Qualitative and Quantitative Changes Observed in the Free α-Amino Nitrogen Fraction of *Tenebrio molitor* Pupal Tissues during Metamorphosis

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(*Received* 19 *October* 1956)

An outstanding feature of the biochemistry of insects is the fact that a very high concentration of free amino acids has been found in the haemolymph of every insect so far investigated. (See Florkin, 1949; Buck, 1953.) This represents about two-thirds of the total free amino acids in the insect, and concentrations of from 20 to 100 times that of the total free α-amino nitrogen content of mammalian blood are generally found.

The chemistry of insect metamorphosis has attracted attention for at least 50 years. Needham (1929) made a comprehensive review of the information available in 1929, and since then more recent work has been summarized by Buck (1953) and Wigglesworth (1950, 1954). The variation in the titre of free amino acids during the metamorphosis of insects has been studied by Heller & Moklowska (1930) in *Deilephila*, by Florkin (1937) in *Bombyx mori* and by Agrell (1949) in *Calliphora*. In the work of Heller & Moklowska (1930) and Florkin (1937), the total free amino nitrogen content of insect blood was measured, and in Agrell's (1949) investigation changes in the concentrations of the free amino acids of pupal tissue breis were observed semi-quantitatively on paper chromatograms. In these three cases it was found that the level of total free amino nitrogen varied little during metamorphosis in spite of the tremendous morphological changes which holometabolous insects undergo at this period of their existence.

The present work describes the variations found in the free α-amino nitrogen content of the pupal tissues of another holometabolous insect, *Tenebrio molitor*, during metamorphosis at 24°, and an attempt is made to relate these findings with the observed changes in various other non-protein tissue fractions.

MATERIALS AND METHODS

Insects. Larvae of *Tenebrio molitor* were kept in a glass aquarium tank, the bottom of which was covered to a depth of 1–2 in. with a diet consisting of 2 parts by weight of bran, 1 part by weight of rolled oats and a supplement of 5% of dried yeast. A supply of water was also available to the insects in the form of a permanently damp pad of cotton wool. By careful daily inspection of the tank fresh pupae were identified and transferred to separate glass jars kept at 24°. The 'period of metamorphosis', which is defined for the present purposes as the time taken for a fresh pupa to become transformed into an adult insect at 24°, lasted 10 days. By making daily collections of fresh pupae it was ensured that samples of pupal tissue of any age were readily available from the beginning of the 10-day period.

Homogenates. All analyses were made on homogenates prepared from pupae with ice-cold water. Chilled pupae were killed by decapitation and homogenized with five times the weight of ice-cold water in a Potter–Elvehjem (1936) homogenizer. Fragments of cuticle were removed by straining through fine gauze.

Total free α-amino N. The ninhydrin–CO_2 titrimetric method of Van Slyke, MacFadyen & Hamilton (1941) was used to estimate the total free α-amino N in 70% (v/v final concn.) ethanolic extracts of pupal-tissue homogenates.

Ethanol was used as a deproteinizing agent because samples of the resulting protein-free filtrates were easily concentrated at comparatively low temperatures and were then suitable for amino acid chromatography. It was also found that the α-amino N contents of 70% ethanolic extracts of homogenates were only about 10% higher than the 1% picric acid extracts favoured by Hamilton & Van Slyke (1943).

Paper chromatography of free amino acids. Samples of the 70% ethanolic extracts obtained at daily intervals during metamorphosis were evaporated to dryness. The residues were freed from fat by extraction with ether and then dissolved in small volumes of water. Samples of the aqueous extracts each approximately equivalent to the same dry weight of tissue were then applied from capillary pipettes as compact spots to the corners of 20 cm. × 20 cm. sheets of Whatman no. 1 paper, and the series of two-dimensional ascending paper chromatograms were developed simultaneously (Datta, Dent & Harris, 1950), first with 80% phenol–ammonia and then 90% Methyl Cellosolve (2-methoxyethanol; Bender, 1951) as irrigating solvents.

