isolated myofibrils as shown in Table 1. Many of the actin preparations have higher ATP values than might be expected in view of the low amount of this nucleotide in the well washed muscle residue from which actin is extracted, and which must consist mainly of myofibril residues. Straub & Feuer (1950) found insignificant amounts of ATP in the acetone-dried fibre from which they extracted G-actin and concluded that ATP was produced during the water extraction of the fibre.

Assuming that the adenine content of the isolated myofibril is compatible with that found *in vivo* it is possible to speculate on the ATP distribution within the muscle cell. Normal muscle contains powerful ADP phosphorylating systems and it is likely that in the resting state all the adenine exists as ATP. This would mean that about 10% of the ATP content of the cell is associated with the myofibrils. As the latter occupy about two-thirds of the cell volume, the concentration of ATP in the extra-myofibrillar space, which is occupied by the sarcoplasm, will be 18 times that in the myofibril, assuming that the myofibrils are not interpenetrated by the sarcoplasm, or alternatively that any ATP which does penetrate into them is broken down. This concentration gradient must be an important factor affecting the accessibility of ATP to the enzyme associated with the contractile structure, the myosin adenosinetriphosphatase.

SUMMARY

1. A non-enzymic method for the isolation of myofibrils from rabbit skeletal muscle has been described.

2. Myofibrils from cardiac and skeletal muscle of the rabbit and skeletal muscle of the rat contained 90–140 μg. of acid-labile phosphorus per gram. Similar values were obtained with rigor muscle.

3. The acid-labile phosphorus of myofibrils isolated from rabbit muscle arises from adenosinediphosphate, and to a lesser extent, adenosinetriphosphate. These nucleotides are bound to the myofibril in such a way that they are not acted upon by the enzymes occurring in myofibril which normally attack them.

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Histidine α-Deaminase and the Production of Urocanic Acid in the Mammal

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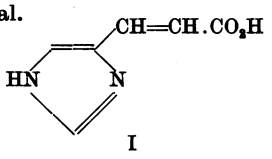
Urocanic acid (β-4(5)-glyoxalinylacrylic acid) (I), was discovered by Jaffe (1874) in the urine of one dog and was considered after its rediscovery by Siegfried (1898) to be a possible normal breakdown product of histidine. Evidence produced by Raistrick (1917, 1919) showing that it was formed from histidine by the action of certain micro-organisms, and the determination of its structure by Hunter (1912) fostered the suggestion that direct deamination played a part in the normal degradation of histidine. This point of view was supported by the later work of Kotake & Konishi (1922), Kiyokawa (1933) and Harrow & Sherwin (1926). Cox & Rose (1926), however, and Darby & Lewis

(1942), came to the conclusion that the animals, in the urine of which urocanic acid had been observed, were abnormal.



I

Edlbacher and his co-workers (see review by Edlbacher, 1943) isolated from liver two enzymes which might play an important part in the breakdown of histidine. One of these, to which he gave the name histidase, degrades histidine to glutamic acid, formic acid and ammonia by fission of the iminazole ring. The other, urocanase, performs a similar function on urocanic acid. Edlbacher did not prove the existence of an enzyme capable of directly removing the amino group from histidine; and although he published a scheme showing the possibility of two distinct pathways for histidine breakdown, he considered that his evidence precluded the existence of a path requiring the intermediary production of urocanic acid.

The gravimetric methods whereby urocanic acid was first isolated were only applicable to the considerable quantities available in large volumes of urine, and could not be used easily on liver tissue or extracts. The method employed by Edlbacher & Bidder (1942), although more sensitive, could not be used to identify urocanic acid in the presence of histidine, since it employed a colour reaction characteristic of glyoxalines generally.

To determine whether histidine is at least partially converted to urocanic acid in the liver and subsequently broken down to glutamic acid by urocanase, it was essential to have available methods for identifying this possible intermediate in small quantities in the presence of histidine. The present paper records the use of two such methods. By these means, although no urocanic acid has been identified in whole liver extracts, presumably owing to the simultaneous presence of urocanase, an enzyme has been demonstrated which is capable of producing the unsaturated acid from histidine.

