^{32}PP and ATP and still found that the sum of the activities with individual amino acids was greater than that with an amino acid mixture. There does not seem to be a readily apparent explanation for this

often observed phenomenon, and it may be an artifact of the enzyme isolation and assay.

Several reports concerning the specificity of amino acid activating enzymes toward L-amino acids differ rather widely in their findings. DeMoss and Novelli (27, p 49-61) stated that D-amino acids were not activated, and did not effect the activation of the L-forms, by the 100,000 g. supernatant from E. coli. Extracts from several bacteria did not activate D-amino acids, according to Ciferri (16, p 411), even though the bacteria produced antibiotics containing these amino acids. Pennington (72, p 205-208) reported that ^{32}PP-ATP exchange in the presence of L-tryptophan was inhibited by the addition of D-tryptophan, but the latter had no effect on L-leucine dependent exchange. However, Baddiley and Neuhaus (6, p 277-279) have purified an enzyme from a sonic extract of acetone dried Lactobacillus arabinosus which produced ^{32}PP-ATP exchange in the presence of D-alanine. This enzyme was inactive toward L-alanine.

When D-valine was incubated with the soluble fraction from Sarcophaga fat bodies, the observed rate of exchange was slightly greater than the endogenous value (Table IX). This may have been due to a very low level of activity towards this amino acid, or possibly to contamination of the D-valine with the L-form.

In an earlier discussion of the incorporation of L-valine-U-^{14}C into fat body protein, it was pointed out that fat bodies from different batches of larvae differed in their ability to

incorporate the amino acid. The ^{32}PP-ATP exchange activity of the fat body soluble fraction also varied from one experiment to the next (Table X), although duplicate assays made with the same enzyme preparation agreed to within plus or minus four percent. The variation in the activities of the different fat body soluble fractions may be due to slight variations in the preparation of the enzyme or they may represent actual differences in the enzyme content of the larvae. Although both of these factors are undoubtedly involved, most of the variation in enzymatic activity probably results from the latter. Since it is known that fat bodies, from larvae which are presumably identical in age and physiological condition, differ in their ability to incorporate amino acids into protein, it does not seem unlikely that they might also exhibit different capacities for the activation of amino acids. In addition, it is improbable that the few steps involved in the routine preparation of the fat body soluble fraction ever varied sufficiently to induce four-fold differences in the specific activity of the enzyme.

In demonstrating the complete complement of amino acid activating enzymes in Sarcophaga bullata fat bodies, two day old larvae were used. This is a period of rapid growth and protein synthesis. It was of interest to know if variations in the rate of protein synthesis during development would be reflected by differences in the level of amino acid activating enzymes. Because of their small initial size and rapid growth rate, larvae were not suited for such an investigation. It was decided,

therefore, to study the amino acid activating enzymes in developing Sarcophaga pupae.

The total ^{32}PP-ATP exchange activity of the pupae decreased with age for the first six days, then increased to a maximum one or two days prior to emergence of the adults (Table XIII and Figure 14). This rise in activity was observed both for the endogenous exchange and for that occurring in the presence of the amino acid mixture. However, the difference between these two activities, designated the amino acid dependent exchange in Figure 14, also increased. The implication from this was that the greater rate of ^{32}PP-ATP exchange in the older pupae was at least partially due to an increased amount of enzyme, and not merely to a higher level of endogenous amino acids. Similar results were reported by Finch and Birt (31, p 59-64) for the pupae of the blowfly Lucilia cuprina. This conclusion was verified when it was demonstrated that the free amino acid concentration of the pupae actually decreased during a period (six to eight days) when the endogenous ^{32}PP-ATP exchange activity was increasing (Figure 17).

The pattern described above for the content of amino acid activating enzymes in developing pupae is consistent with evidence that during the pupation of holometabolous insects, breakdown of larval tissue is followed by the formation of adult organs and tissues (9, p 822). McAllan and Chefurka (64, p 290-299) have also concluded, from studies of amino acid transaminases,

that protein synthesis increases significantly during the late stages of pupation. The level of amino acid activating enzymes in Sarcophaga pupae decreased to a minimum value over the first six days, a period during which breakdown of larval tissue was probably the predominant process. The increase in activating enzymes from older pupae was undoubtedly related to the rapid synthesis of adult protein.

The pattern of the specific activity of the amino acid activating enzymes in developing pupae was similar to that of the total enzyme content for the first seven days (Figure 14 and Figure 16). However, increases in the specific activity were much greater than those for the total enzyme in the eight and nine day pupae, and specific activities reached their highest level in the soluble fraction of newly emerged flies. These high specific activities in the older pupae and adults may be correlated with an increased amount of the activating enzyme (Figure 14) and a pronounced decrease in the soluble protein (Figure 15). The latter factor more than compensated for a decrease in enzyme upon emergence of the flies, to give the soluble fraction from adult Sarcophaga the highest observed specific activity.