Citrate. The microcolorimetric method of Weil-Malherbe & Bone (1949) was used with the modification that the final colour production was carried out by extracting the light petroleum solution of pentabromoacetone with a solution of thiourea buffered with borax (Natelson, Pincus & Lugodvy, 1948). Citrate was estimated in 5% (w/v final concn.) trichloroacetic acid extracts of the tissue homogenates.

Uric acid. Tenfold dilutions of the 1/5 tissue homogenates were deproteinized by suspending 1 ml. in 5 ml. of water

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and then adding 1 ml. each of 10% (w/v) sodium tungstate solution and 0·67 N-H_2SO_4. After making the volume up to 10 ml. and mixing thoroughly, the filtrate finally obtained was used for the estimation of uric acid by the Dresel & Moyle (1950) modification of the method of Brown (1945).

Nucleic acids. Protein and nucleic acids were first precipitated from samples of homogenates by ice-cold 5% (w/v final concn.) trichloroacetic acid. The precipitate obtained by centrifuging was allowed to drain thoroughly, washed with cold 5% trichloroacetic acid and then extracted with 5% trichloroacetic acid at 90° for 20 min. This extract was then used for the colorimetric estimations of deoxyribose nucleic acids (DNA) and ribosenucleic acids (RNA).

DNA was estimated in the trichloroacetic acid extract by the diphenylamine reaction of Dische (1930, 1955).

RNA was estimated by the Mejbaum (1939) modification of the Bial orcinol reaction for pentoses (see also Dische, 1955).

By preparing calibration curves for DNA and RNA from nucleic acid samples of known phosphorus content, it was possible to express results in terms of either nucleic acid or nucleic acid phosphorus content.

RESULTS

The total free α-amino N content of *Tenebrio* pupal tissue was determined in 70% ethanolic extracts of 1:5 homogenates. Since it is generally found that the free amino acid content of insect blood constitutes between 50 and 85% of the total non-protein nitrogen (Buck, 1953), the measurement of total free α-amino N was taken to be a measure of the 'pool' of free amino acids in *Tenebrio* pupal tissues. Two or three samples of homogenate were analysed in duplicate at the different stages of metamorphosis (in the present context, a stage is equivalent to the pupal age in days at 24°).

The average values obtained are given in Table 1, and it is seen that the free α-amino N fraction represented between 2·6 and 4·6% of the total N content of the homogenate. After an initial sharp decrease in the total free α-amino N, the level remained approximately constant from about day 3 to day 7. Subsequently and at about the time of ecdysis (day 10) the free amino acid content tended to rise once more. This is essentially the same kind of result that Florkin (1937) obtained when he measured the variation in total amino N in the blood of *Bombyx mori* pupae with a modification of Folin's colorimetric method. That is to say, at least in the holometabolous insects *Bombyx* and *Tenebrio*, there is a free amino acid 'pool' of approximately constant size during a considerable part of metamorphosis. At the beginning and end of pupal life, however, certain fluctuations are noticed in both these insects and also in *Deilephila* (Heller, 1924), where a decrease in free amino N was observed at the end of metamorphosis.

One-dimensional paper chromatography of the free amino acids in breis of *Calliphora* was described by Agrell (1949). Here a semi-quantitative evaluation of the variation in the quantities of free amino acids was made and it was found that, apart from a fall in concentration at about one-fifth of the way and another at about three-quarters of the way through metamorphosis, there was an approximately constant level of free amino acids throughout pupal life. During the present work on *Tenebrio* homogenates a semi-quantitative investigation was made with two-dimensional paper chromatography.

After development of the chromatograms and after ensuring complete removal of irrigating solvents, the paper sheets were uniformly sprayed with 0·5% solution of ninhydrin in butanol, and the amino acid spots were revealed by heating in an electric oven at 110° for 10 min. The amino acid spots were identified by comparison with a 'map of spots' which had been obtained when standard solutions of known amino acids were chromatographed both singly and as mixtures. The two-dimensional array of spots was similar to that described by Dent (1948), except that R_F values of amino acids in the direction of the development with 90% Cellosolve were found to be generally greater (cf. Bender, 1951).

