Chibnall, A. C., Mangan, J. L. & Rees, M. W. (1958*b*). *Biochem. J.* **68**, 114.
Chibnall, A. C. & Rees, M. W. (1951). *Biochem. J.* **48**, xlvii.
Chibnall, A. C. & Rees, M. W. (1958). *Biochem. J.* **68**, 105.
Clayton, R. A. & Strong, F. M. (1954). *Analyt. Chem.* **26**, 1362.
Conway, E. J. (1935). *Biochem. J.* **29**, 2755.
Craig, L. C., Gregory, J. D. & Hausmann, W. (1950). *Analyt. Chem.* **22**, 1462.
Craig, L. C., Konigsberg, W. & Hill, R. J. (1958). In *Amino Acids and Peptides with Antimetabolic Activity*, p. 226. London: J. and A. Churchill Ltd.
Craig, L. C., Weisiger, J. R., Hausmann, W. & Harfenist, E. J. (1952). *J. biol. Chem.* **199**, 259.
Crawhall, J. C. & Elliott, D. F. (1955). *Biochem. J.* **61**, 264.
Flynn, F. V. & de Mayo, P. (1951). *Lancet*, **261**, 235.
King, F. E. & Spensley, P. C. (1950). *J. chem. Soc.* p. 3159.
Lockhart, I. M. & Abraham, E. P. (1954). *Biochem. J.* **58**, 633.
Lockhart, I. M., Abraham, E. P. & Newton, G. G. F. (1955). *Biochem. J.* **61**, 534.
Lyttle, D. A., Jensen, E. H. & Struck, W. A. (1952). *Analyt. Chem.* **24**, 1843.
Markham, R. (1942). *Biochem. J.* **36**, 790.
Newton, G. G. F. & Abraham, E. P. (1950). *Biochem. J.* **47**, 257.
Newton, G. G. F. & Abraham, E. P. (1953*a*). *Biochem. J.* **53**, 597.
Newton, G. G. F. & Abraham, E. P. (1953*b*). *Biochem. J.* **53**, 604.
Newton, G. G. F. & Abraham, E. P. (1954). *Biochem. J.* **58**, 103.
Nystrom, R. F., Chaikin, S. W. & Brown, W. G. (1949). *J. Amer. chem. Soc.* **71**, 3245.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
Rees, M. W. (1958). *Biochem. J.* **68**, 118.
Smith, I. (1953). *Nature, Lond.*, **171**, 43.
Sörensen, S. P. L. (1905). *Bull. Soc. chim. Fr.*, **33**, 1052.
Sörensen, S. P. L., Höyrup, M. & Andersen, A. C. (1911). *Hoppe-Seyl. Z.* **76**, 44.
Swallow, D. L. & Abraham, E. P. (1957). *Biochem. J.* **65**, 39P.
Swallow, D. L. & Abraham, E. P. (1958). *Biochem. J.* **70**, 364.
Synge, R. L. M. (1951). *Biochem. J.* **49**, 642.
Underwood, J. C. & Rockland, L. B. (1954). *Analyt. Chem.* **26**, 1553.
Weisiger, J. R., Hausmann, W. & Craig, L. C. (1955*a*). *J. Amer. chem. Soc.* **77**, 731.
Weisiger, J. R., Hausmann, W. & Craig, L. C. (1955*b*). *J. Amer. chem. Soc.* **77**, 3123.
Woiwod, A. J. (1949). *J. gen. Microbiol.* **3**, 312.
Wright, B. E. & Stadtman, T. C. (1956). *J. biol. Chem.* **219**, 863.

