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)-glyoxalinyllacrylic acid) based on its ultra-violet 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

By VIVIEN MOYLE AND E. BALDWIN*

The Biochemical Laboratory, University of Cambridge

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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

* Present address: University College, London.

are branched-chain compounds and, in the case of the valeric fraction (m.p. of anilide 98°), that it was neither the *n*-acid (m.p. of anilide 63°) nor the *iso*-acid (m.p. of anilide 112°), but must probably be the optically active methylethylacetic acid (m.p. of anilide of the racemic acid, 108°). Trimethylacetic acid was excluded on the grounds that it is solid at room temperature, while Kruger's fraction was liquid. The only confirmation in support of his identification of the optically active acid was the fact that mixtures of the anilides of the natural product and synthetic racemic acid, melted between 98 and 108°.

As we were able to obtain considerable amounts of the acids in the course of other investigations, we felt it was worth while to attempt a more positive identification. The work reported here was completed in 1949, but remained unpublished because we hoped at that time to carry it further. But in view of the recent work of Bueding & Yale (1951), it has seemed to us proper to record our observations. A brief account of this work was communicated to the Biochemical Society in London on 24 March 1950 (Baldwin & Moyle, 1950).

MATERIAL AND METHODS

Worms were obtained from the St Edmundsbury Co-operative Bacon Factory, to the manager of which we wish to express our gratitude for a regular and abundant supply of material. After washing in warm water, the specimens were conveyed to Cambridge in large thermos vessels containing the 'keeping medium' described elsewhere (Baldwin & Moyle, 1947), previously heated to 38°. On arrival in the laboratory the worms were drained, dried on filter paper, opened by a longitudinal incision and allowed to drain on a funnel, the stem of which was loosely plugged with glass wool. The clear, deep-red perienteric fluid thus collected, accounted for approximately one-third of the live weight of the worms and contained on the average about 40 m-moles of steam-volatile fatty acids per litre.

Proteins were precipitated by the addition of 0.8 vol. of 10N-H₂SO₄ and 1.0 vol. of 30% Na tungstate. The precipitate was removed in some cases by filtration and in others by

centrifugation. To each 100 ml. of the clear, colourless fluid 25 g. MgSO₄·7H₂O were added and allowed to dissolve. The whole was then distilled until crystallization began in the hot. The distillates were titrated with N-NaOH (phenolphthalein); the average yield was equivalent to 38.0 ml. N-NaOH/l. of perienteric fluid. A 10% excess of N-NaOH was added before evaporating the distillates *in vacuo* to about 2 ml. An excess of finely powdered KHSO₄ and two drops of 10N-H₂SO₄ were added, and the resulting dry powder was extracted in each case with about 50 ml. of CHCl₃ containing 5% (v/v) *n*-butanol in small successive portions.

Attempts were at first made to analyse the mixed acids by the distillation method of Friedemann (1938), and by fractional crystallization of the phenylhydrazides and piperazinium salts, before and after fractional distillation. The results obtained were discouraging, and we next tried the method of partition chromatography of Elsdon (1946). This method, however, did not allow separation between caproic and valeric acids or between these and butyric acid. We were accordingly led to investigate the possibility of using heavily buffered silica partition columns. Details for their preparation and operation have already been described (Moyle, Baldwin & Scarisbrick, 1948), and in particular the performance of columns I-III. In Table 1

Table 1. *Behaviour of aliphatic acids (C₂-C₆) on column IV*

Butanol (%)	Eluted	By-passed	Retained
1	C ₅	C ₅ (part) and higher	C ₂ and lower
10	C ₄		
30	C ₃		

characteristics of column IV, which uses as 'buffer' KH₂PO₄ only, are given. This method was applied to our present problem, after preliminary distillation of the aqueous solution in presence of HgO (Friedemann, 1938) to remove formic acid. The results obtained are summarized in Table 2.

RESULTS

Fraction C₁. It was found in preliminary experiments that the mixed acids, in the form of their sodium salts, decolorized iodine and bromine water, indicating the presence of small quantities of un-

Table 2. *Steam-volatile acids obtained from A. lumbricoides*

(F₁ and F₂ were duplicate analyses carried out on one sample.)