Table 1. *Some changes in the chemical composition of* Tenebrio *pupal tissues during metamorphosis at* 24°

Stage of metamorphosis (days)	Average total free α-amino N (μg./pupa)	Average citrate content (μg./pupa)	Ratio α-amino N: citrate	α-Amino N as % of total N (average)	Average uric acid N content (μg./pupa)	Average RNA content (μg./pupa)	Average DNA content (μg./pupa)	Ratio RNA:DNA
Pupa 0	300	109	2·8	4·6	139	494	107	4·6
1	219	146	1·5	3·1	145	527	113	4·7
2	208	157	1·3	2·6	172	408	158	2·6
3	242	184	1·3	3·0	171	387	136	2·8
4	258	172	1·5	3·0	174	328	133	2·5
5	256	160	1·6	3·2	184	305	163	1·9
6	263	158	1·7	3·3	178	354	172	2·1
7	267	144	1·9	3·5	170	319	159	2·0
8	333	147	2·3	3·1	198	356	167	2·1
9	267	105	2·5	3·2	216	322	118	2·7
10	354	118	3·0	4·3	252	414	152	2·7
Adult 1	—	—	—	4·2	—	569	131	4·3

By comparing twelve chromatograms representing samples of tissue obtained at each day of pupal life and on the first day of adult life, at the same time in reflected daylight, a record was made in arbitrary units of the variations observed in the concentrations of the individual amino acids comprising the free amino-acid 'pool'. No attempt was made to relate concentrations of amino acids to one another. The figures given in Table 2 show how the different amino acids were found to vary in concentration during metamorphosis with this procedure. Since the free amino acids were not completely resolved on these chromatograms, two of the twelve spots obtained represent mixtures of two or three amino acids, and variations in concentration of these composite spots may not be attributed to changes in concentration of any specific amino acids.

Certain striking changes in the qualitative composition of the free amino acid pool were seen during the metamorphosis of *Tenebrio*. Aspartic acid, glycine, glutamic acid, proline and glutamine showed a general downward trend. One or both of the components of the arginine–lysine spots and one, two or all of the amino acids valine, methionine and tryptophan also decreased in concentration. Tyrosine gradually built up to a very high concentration, and was probably mobilized as a substrate for synthesis of cuticle melanin pigment as reported by Fraenkel & Rudall (1947) in the pupa of *Sarcophaga*. Concentrations of alanine and the leucines remained almost unchanged, whereas the hydroxy-amino acids serine and threonine increased in concentration at first and then began to decrease from day 5 or 6 onwards. A number of amino acids were seen to decrease in concentration just before ecdysis (days 9 and 10), but afterwards increased concentrations were seen in most cases. This corresponded with the terminal increase observed in the total free α-amino N concentration.

Until recently, the only citrate analysis reported in insects was due to Levenbook (1950). The blood of the larva of *Gastrophilus intestinalis* was examined exhaustively by this author and found to contain 45 mg. of citrate/100 ml. This insect, like all others so far investigated, also possessed a high free amino N concentration (93·8 mg./100 ml.). Examination of the blood of *Rhodnius prolixus* and *Tenebrio molitor* yielded similar results (Patterson, 1956). The concentration of citrate in the haemolymph of *Rhodnius* was found to be 44 mg./100 ml. and in *Tenebrio* 97 mg./100 ml. The corresponding free α-amino N concentrations were 75·3 and 180·5 mg./100 ml.

At first sight it appeared that there might be a direct relationship between the accumulation of the free amino acid and citrate. In all three insects for which figures were available the ratio of total α-amino N to citrate concentration was between 2·1

Table 2. *Daily semi-quantitative analyses of the free amino acids in* Tenebrio *pupal tissues during metamorphosis at 24°*

Concentrations of individual amino acids and mixed spots are expressed in arbitrary units. Tr., trace detected.