METHODS

Measurement of ultraviolet absorption

All the ultraviolet absorption data were obtained on a Beckman spectrophotometer, model DU. All the solutions were aqueous, and where values are given indicating differences in absorption between a test solution and a control, the absorption figures for each were measured separately against water as a blank and the difference obtained by subtraction.

Paper chromatography

Paper chromatograms of pure substances and of preparations from enzyme solutions were carried out in an upward direction (Williams & Kirby, 1948) on Whatman no. 4 paper. Two solvent mixtures were employed: (*a*) butanol, 50 ml.; water, 50 ml.; glacial acetic acid, 12 ml., the alcoholic layer being used after equilibration; (*b*) a miscible mixture; butanol, 40 ml.; ethanol, 10 ml.; water, 50 ml.

The chromatograms were developed, after a run of 2–4 hr. at 30°, by spraying successively with a cold aqueous solution of diazotized *p*-chloroaniline (Edlbacher, Baur, Staehelin & Zeller, 1941) and N-NaOH. The latter spray could be replaced by suspension of the paper in an atmosphere of NH_3 vapour. The colours which varied between yellow and red persisted strongly, assuming a more intense red colour with age.

Preparation of urocanic acid

Urocanic acid was prepared by decarboxylation of a sample of 4(5)-glyoxalinylmethylenemalonic acid, which was kindly supplied by Dr A. Neuberger, F.R.S. The method of decarboxylation was essentially that of Akabori, Ose & Kaneko (1940) employing boiling pyridine. Most of this reagent was removed by distillation under reduced pressure and impure urocanic acid precipitated on pouring the residue into ether. Persistent washing with ether was necessary to ensure the removal of all the pyridine; the acid was then recrystallized from water to give material with a m.p. (uncorr.) of 226–228°. The absorption curve for this material is given in Fig. 1. Purity of the sample was determined in two ways. Freedom from pyridine was proved by the absence of the characteristic absorption peak of pyridine at 256 mμ. Freedom from malonic acid derivative was proved by partition chromatography. The R_F values of 4(5)-glyoxalinylmethylenemalonic acid and urocanic acid in butanol-acetic acid are respectively 0·30 and 0·52; thus separation is complete and small amounts of the malonic acid derivative can be detected in the presence of large quantities of urocanic acid.

A further sample of urocanic acid was obtained by an adaptation of the method of Engeland & Kutscher (1913) for the production of histidine betaine, and its subsequent breakdown to urocanic acid and trimethylamine by the method of Barger & Ewins (1911). The material prepared by this method had properties identical with those of the sample prepared from 4(5)-glyoxalinylmethylenemalonic acid.

Identification and estimation of urocanic acid

Histidine at pH 7·4 has a relatively low absorption peak at 262 mμ., and the molecular extinction coefficient of urocanic acid at the same pH is 200 times as great. Thus it is possible to use ultraviolet absorption as a method for the identification of urocanic acid in the presence of histidine.

The variation in intensity of absorption and in the position of the peak with changes in pH are such as could be expected with an amphoteric substance. The four curves in Fig. 1 demonstrate the magnitude of these pH variations. The curve at pH 4·5 represents the absorption of urocanic acid in aqueous solutions at a concentration of 4 μg./ml. The other curves are those for the same concentration of the acid brought to the stated pH by the addition of NaOH or HCl. It was found that in the presence of acid there was much irrelevant absorption with the systems under investigation and pH values around neutrality were, therefore, always employed when examining solutions for the presence

of urocanic acid. For although the peak at 278 mμ. is lower it is well defined owing to the pronounced minima at nearby wavelengths.

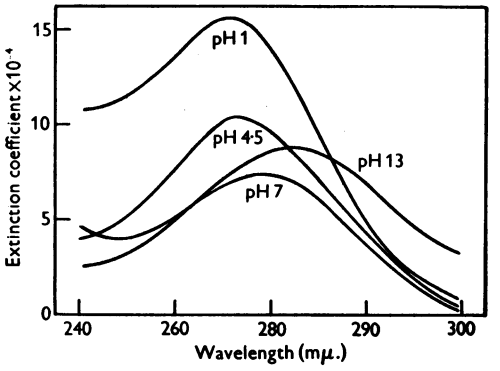


Fig. 1. Absorption curves of urocanic acid (4 μg./ml.), brought to the pH values indicated by the addition of appropriate amounts of sodium hydroxide and hydrochloric acid. The curve of pH 4·5 represents the actual curve of the acid dissolved in distilled water.