Sample	Percentage of total steam-volatile acids					
	C ₈	C ₉	C ₅	C ₄	C ₃	C ₂
B	1.48	40.9	40.0	1.14	4.06	12.4
C	0.78	41.2	43.5	1.72	4.73	8.23
D	29.8		50.8	2.75	4.31	12.6
E	0.37	34.1	46.2	1.89	5.12	12.6
F ₁	33.8		49.4	—	3.78	—
F ₂	33.7		49.3	—	3.93	—
Averages	35.7		46.0	1.9	4.4	11.1

saturated or reducing substances. Preliminary distillation of the acids in the presence of HgO , as recommended by Friedemann (1938) for removal of formic acid, yielded distillates which failed to react with either iodine or bromine water. It seems possible that the reducing material consisted of formic acid, small amounts of which have been found in *Ascaris* by other workers, e.g. Flury (1912). The amount present, calculated on the basis of iodine value, corresponded to only about 2% of the total acids present, and we therefore did not concern ourselves further with this fraction.

Fractions C_2 and C_3 . C_2 and C_3 together accounted for 15.5% of the total acids and corresponded in their chromatographic behaviour to acetic (11.1%) and propionic (4.4%) acids respectively.

Fraction C_4 . To obtain enough of the C_4 acid for identification we used silica partition columns 30 mm. in diameter, each containing 20 g. of silica and proportionate quantities of the other reagents. The original CHCl_3 -butanol extract (80 ml.) was placed on a column of type II and the large C_5 and C_6 fractions eluted, the first with 1% butanol in CHCl_3 , and the second with 10% butanol in CHCl_3 . The column was then allowed to run dry, the silica removed, and, after intimate admixture with an excess of finely powdered KHSO_4 , extracted with 100 ml. of 10% butanol in CHCl_3 , in successive portions. The extract was concentrated by adding a small excess of N-NaOH , evaporating to dryness, acidifying in the usual way and extracting with 20 ml. of 5% butanol in CHCl_3 . The whole extract was rechromatographed on a type IV column of standard size (5 g. silica) and the C_4 fraction eluted. A second 80 ml. sample of the original CHCl_3 -butanol extract was worked up in the same way. The combined C_4 fractions were now neutralized, recovered and again chromatographed on a type IV column. Titrations were carried out in the usual manner until the C_4 acid began to be eluted and subsequent samples were collected without titration until the whole fraction had been eluted. Titration of a small sample indicated that, in all, 19 mg. of the C_4 acid had now been obtained in 60 ml. of extract. The theoretical amount of piperazine hexahydrate in ethanol was now added and the whole evaporated under reduced pressure. On addition of a few drops of acetone to the resulting oil immediate crystallization took place. After standing overnight at 3° the product was filtered off (m.p. $115\text{--}117^\circ$; mixed m.p. with the piperazinium salt of *n*-butyric acid $116\text{--}117^\circ$). The product was recrystallized from acetone (m.p. $117\text{--}118.5^\circ$; mixed m.p. with the derivative of isobutyric acid 65° approx.).

The C_4 acid is thus identified as *n*-butyric acid. (All m.p.'s uncorr.).

Fractions C_5 and C_6 . Samples of the mixed acids were put on to 20 g. columns of type II. The C_5 fraction was eluted with 1% butanol in chloroform and the C_6 acids with 10% butanol in chloroform. Fractions obtained in this way from each of several columns were massed together and purified on 20 g. columns of type III, from which the C_5 acids were eluted with 10% butanol in chloroform, and C_6 acids with 30% butanol in chloroform.

After recovery of the acids in the usual way, the fractions were separately treated with the theoretical amounts of piperazine hexahydrate, in repeated attempts to obtain crystalline derivatives, but in every case oily products were obtained which failed to crystallize, even on prolonged standing. Since the piperazinium derivatives of all four isomers of valeric acid and of *n*- and *iso*-caproic (hexanoic) acid were readily obtained individually from pure samples of each, we concluded that the natural C_5 and C_6 fractions consisted of mixtures containing more than one isomer in each case. Further chromatographic experiments were therefore made in the hope of resolving the mixtures.