Stage of metamorphosis (days)	Aspartic acid	Glutamic acid	Serine	Glycine	Threonine	Alanine	Tyrosine	Glutamine	Arginine + lysine	Proline	Valine + methionine + tryptophan	Leucines
Pupa 0	1	3	1	5	1	1	2	3	4	2	3	3
Pupa 1	1	3	1	3	1	1	1	2	4	2	3	3
Pupa 2	1	3	2	2	2	1	1	3	3	2	3	2
Pupa 3	1	3	2	2	2	1	1	2	4	2	2	2
Pupa 4	0	3	2	2	2	1	1	2	4	2	2	2
Pupa 5	0	3	3	2	2	1	1	2	4	2	2	2
Pupa 6	0	3	3	3	1	1	2	1	3	1	1	2
Pupa 7	0	2	2	2	1	1	2	1	3	1	1	2
Pupa 8	Tr.	2	2	2	1	1	3	1	3	1	1	2
Pupa 9	0	2	1	1	0	1	4	1	2	1	1	2
Pupa 10	0	1	1	1	0	1	4	1	2	1	1	2
Adult 1	Tr.	2	1	3	Tr.	1	5	1	2	2	3	2

and 2·4. And in order to test this possibility, citrate was estimated in duplicate on two to four samples of *Tenebrio* pupal tissue taken at different stages of development. Total free α-amino N was also estimated on the same tissue samples. The ratio of total free α-amino N:citrate was calculated for each sample of tissue and average values obtained for the daily stages of metamorphosis are given in Table 1; it is concluded from these figures that there is no evidence to suggest that a direct relationship obtains between the two concentrative processes.

Two major pathways for the utilization of amino acids are the synthesis of proteins from amino acids and the deamination of amino acids before utilization either for the synthesis of non-protein molecules or for eventual oxidation to yield energy. Deamination may give rise either to ammonia or to a transferable amino group. The former product is detoxicated in animals with a restricted supply of water, and in insects it is well known that uric acid is the final end-product of this process (Baldwin, 1940; Florkin, 1949). In the *Tenebrio* pupa, which is an enclosed system from the point of view of nitrogen metabolism (i.e. it neither feeds nor excretes during the whole period of metamorphosis); and in the absence of further breakdown of uric acid (D. S. P. Patterson, unpublished results) this substance accumulates in the pupal tissues. The rate of uric acid accumulation must be largely dependent upon the rate of deamination and its measurement may be legitimately used as an approximate measure of deaminase activity during given phases of metamorphosis.

The uric acid content of samples of *Tenebrio* pupal tissue homogenates was estimated at different stages of metamorphosis and the average figures obtained (four or five samples at each stage) are recorded in Table 1. From the results it appeared that the rate of accumulation went in three phases during metamorphosis. The first phase ended at day 2, the second at day 7 and the third ended with ecdysis. Since the average uric acid content of a *Tenebrio* larva was found to be about 75 μg., the total amount of uric acid in each insect is doubled during the first 2 days of pupal life. This, then, is a period of very rapid deamination. From day 2 to day 7 the average uric acid content of the pupa is nearly constant and consequently deamination must proceed at a minimal rate. Thereafter a second period of rapid deamination is observed, the level of uric acid being increased by about another 50% during the last 3 days.

There is a well-known correlation between protein synthetic activity and an increase in the RNA content of tissues (Brachet, 1955). Consequently, provided that the number of cells remains unchanged in samples of insect tissue taken at different stages of development, an observed increase in RNA at any particular stage would probably correspond with a period of pronounced protein synthesis. In the absence of cytological techniques for counting the nuclei present in samples, it is permissible to assume, provided that the DNA content of tissue samples is constant, that an increase in the RNA:DNA ratio (which represents a true increase per unit of nuclear material) would have the same significance.

Therefore RNA and DNA estimations were made on from two to five samples of homogenates of pupal tissue taken at different stages of metamorphosis. The ratio of RNA content to DNA content was calculated and expressed in terms of nucleic acid phosphorus. Average values quoted in Table 1 represent the averages of the various values of this ratio obtained for individual tissue samples at the same stage of development. Whereas the average DNA content appeared to be evenly distributed about a mean value of 140 μg./pupa, the average RNA fell from about 510 μg./pupa at the beginning of pupal life to about 330 μg./pupa during days 4 to 6. Thereafter, it rose to about 570 μg. of RNA/insect in the first day of adult life. The ratio RNA:DNA also exhibited a U-shaped variation during metamorphosis, so that taking into account the approximately constant concentration of DNA (a measure of nuclear material) it appears that at the beginning and at the end of metamorphosis there was a high level of RNA per cell, and therefore these two periods of development might well be associated with intensive protein synthetic activity.