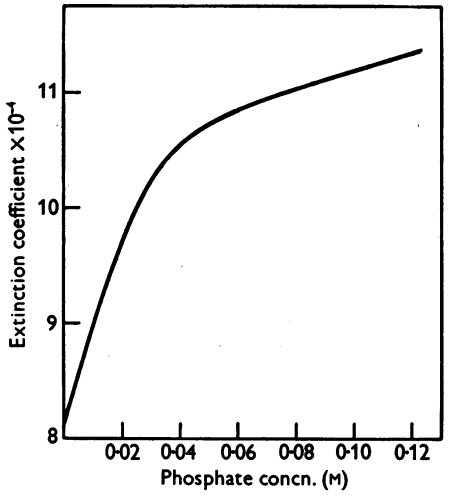


Fig. 2. The effect of phosphate ion concentration on the extinction coefficient at $\lambda_{max.}$ (278 mμ.) for solutions of urocanic acid brought to pH 7 with sodium hydroxide.

Since in many of the systems to be examined, phosphate was present, its effect on the absorption was examined. As can be seen from Fig. 2, the absorption is considerably increased by the presence of even small concentrations of phosphate. Although the rate of increase falls off above a phosphate concentration of more than 0·05 M, there is a slight but steady increase at greater concentrations. The graph shows the absorption at 278 mμ., and pH 7·6. This effect was also observed with borate; but not with other commonly occurring ions.

Solutions of urocanic acid, kept at normal temperatures in diffuse daylight, show variable changes in absorption. The value may fall by as much as 20% in 3–4 days, but the presence of phosphate appears to retard the diminution of absorption.

Owing to the variations in absorption which are mentioned in the two preceding sections, it is impossible to obtain quantitative comparisons between solutions with entirely different pre-treatment, but for comparison of two solutions which have been treated in an identical manner, the method is regarded as quantitative. Over the range of 1–9 μg./ml. in aqueous solution, the relationship between absorption and concentration of urocanic acid is linear. This covers the whole effective range of the spectrophotometer, using a 10 mm. cell. Higher concentrations can be read after dilution.

Examination of liver extracts

Choice and preparation of material. In most of the work reported here, cat-liver extracts were employed, but the technique was identical with extracts prepared from the livers of other animals. Livers were removed from cats killed by severance of the carotid artery and were washed free of superficial blood. The tissue was macerated to a fine cream in 2·5 times its weight of physiological saline. The grosser debris after 20 min. in the macerator (Townson & Mercer top-drive type) was removed by filtration after addition of kieselguhr equivalent to half the wet weight of the liver. The resultant opalescent solution was poured into 2 vol. of acetone at a temperature of less than 5°. The precipitate was filtered off at the pump. The cake, washed once with acetone, was dried at room temperature and stored at 0°. The powder was not completely soluble in water or the buffer used, but by agitation could be brought into a sufficiently fine suspension to withstand centrifuging under a force of 3500 *g*.

Properties of the extract powder. A sample of the original macerate, filtered through a 3 mm. layer of paper pulp on a sintered funnel, gave an indeterminate curve showing marked absorption in the range 240–300 mμ., but no peak at 278 mμ. After ultrafiltration through a collodion membrane most of the absorption disappeared, showing it to be due to material of large mol.wt. There was no peak in the region of that associated with urocanic acid. Similar experiments on an aqueous or phosphate buffer extract of the acetone-dried powder prepared from the macerate failed to demonstrate the presence of any material with properties similar to urocanic acid. A sample of the extract from an acetone-dried powder that had been kept for 2 months at 0°, showed a marked peak in the neighbourhood of 278 mμ. Dialysis of the sample against running water for 24 hr. completely removed the peak, showing the material responsible for the absorption to be water-soluble and of low mol.wt.