Chromatographic examination of C_5 fraction

Partition analysis. The behaviour of the C_5 fraction on our usual buffered columns (type III) is depicted in Fig. 1, which also shows the results of running a series of mixed chromatograms with known acids. As previously shown (Moyle *et al.* 1948), *n*-valeric and trimethylacetic acids are separable from *isovaleric* and methylethylacetic acids, but neither pair can be resolved by this method. The C_5 fraction from *A. lumbricoides* behaves in the same way as *isovaleric* and methylethylacetic acids, whether separately or in mixtures, and comes through significantly later than *n*-valeric and trimethylacetic acids. This led us to the conclusion that the C_5 fraction must include one or more of three compounds, namely, *isovaleric*, (+)- and (−)-methylethylacetic acids.

Adsorption analysis. Following the indications given by Claesson (1946), we carried out experiments on short adsorption columns of silica, previously dried at 800° , using pure specimens of the valeric acid isomers dissolved in cyclohexane. In this way it was found that *n*-valeric and trimethylacetic acids could be separately estimated by frontal analysis; mixtures of either, with other isomers or with the worm acid(s), could not be analysed. Mixtures of *isovaleric* and methylethylacetic acids showed no signs of separation.

Although this part of our work failed to carry us any further, it is mentioned here since *n*-valeric and trimethylacetic acids are inseparable by our partition columns. Their separation and estimation by frontal analysis on adsorption columns thus forms a useful supplement to the partition technique.

Further examination of C_5 and C_6 fraction. Having failed so far in our attempts to resolve either the C_5 or C_6 fractions into their isomeric components by chromatographic methods, we now reverted to more classical techniques involving the use of larger quantities of material. The acids used in this part of the work were obtained by steam distillation of large, bulk samples of intact worms previously submitted to digestion by 30% (w/v) sodium

Fig. 1. Chromatographic behaviour of C_5 worm acid and isomers of valeric acid; mixed chromatograms.

hydroxide, first for several weeks at room temperature and then on the boiling-water bath, and subsequently acidified with sulphuric acid.

Approximately 9 g. of the mixed worm acids were refluxed for 5 hr. with 40 ml. dry methanol containing 0.5 ml. conc. H_2SO_4 . Methanol (30 ml.) was removed by distillation and the residual mixture of methyl esters extracted with ether. The ethereal layer was washed five times with 2% aqueous K_2CO_3 , dried over K_2CO_3 and distilled to remove ether. The mixed esters were now fractionally distilled from a small flask of about 25 ml. capacity. The following fractions were collected:

Fraction	B.p. (°)	Weight (g.)	Probable composition
1	34–36	—	Ether
2	36–100	0.86	Methyl esters of acetic, propionic and butyric acids
3	100–120	2.02	Methyl esters of valeric acids
4	120–132	2.43	Methyl esters of caproic acids

Since fraction 4 contained two main sub-fractions, boiling at 129° and 131–132°, it seems probable that two isomeric forms of caproic acid are present. These cannot be either *n*-caproic (b.p. of methyl ester 150°) or methylethylpropionic acid (b.p. of methyl ester 141°) and must therefore be other branched-chain caproic acids. The fraction was found to be optically active ($[\alpha]_D = -2.245^\circ$) and must therefore contain at least one optically active acid.

The main part of fraction 3 came over at 115–117° and might consist of the methyl esters of *isovaleric* (b.p. 116°) and (+)- and (-)-methylethylacetic acids (b.p. 113–115°). This fraction, too, proved to be optically active ($[\alpha]_D = -1.405^\circ$) and must, therefore, contain at least some methylethylacetic acid. Because their boiling points lie so close together, there seemed little prospect of analysing this mixture of esters by fractional distillation, and still less of analysing the corresponding mixture of acids ((±)-methylethylacetic acid, b.p. 173°; *isovaleric* acid, b.p. 174°). As our supplies of the acids were now running low we made no further attempts to fractionate the C_6 acids by distillation methods, but turned instead to infrared spectroscopy. The C_6 fraction of the worm acids (1–2 g.) was obtained in the pure state by repeated chromatography as for the C_4 fraction, described above. The acids were titrated with *N*-sodium hydroxide, the sodium salts thus obtained distilled to dryness, and dried *in vacuo* over phosphorus pentoxide. A pure, dry sample of the free acids was obtained by intimately mixing the dry sodium salts with finely powdered dry potassium hydrogen sulphate and distilling the acids in a flask similar to that used by Ellis (1934).