DISCUSSION

During the metamorphosis of holometabolous insects considerable morphological changes take place, and proteolysis of larval proteins and subsequent or even concurrent synthesis of pupal and adult tissue protein must be expected. With the continuous re-utilization of amino acid molecules derived in the first place from larval proteins it might be anticipated that rather considerable fluctuations in the level of total free α-amino nitrogen would be observed. In fact, it has been shown that for a considerable part of the metamorphosis period of *Tenebrio molitor* the total free α-amino nitrogen content of pupal tissue is approximately constant. Essentially the same observation was made by Florkin (1937), who analysed the blood of *Bombyx mori* pupae, and by Heller (1924) and Heller & Moklowska (1930) who examined *Deilephila* pupal tissues.

Agrell (1949) made a semi-quantitative investigation of the free amino acids in *Calliphora* pupal breis and also observed the approximately constant size of the free amino acid 'pool'. While considering the concentration of free amino acids to be maintained

by a balance of protein synthesis and protein breakdown, Agrell suggested that quantities in excess of an equilibrium titre of free α-amino nitrogen were oxidized, especially at the beginning and at the end of metamorphosis. These periods of more intensive amino acid oxidation corresponded with the peaks of the U-shaped oxygen utilization and various amino acid dehydrogenase-activity curves. The present results which indicate a maximal deaminase activity in *Tenebrio* pupal tissues at the same periods of pupal life are in some agreement with Agrell's observations, although the approximately constant level of α-amino nitrogen actually corresponded with a period of minimal deamination (day 3 to day 7).

The initial drop observed in the total free α-amino nitrogen content of pupal tissue coincided understandably with a phase of rapid deaminase activity, whereas the final increase in the free α-amino nitrogen titre and the general increase in concentrations of individual amino acids separated by paper chromatography also occurred at a time when the deamination was intensive. This apparent incompatability between the two sets of data may, however, be understood once it is realized that towards ecdysis, amino acid metabolism is complicated by the fact that a large proportion of the protein of the pupal cuticle is actually absorbed by the insect (Evans, 1938). In this event, cuticle protein, probably absorbed as amino acids, may easily be in excess of the capacity of the deaminating enzymes, and therefore in spite of the observed rapid rate of accumulation of uric acid at this period the total free α-amino nitrogen level tends to rise.

The 'pool' of free amino acids represents a balance between protein breakdown and amino acid utilization, but mechanisms which control the extent to which amino acids accumulate are not understood. The accumulation of high concentrations of amino acids in certain tissues may possibly be associated with high protein synthetic activity. Thus it has been observed by Van Slyke & Meyer (1913) that mammalian hepatic tissue has a greater ability to concentrate amino acids than skeletal muscle. (Dog muscle saturation value was about 70 mg./100 g. Liver was saturated at about 157 mg./100 g.) Awapara (1952) pointed out that in the ventral prostate of the dog, an elevated level of free amino acids was accompanied by an increased citric acid content and thought it possible that the two concentrative processes were interrelated.

The elevated level of citrate in *Tenebrio* pupal tissue was not found to be directly related to the accumulation of free α-amino nitrogen. There was, however, a noticeable variation in tissue-citrate concentrations, and the maximum citrate accumulation occurred at about the time of least oxygen utilization. In fact there has been shown to be an approximately linear relationship between tissue-citrate concentration and the rate of oxygen utilization by live pupae (Patterson, 1956). This led to the speculation that during the metamorphosis of *Tenebrio molitor* the reactions of the tricarboxylic acid cycle functioned more or less efficiently according to whether relatively low or very high concentrations of citrate were to be found in the tissues.

Since certain keto acids and especially α-oxoglutarate are synthesized by means of the reactions of the tricarboxylic acid cycle it would not be unreasonable to suppose that at a period of the cycle's least efficiency (at about day 3), deamination, probably initiated by α-amino group transfer to α-oxoglutarate, would be carried out at a slow rate. In fact, at this phase of pupal life, the rate of accumulation of uric acid (the end-product of deamination) was minimal. Moreover, a decrease in the concentrations of certain individual amino acids such as aspartic acid, glutamic acid, glutamine, threonine and proline was more noticeable (Table 2) as the concentration of citrate fell from the maximum value, i.e. from day 4 onwards.