Activity of liver powder

The incubation of a fresh acetone-dried liver extract with histidine caused a marked increase in absorption with a peak at 278 mμ. Such an increase was produced by a heat-labile system, no increased absorption being observed when histidine was incubated with boiled preparations. The same histidine α-deaminase is suggested for the enzyme concerned.

Method of testing enzyme activity. Except when various concentrations of enzyme and substrate were examined for the specific purpose of determining concentration effects,

the following procedure was employed for assessing the amount of enzyme in a given preparation. Enzyme powder (50 mg.) and solid phosphate buffer mixture, pH 7·6, (50 mg.) were dissolved in 5 ml. of water. To this mixture was added 5 ml. of a solution of histidine hydrochloride in water (1 mg./ml.) brought to pH 7·6 with NaOH. Water (5 ml.) was added in the case of the blank. The preparations were incubated at 30° for 18 hr. in the presence of 0·2 ml. of toluene in 50 ml. flasks covered with a 30 ml. beaker. After incubation the flasks were placed in boiling water for 5 min. to inactivate the enzyme, coagulate the protein and remove the small quantity of toluene. The solid was then removed by centrifuging, and the volume of the supernatant made up to 10 ml. 1 ml. of this solution was diluted with water to 50 ml., to give the solution for measurement in the spectrophotometer. The curves observed on incubation of the enzyme in the presence and absence of histidine are given in Fig. 3.

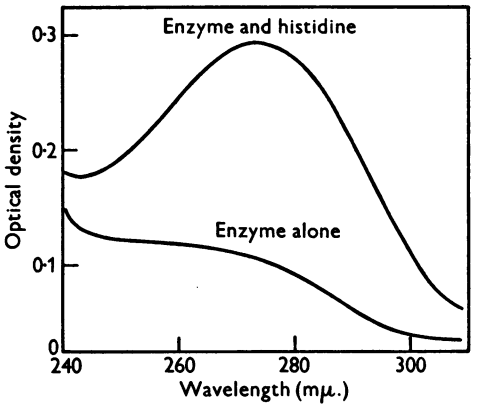


Fig. 3. Absorption curves of enzyme and enzyme-substrate mixtures after incubation at pH 7·8 for 18 hr. 50 mg. liver powder, 50 mg. phosphate buffer powder, and either 10 ml. of a 0·1% solution of histidine hydrochloride or 10 ml. of water.

Evidence for the identification of the enzyme product as urocanic acid

Examination of the material produced during the incubation of the enzyme preparation with histidine showed that changes in absorption brought about by pH were identical with those observed in pure solutions of urocanic acid. For this experiment buffer solutions or acid or alkali were used instead of water for the 50-fold dilution prior to measurement of the absorption. The changes observed are exactly as those shown in Fig. 1.

For the chromatographic identification of the material as urocanic acid all the quantities employed in the normal test were multiplied ten times. After the completion of the incubation period and after the coagulation and removal of most of the protein by filtration, the solution was concentrated to 3 ml. under reduced pressure, and the smaller molecules separated from the residual protein by ultrafiltration through a collodion membrane. A chromatogram of this material in butanol/acetic acid showed two main spots and two fainter ones. The R_F values of these and of a number of other imidazole derivatives are given in Table 1. In butanol/ethanol/water the movement of the lower two spots was only slight, and only after very long runs did separation occur. The two main spots coincide with those for histidine and urocanic acid; the other two have not yet been identified.

Table 1. *R_F values of certain glyoxaline derivatives in butanol/acetic acid/water and butanol/ethanol/water mixtures*

(The two unknown substances 1 and 2, were produced by the action of the liver preparation on histidine.)

Substance	R_F Butanol/acetic acid/water	R_F Butanol/ethanol/water
Urocanic acid	0·50	0·43
Histidine	0·11	0·10
β-Glyoxalinylpropionyl chloride	0·25	0·37
Glyoxalinylmethylene-malonic acid	0·30	—
Methylglyoxaline	0·39	0·52
Dicarboxyglyoxaline	0·0	0·09
Unknown substance 1	0·29	0·35
Unknown substance 2	0·13	0·14

Distribution of properties of the enzyme

The enzyme has been shown to be present in the livers of cats, rats, rabbits and dogs, but not in the following tissues: the pancreas, kidney, duodenum, stomach, spleen and thyroid of the pig or heart of the horse. Nor is it present in *Escherichia coli* cells, although a casual contaminant of a histidine solution, which proved to be a Gram-negative diplococcus, produced a filtrate containing urocanic acid after 3 weeks at 18°.