In order to determine whether the C_6 acid obtained above was methylethylacetic acid or *isovaleric* acid, the infrared spectra of pure samples of these acids and of the unknown were determined. The spectrometer used was a Perkin Elmer Type 12B, and the spectra were obtained between 1800 and 700 cm^{-1} using a rocksalt prism. The liquid samples were approximately 0.005 mm. in thickness. The absorption curves obtained are shown in Fig. 2.

Fig. 2. Infrared absorption spectra of C_6 acids.

Although the two pure acids have a number of absorptions in common, there are considerable differences in their spectra, notably in the region 1550–1150 cm^{-1} . In particular, *isovaleric* acid has strong absorption peaks close to 1300 and 1220 cm^{-1} , whereas methylethylacetic acid has a marked absorption distinct from those of *isovaleric* at about 1230 cm^{-1} . It can be seen immediately from the figure that the spectrum of the unknown material very strongly resembles that of methylethylacetic acid throughout the region investigated, and differs in many respects from that of *isovaleric* acid. The only detailed difference between the spectrum of the unknown acid and of pure methylethylacetic acid is an additional faint absorption in the former, which shows as a shoulder in the spectrum near 1210 cm^{-1} . This presumably corresponds to a small amount of impurity, which might possibly consist of a little *isovaleric* acid. It is clear, however, that the unknown acid is very nearly pure methylethylacetic acid, with an impurity content of probably less than 10%.

It is not possible, of course, to distinguish by spectroscopic means between the (+)- and (-)-forms of this acid. The polarimetric data already mentioned indicate that, in the acid as analysed, there was a small preponderance (about 6%) of the (-)-isomer. There remained, however, the possibility that extensive racemization might have taken place in the course of preparation of these particular batches of acids. The most likely step at which racemization might have occurred was during the digestion of the worms with 30% sodium hydroxide for some weeks in the cold, followed by several hours in the hot. This possibility was investigated in the following manner.

Approximately 50 g. of synthetic ($\pm$)-methylethylacetic acid was partially resolved with brucine, according to the procedure used by Schutz & Marckwald (1896). The product, after recovery, showed $[\alpha]_D = -9.6^\circ$, indicating the presence of the (+) and (-) acids in the proportion of 20 and 80 % approximately. This material was heated on a boiling-water bath for 5 hr. with 30 % NaOH and then allowed to stand at room temperature for some weeks. Despite this treatment there were no signs of racemization, and we therefore think that the optical properties of the products of fractional distillation of the methyl esters of the worm acids, correspond to those of the natural material.

Accordingly, we have to conclude that at least 90 % of the C_6 fraction consists of optically active methylethylacetic acid, with the (-)-form slightly preponderating over the (+)-acid.

Fraction C_6 . As has been pointed out, we were unable to resolve the C_6 acids whether by chromatography, fractional crystallization of the piperazinium salts, or fractional distillation of the methyl esters. The possibilities of achieving separation of the acids themselves by fractional distillation seemed remote and the only promising line for further approach appeared to lie in infrared spectroscopy. This method, however, could not be applied, since the necessary reference substances were not available to us. The nature of the C_6 acid(s) therefore remains unknown for the present.

Fraction C_8 . This fraction consisted of a mixture of acids containing more than six carbon atoms, and, since it accounted for only 1 % of the total acids, was not examined further.

DISCUSSION

The presence of formic, acetic, propionic and *n*-butyric acids in a wide range of biological materials is well known, and they are, moreover, among the principal products of the digestion of cellulose by the rumen contents of the sheep and other herbivores (McAnally & Phillipson, 1944). That these acids may also be formed by bacterial activity in the alimentary tract of the pig is possible, and the fact that they occur in laboratory media in which specimens of *Ascaris* have been incubated has led many workers to the conclusion that they are bacterial in origin. But these acids are present in relatively high concentrations in the perienteric fluid of freshly collected worms, suggesting that they escape into the keeping medium by diffusion or, perhaps, by excretion. However, these lower acids together account for less than 20 % of the total volatile acids present in the perienteric fluid. The residual methylethylacetic acid (46 %) is optically active, and the C_6 acid (35 %) also appears to be optically active. Optically active isomers of valeric and caproic acids are not known as products of microbial digestion, at any rate of cellulose, nor, so far as we are aware, have they heretofore been dis-

covered as constituents of any biological material. This adds some weight to the opinion that these, and probably the shorter chain acids also, are peculiar products of the metabolism of *Ascaris* itself and not of any bacteria with which it normally is, or experimentally may become, associated.