The variations in the RNA:DNA ratio which have been seen during the pupal period have not been observed before in insect development. In 1952, however, Agrell reported complementary variations in the RNA phosphorus and DNA phosphorus contents of *Calliphora* pupae during metamorphosis and deduced that the two nucleic acids were interconverted by this insect. The ratios RNA P:DNA P were not recorded in this paper by Agrell (1952), but calculations made from his data show that there was also a similar U-shaped variation in this case of insect development. The actual figures so obtained for RNA P:DNA P are somewhat low compared with those recorded in mammalian tissues, and in the present work on *Tenebrio* pupal tissue (Table 3), but they may have been influenced by his use of the method of Ogur & Rosen (1950) for estimating nucleic acids. Davidson (1953) has claimed that results obtained by this method are misleading owing to contamination by inorganic phosphate unless prior ionophoretic separation of the soluble nucleotides is undertaken.

From the beginning of pupal life the value of RNA:DNA was seen to fall to a fairly uniform value of about 2·0, which was estimated on tissue samples from day 5 to day 8, the value increasing again thereafter. It was supposed, then, that at the beginning of the pupal period protein synthesis was more rapid than elsewhere during metamorphosis. The increasing value obtained for the ratio at about the time of the emergence of the adult was probably associated with protein synthesis in the new adult cuticle. Both phases of increased protein synthetic activity occurred at times when the titre of free amino acids was relatively high and clearly this is an advantageous situation.

Table 3. *Ratio of RNA phosphorus:DNA phosphorus in various animal tissues*

Tissues	RNA P:DNA P	Source
Calliphora (pupae)		
Beginning of pupal life	0·71	Agrell (1952)
Middle of pupal life	0·30	
At ecdysis	0·60	
Tenebrio molitor (pupae)		
Day 0	5·09	Present work
Day 5	2·14	
Day 10	3·02	
Tenebrio molitor (young adult)	4·83	
Rat liver	4·0	Davidson (1953)
Cat liver	2·3	
Human liver	3·0	
Cat pancreas	3·4	
Rat mammary gland (lactating)	2·7	T. F. Slater (private communication)

The period of intensive protein synthesis which is indicated at the very beginning of pupal life (RNA:DNA = about 4·6 at day 0 and day 1) is probably associated with the formation of adult structures within the pupa, which has been held by Dobzhansky & Paulson (1935) to be often completed in *Drosophila* before the period of minimum oxygen utilization. If this is a general feature of holometabolous insect development it means that the 'metamorphosis' hitherto referred to in this work is, in fact, largely the development of the adult within the pupal skin, the actual metamorphosis or transformation of larval structures having been undergone predominantly in the pre-pupal stage.

SUMMARY

1. The total free α-amino nitrogen content of *Tenebrio molitor* pupal tissues was measured during metamorphosis. For a considerable period (5 days), the level of α-amino nitrogen was approximately constant.

2. A decrease in the free amino acid titre observed at the beginning of metamorphosis is associated with high deaminase activity, and the increase which was seen at about the time of ecdysis is probably a result of the absorption of pupal cuticle protein.

3. Semi-quantitative examination of the free amino acids by means of two-dimensional chromatography generally reflected the changes in total free α-amino nitrogen.

4. The rate of deamination measured by the rate of accumulation of the ultimate nitrogenous end-product uric acid in the pupal tissues was noticed to be greatest at the very beginning of the metamorphosis, constant during the middle and elevated towards the end of pupal life.

5. There was no evidence for the accumulation of citrate in *Tenebrio* pupal tissues being directly related to synchronous accumulation of free amino acids.

6. The ratio RNA:DNA decreased after the beginning of the pupal period and increased towards its end. The maxima were thought to correspond with intensive protein synthetic activity during the formation of adult tissues and when the adult cuticle protein was being synthesized.