Table 2. *The effect of changes in concentration of enzyme and substrate on the ultraviolet absorption of histidine/histidine α-deaminase systems, after incubation at 30° for 18 hr. at* pH 7·6

(The difference in $E_{max.}$ at 278 mμ. was calculated by subtracting the curve obtained from the control enzyme preparation incubated without histidine, from that obtained after incubation in the presence of histidine. This also applies to the optical density figures in Table 3.)

Concentration of liver powder added (mg./ml.)	Difference in $E_{max.}$ Concentration of added histidine (mg./ml.) 0·1	0·2	0·5	1·0
0·5	0·007	0·010	0·018	0·028
1·0	0·009	0·013	0·023	0·039
2·0	0·019	0·026	0·038	0·056
5·0	0·027	0·051	0·084	0·114

In Table 2 are shown the results of a typical experiment in which both the concentrations of the

enzyme and of the substrate have been altered. The constants of the enzyme reaction have not yet been determined, since they will have little significance until purer preparations are obtained.

Enzyme activity was completely suppressed at pH values below 5; the pH optimum was found to be in the neighbourhood of 7·8. Citrate and phosphate buffers were employed to cover the whole pH range, and the figures for those experiments with phosphate were corrected for the enhancing effect of that ion on absorption (Table 3).

Table 3. *The effect of changes in* pH *on the ultraviolet absorption at* 278 *mμ. of histidine/histidine α-deaminase systems incubated for* 18 *hr. at* 30°. *The figures for the experiments with phosphate buffer are corrected for the enhancing effect of the phosphate ion (see Fig.* 2)

Buffer	pH	Differences in optical density
Citrate	3·0	0·012
	3·9	0·016
	5·1	0·003
	6·1	0·019
Phosphate	7·0	0·012
	7·2	0·008
	7·4	0·019
	7·6	0·098
	7·8	0·166
	8·0	0·144
	8·2	0·092

Old enzyme preparations which show the marked increase in urocanic acid content mentioned above are almost inactive, measured by increase of absorption on incubation with histidine. However, after dialysis and the removal of the urocanic acid the activity of the system increases to its normal value (Fig. 4).

DISCUSSION

Since it contains three conjugated double bonds, urocanic acid is expected to show considerable absorption in the ultraviolet. In fact the increase over histidine with only two double bonds is about 200-fold. This raises the molecular extinction coefficient to a level at which it is of use in the estimation of the acid in biological materials. The variability of the absorption renders the method not fully quantitative, but it is still of use in comparing similarly treated solutions. When associated with partition chromatography, ultraviolet absorption gives an admirable method of identifying the acid with a reasonable degree of certainty, but awaiting chemical characterization for absolute proof.

The reason for the decay of absorption on standing may be associated with the possibility of *trans-cis* interconversion which Edlbacher & Heitz (1943) have shown to occur quite rapidly at 100° and to be photocatalysed. Whether phosphate acts as a stabilizer of the *trans* form or enhances the absorption in some other fashion cannot yet be decided.

The absence of urocanic acid from fresh liver extracts is consistent with urocanic acid being on the pathway of histidine metabolism. Assuming that urocanase catalyses a faster reaction in the destruction of urocanic acid than histidine deaminase does in its production, not much urocanic acid would be expected to accumulate. The few