The results of our analyses, carried out on freshly collected perienteric fluid from worms collected only a few hours before, differ substantially from those recently published by Bueding & Yale (1951), who worked up the acids from saline media in which the worms had been kept:

Percentage of total steam-volatile acids					
C_6	C_5	C_4	C_3	C_2	
36	46	2	4	11	Present work
20	40	2-5	10-15	15-25	Bueding & Yale (1951)

But these differences are less important than those between our respective conclusions regarding the nature of the C_5 acid; for whereas we believe it to consist almost in its entirety of methylethylacetic acid, Bueding & Yale (1951) state that about one-half consists of *n*-valeric acid and the other half of a mixture of *isovaleric* and methylethylacetic acids. Small amounts of an $\alpha\beta$ -unsaturated C_5 acid were also reported. We ourselves found traces of a substance which decolorizes bromine water and which might be formic or some unsaturated acid. It is difficult to reconcile our respective conclusions or to explain the differences that exist; but it is conceivable, since much of the C_6 acid has apparently been transferred to the lower fractions in Bueding & Yale's experiments, that these workers were, in fact, dealing with partial degradation products formed—most probably by some kind of microbial activity—from acids which had diffused out of the worms into the culture medium. They themselves deny that bacterial activity was involved in their experiments, on the grounds that the worms used were treated with antibiotic substances. But antibiotics in general are more or less specific in their action, so that these allegedly sterile experiments may reasonably be regarded with some suspicion.

The origin and mode of formation of these acids is wholly obscure. In the past they have often been compared with the lactic acid which can arise anaerobically from carbohydrate, e.g. in muscle and in a variety of bacteria, for it is generally supposed that *Ascaris* itself lives under conditions that approximate fairly nearly to anaerobiosis (see Hobson, 1948). However, the structure of methylethylacetic acid, which contains an asymmetrically branched carbon chain and accounts for practically one-half of the total acids, suggests that the latter can hardly arise from carbohydrate sources. The branched chain of methylethylacetic acid occurs,

however, in isoleucine; it is therefore conceivable that our C_5 acid might arise by oxidative deamination of isoleucine, followed by oxidative decarboxylation of the resulting α -keto acid. These are reactions that are known to occur among vertebrates and probably go on in invertebrates also. Perhaps they are less likely to take place in an animal that leads what appears to be a largely or mainly anaerobic existence, though even here oxidative processes might occur by coupling to simultaneous reductive reactions. But in any case, unless we assume that isoleucine alone is singled out for this kind of treatment we should expect leucine itself to give rise through similar reactions to *isovaleric* acid, and the latter cannot account for more than 10% of the total valeric fraction, according to the spectroscopic evidence. Valine, moreover, would be expected to give rise in a similar way to *isobutyric* acid, whereas our C_4 acid is the *n*-compound. Nor does such a mechanism allow us to explain in any simple manner the relatively large-scale formation of a C_6 acid.

Other mechanisms might be invoked to account for the formation of methylethylacetic acid from isoleucine, the only other commonly occurring biological compound known to contain the appropriate branched chain. We have considered several possibilities of this kind, all of which lead to conclusions that are inconsistent within themselves,

like the scheme discussed here. We must at present confess to complete ignorance of the origin, mode of formation and function of these curious compounds.

SUMMARY

1. The steam-volatile fatty acids of the perienteric fluid of *Ascaris lumbricoides* have been analysed by chromatographic methods.

2. Acetic, propionic and *n*-butyric acids have been identified by their chromatographic behaviour and by the piperazinium salt of *n*-butyric acid. Small quantities of formic acid also appear to be present.

3. Infrared spectroscopy showed that the C_5 fraction consists, almost in its entirety, of the optically active methylethylacetic acid (2-methylbutanoic acid). There is a slight preponderance of the laevorotatory form.

4. The C_6 fraction has not so far been identified.

5. It is concluded that these acids are products of the undoubtedly peculiar metabolism of *A. lumbricoides* and are not formed by microbial activity. Their origin, function and mode of formation are unknown.

We wish to thank Dr N. Sheppard for determining the infrared absorption spectra of the acids examined by this method.

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