It is a pleasure to acknowledge the interest and encouragement shown by Professor E. Baldwin during the course of this work, which was made possible by the receipt of an Agricultural Research Council grant. The author also wishes to thank Mr F. L. Baxter for his help in maintaining insect cultures.

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The Effects of Phosphates, Arsenates and Nucleotides on L-Amino Acid Decarboxylases

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(*Received* 26 *September* 1956)

Gale & Epps (1944) and Gale (1945) observed that the lysine decarboxylase activity of *Escherichia coli* '86' was increased by inorganic phosphate (0·1–0·5 M), and that the decarboxylation of ornithine by *Clostridium septicum* was more rapid in phosphate buffers than in citrate buffers of comparable pH. Krebs, Eggleston & Knivett (1955) recently confirmed that the decarboxylation of L-ornithine and L-lysine by *Esch. coli* was accelerated by the addition of phosphate at pH 6·5–6·8. The present paper is concerned with a more detailed study of the effects of inorganic and organic phosphates and related substances on the activity of amino acid decarboxylases.

EXPERIMENTAL

Materials

Buffers. The following buffers were used: phosphate, made from molar stock solutions of Na_2HPO_4 and NaH_2PO_4; arsenate, made from 0·5 M-Na_2HAsO_4 and N-HCl; citrate made from 0·2 M sodium citrate and N-HCl or N-NaOH; tris, made from M aminotrishydroxymethylmethane and N-HCl; acetate, made from sodium acetate and acetic acid; acetate–veronal (Michaelis, 1931), and tris–maleate (Gomori, 1955). A glass electrode and a Pye pH meter were used to measure the pH of these buffers after dilution to the concentrations used in experiments. The additions of enzyme and substrates had little or no effect on the pH.

Nucleotides. Adenosine triphosphate (ATP) was prepared in this laboratory by Mr R. Hems. The barium salt from rabbit muscle (LePage, 1949) was dissolved in 0·1 N-HCl and passed through a column of Amberlite resin IR-120 (H) in the H^+ form. The free acid was neutralized with NaOH and concentrated by freeze-drying. Analysis by the method of Krebs & Hems (1953) showed it to be at least 98% ATP, with a trace of adenosine diphosphate (ADP), and no other detectable phosphate impurity. In some experiments two commercial samples of ATP were used; analysis showed them to be mixtures of ATP and ADP (about equal quantities) with about 15% of other impurities including inorganic phosphate, adenosine 5′-phosphate (AMP-5′) and some other unidentified organic phosphates.

Barium ADP was obtained from Schwarz Laboratories Inc. It was found to contain less than 5% ATP.

Muscle adenylic acid (AMP-5′) from Roche Products Ltd., and yeast adenylic acid from L. Light and Co. Ltd., were both found to be 98–100% pure when examined by the chromatographic techniques of Krebs & Hems (1953).

Adenosine 2′-phosphate (AMP-2′) and adenosine 3′-phosphate (AMP-3′) were obtained from L. Light and Co. Ltd. Examination of these compounds by the chromatographic method of Carter (1950) showed no phosphate impurities. Guanylic, uridylic and cytidylic acids, which are known to be mixtures of the 2′- and 3′-phosphates, were also obtained from L. Light and Co. Ltd.

Inosine triphosphate and inosine 5′-phosphate from Sigma Chemical Co. showed no phosphate impurities when tested by the method of Krebs & Hems (1953).

Flavin mononucleotide was a gift from the Sigma Chemical Co.

Other compounds. Barium ribose 5-phosphate was prepared according to Long (1955). Chromatographic analysis according to Eggleston (1954) revealed traces of AMP-5′, but no other impurity. For use it was dissolved in dilute HCl and a calculated amount of Na_2SO_4 was added to precipitate the barium; after centrifuging, the supernatant solution was adjusted to pH 6·8 with NaOH. Glucose 1-phosphate, glucose 6-phosphate and sodium arsonoacetate ($Na_2O_3.AsCH_2.CO_2Na$) were prepared by Mr D. H. Williamson. Other organic arsenicals were obtained from British Drug Houses Ltd. Organophosphorus insecticides were obtained