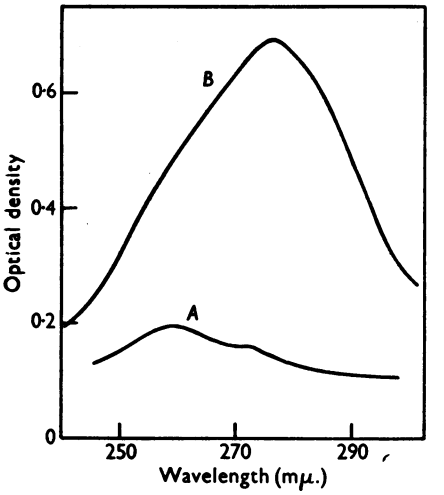
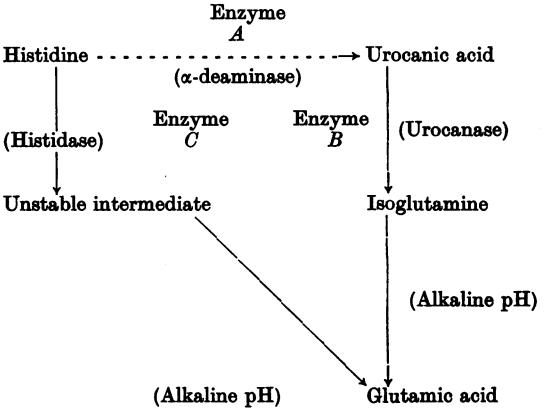


Fig. 4. Effect of dialysis on aged preparations. *A*, difference in optical density between enzyme and enzyme-substrate mixture of aged preparation before dialysis; *B*, similar curve obtained after dialysis of enzyme powder for 2 days against running water. The conditions of the experiments were in each case as given in legend to Fig. 3.

animals which have been shown to produce urocanic acid may not, therefore, have called into play a fresh metabolic pathway, but may merely have suffered the blocking of a single step in an enzymic chain. Until it is possible to measure quantitatively



the amount of urocanic acid produced it will be impossible to assess what fraction of the histidine is metabolized by the route involving urocanic acid.

The hypothetical system, shown on the previous page, proposed by Edlbacher would, however, appear to have more foundation in fact than he thought, since both enzymes *A* and *B* as well as *C* have now been shown to exist side by side in liver.

SUMMARY

1. Methods for the identification of urocanic acid (β-4(5)-glyoxalinylacrylic acid) based on its ultraviolet absorption spectrum and on its separation from other iminazole derivatives by partition chromatography are described.

2. No urocanic acid has been observed in fresh liver homogenates or extracts from acetone-dried powders obtained from aqueous extracts of liver.

3. An enzyme capable of producing urocanic acid from histidine has, however, been shown to be present in such aqueous extracts.

4. The name histidine α-deaminase is suggested for this enzyme.

5. The enzyme has an optimum pH in the region of 7·8.

6. It appears to be completely inhibited by the product of its reaction.

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Volatile Fatty Acids of *Ascaris lumbricoides* from the Pig

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(*Received* 9 *October* 1951)

During a series of investigations of the pharmacology and physiology of *Ascaris lumbricoides*, extending from 1940 to 1949, our attention was constantly drawn to the peculiar and characteristic odour emitted by these parasites and by saline media in which specimens had been kept. Bunge (1890) appears to have been the first to attribute this odour to lower, steam-volatile fatty acids, but it was left to Weinland (1901, 1904) to show that the principal acids present are a valeric and a caproic acid. Similar substances are present in another parasitic nematode, *Parascaris equorum* (Schimmelpfennig, 1903). Flury (1912), working with *A. lumbricoides*, demonstrated the presence of volatile fatty acids, especially valeric and caproic, in the tissues of this worm as well as in saline media in which it had been kept. There has, in the past, been a good deal of discussion regarding the origin of these acids, some attributing them to bacterial activity, while others maintain that they are produced by the worms themselves. The presence of relatively very high concentrations of these compounds in the body fluid and throughout the tissues would make it appear certain that the acids are produced by the worms themselves. A comprehensive review of earlier work on these acids has been given by Hobson (1948).

Although a dozen or more authors have studied the chemistry of these substances, their precise nature is still not definitely known. The results of Kruger (1936) were perhaps the most conclusive data published when our own work began, but even these are unconvincing. He collected the acids by steam-distillation of large volumes of saline in which his specimens had been accommodated. By fractional distillation of the product he obtained two main fractions, one consisting of a valeric and the other of a caproic acid, from both of which he prepared the anilides. He concluded that both acids

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