The decrease in the soluble protein and the increase in amino acid activating enzymes, beginning at around seven or eight days, may well represent the conversion of soluble products from the breakdown of larval tissue into adult protein. This would suggest the complete degradation of soluble proteins to amino

acids, followed by their activation and incorporation into insoluble adult protein. Although the data on soluble proteins and ^{32}PP-ATP exchange activity of the developing pupae are consistent with this hypothesis, the low level of free amino acids in the eight day pupae (Figure 17) appears to contradict it. A closer examination of Figure 14 and Figure 15, however, indicates that the pattern of free amino acid concentration may agree very well with those for enzymatic activity and soluble protein concentration. Any protein synthesis during the early pupal period would consume free amino acids. If there were little degradation of soluble protein to replace these compounds, the amino acid concentration would decline. The activating enzymes began to increase on the seventh day, making increased demands on the free amino acid pool, while the soluble protein concentration remained constant. Still greater amounts of enzyme in the eight day pupae, coupled with only a small decrease in soluble protein, would result in a further decrease in the amino acid concentration. Degradation of the soluble protein was much greater by nine days, and the activating enzymes had leveled off, so there was a marked increase in the free amino acid concentration. Therefore, the patterns of ^{32}PP-ATP exchange, soluble protein concentration, and free amino acid concentration in the soluble fraction of developing Sarcophaga pupae are consistent with one another and with the morphogenetic changes occurring during this period.

As mentioned earlier, Finch and Birt (31, p 59-64)

observed similar changes in the content of activating enzymes in blowfly pupae and also concluded that these changes were related to the synthesis of adult protein in the older pupae. With the blowfly pupae, however, the patterns of soluble protein and free amino acid concentration were of the same general shape as the plot of the ^{32}PP-ATP exchange activity and had maxima and minima which corresponded to those on the curve for enzymatic activity.

A striking feature of Figure 17 is the peak occurring at five days. This high level of free amino acids was probably associated with a rapid rate of proteolysis at this particular stage of pupal development. The amino acid concentration in five day pupae was more variable than that observed at any other age. It is not known whether this signified an unusual amount of variation at five days, or whether the proteolysis occurred so rapidly that in some measurements the degradation of protein had proceeded much further than in others. Whatever the explanation, it is not felt that this significantly affects the general picture described earlier for the biochemical changes occurring in Sarcophaga pupae. However, the high concentration of amino acids in the soluble fraction from five day pupae was undoubtedly responsible for the increased endogenous ^{32}PP-ATP exchange observed with this enzyme (Figure 14).

SUMMARY

The investigation of protein synthesis in the flesh fly, Sarcophaga bullata, was undertaken because it was felt that certain characteristics of the developing organism would influence the formation of protein.

Incubation of ^{14}C-labeled amino acids with intact larval fat bodies indicated that this tissue was capable of incorporating carbon atoms from amino acids into proteins and lipids, and that it contained enzymes required for the degradation of amino acids to carbon dioxide. All of these activities were higher in fat bodies from two or three day larvae than in those from six day larvae. This indicated that not only the synthetic processes, but also the energy requirements, were greatest in the young, rapidly growing larvae. The difference with age was most extensive for degradation of amino acids to carbon dioxide and least for the incorporation of radioactivity into lipids.

The ^{32}PP-ATP exchange method was used to study amino acid activating enzymes in sub-cellular fractions from larval fat bodies and in pupae at different stages of development. The soluble fraction from the fat bodies, which contained enzymes exhibiting activity toward nineteen individual amino acids, was investigated most extensively. Various properties and requirements were determined for these enzymes, and the activation of amino acids by the soluble fraction was discussed with regard

to variations in the activity shown toward different amino acids, the high level of endogenous activity, differences in the activity observed for a mixture of amino acids as compared to that for the same amino acids individually, variability in the enzymatic activity toward a D-amino acid, and differences observed for several amino acids between their relative rates of activation and incorporation into fat body protein. The larval fat body soluble fraction could be further fractionated by isoelectric precipitation, resulting in some separation of activity toward specific amino acids.

The amino acid activating enzymes, soluble protein concentration and level of free amino acids were determined for the soluble fraction from pupae at different stages of development. All of these parameters were consistent with known changes occurring during pupation, involving the initial degradation of larval tissue, followed by the synthesis of adult proteins from amino acids produced as breakdown products of the larval proteins.

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