Studies in the Biochemistry of Micro-organisms

106. METABOLITES OF *ALTERNARIA TENUIS* AUCT.: THE STRUCTURE OF TENUAZONIC ACID*

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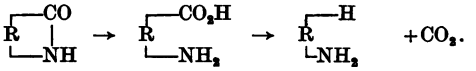
Rosett, Sankhala, Stickings, Taylor & Thomas (1957) described the isolation, from culture filtrates of *Alternaria tenuis* auct. grown on a glucose medium, of a number of metabolic products. Several of these appeared to be related to alternariol, a mycelial product formed by the same strains, and shown by Raistrick, Stickings & Thomas (1953) to be 3:4′:5-trihydroxy-6′-methyldibenzo-α-pyrone. The exception was tenuazonic acid, $C_{10}H_{15}O_3N$, the only nitrogen-containing metabolite among the new products; this was quite different in properties. This paper describes the work carried out on this acid and advances a structural formula. Rosett *et al.* (1957) described tenuazonic acid as an optically active viscous liquid, giving a strong orange–red ferric reaction and a green complex copper salt. It behaved as a monoketone and a monobasic acid, and was converted into an optical isomer on boiling with aqueous alkali, or on long keeping.

It has now been shown that hydrolysis of tenuazonic acid with 0·1 N-mineral acid gives good yields of acetic acid and a colourless crystalline monobasic acid, m.p. 117·5–119·0°, which has the formula $C_8H_{13}O_2N$. This substance, shown below to be an epimeric mixture, will be called the deacetyl compound; it yields a mono-2:4-dinitrophenylhydrazone. In addition, tenuazonic acid gives iodoform with alkaline iodine. These reactions show the presence of both a $CH_3 \cdot CO$ and a CO group in the molecule.

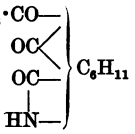
Hydrolysis of tenuazonic acid with 2 N-mineral acid gives high yields of acetic acid, carbon dioxide and a basic compound, isolated as a benzenesulphonyl derivative, $C_7H_{14}ON \cdot SO_2C_6H_5$, m.p. 123·5–125·0°, which also has ketonic properties.

* Part 105: Birkinshaw & Chaplen (1958).

The benzenesulphonyl derivative is soluble in cold aqueous sodium hydroxide, hence the base is a primary amine; it is therefore an aminoheptanone; this substance is also produced in small amounts in the above-mentioned hydrolysis with 0·1 N-acid. The deacetyl compound, and tenuazonic acid itself, are both essentially non-basic. Together with the elimination of carbon dioxide, this suggests a lactam structure:



Tenuazonic acid can now be written

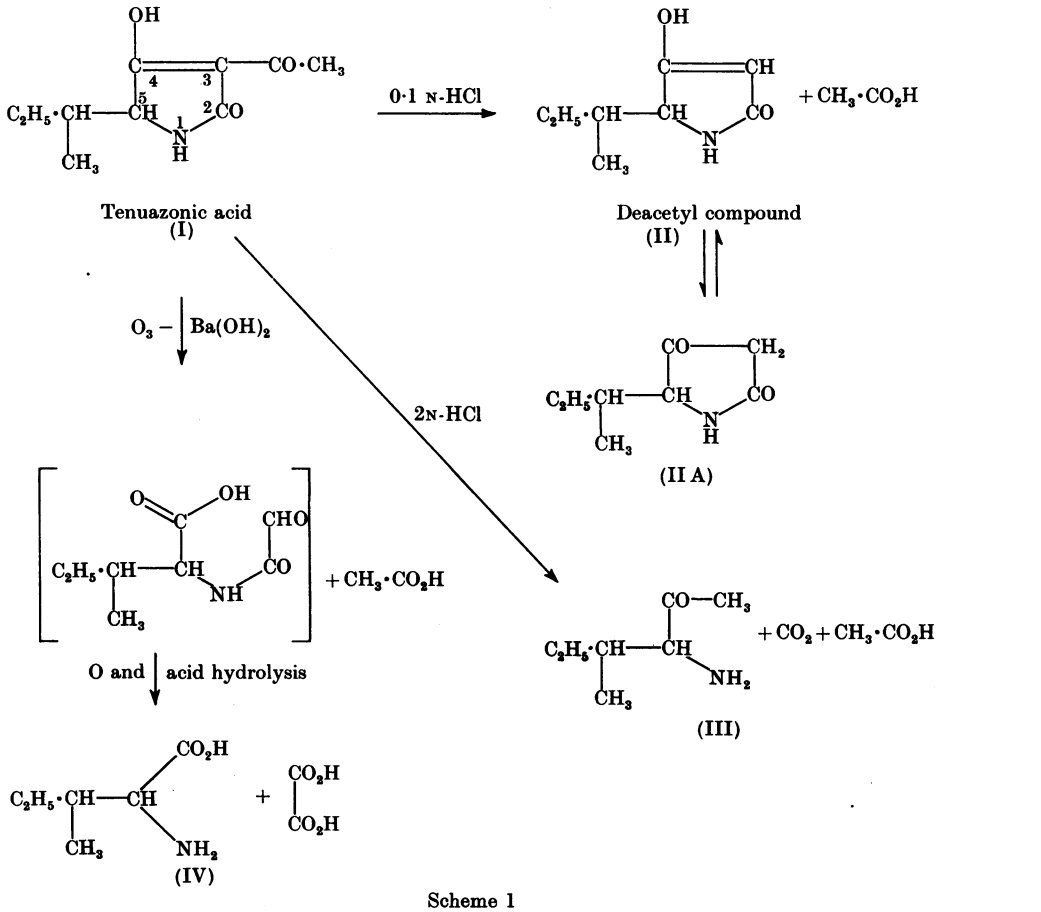


This accounts for all the oxygen and nitrogen atoms: the remainder of the molecule must consist of saturated hydrocarbon links and chains.

Ozonolysis of tenuazonic acid in aqueous alkali, followed by acid hydrolysis, yields acetic acid, oxalic acid and an amino acid. The latter is shown to be L-isoleucine by elementary analysis, optical rotation, paper-chromatographic behaviour and degradation by ninhydrin to a volatile aldehyde giving a 2:4-dinitrophenylhydrazone indistinguishable from (+)-2-methylbutanal 2:4-dinitrophenylhydrazone (cf. Fones, 1954).

Of the C_6 residue in the above partial formula for tenuazonic acid, five carbon atoms are accounted for by the branched hydrocarbon chain in isoleucine, and this chain must be attached to the nitrogen atom in tenuazonic acid. The base produced by strong acid hydrolysis must therefore be the methyl ketone corresponding to isoleucine, i.e. 3-amino-4-methylhexan-2-one (Scheme 1, III). This formulation is supported by the strong iodoform reaction given by the benzenesulphonyl derivative. The methyl in this $CH_3 \cdot CO$ group must arise from the other carbon atom in the C_6 residue.

The original acid is a strong monobasic acid (pK about 3·35), and the deacetyl compound is also a



Scheme 1

monobasic acid, though less strong. No carboxyl group is possible, hence there must be an enolic hydroxyl group in tenuazonic acid, i.e. a CH or CH_2 group α to at least two CO (or CO·NH) groups. This argument leads to the formula II A or II for the deacetyl compound, and I for tenuazonic acid. Attachment of the acetyl group at position 3 would account for the stronger acidity of tenuazonic acid as compared with the deacetyl compound, the intense ferric reaction of tenuazonic acid and the formation of a complex copper salt.

Tenuazonic acid is thus α-acetyl-γ-*sec*-butyltetramic acid. This formulation explains most of the known facts. Thus ozonolysis in alkaline solution oxidizes the double bond, acetic acid is lost by hydrolysis and the terminal –CHO group is further oxidized to $-CO_2H$. Hydrolysis then yields oxalic acid and isoleucine (IV).

Hydrolysis under mild acid conditions leads to partial breakdown of the β-diketo-amide system, and with stronger acid the degradation is exactly comparable with that of α-acyltetronic acids under the same conditions (Clutterbuck, Raistrick & Reuter, 1935*a*, *b*). The chemistry of tenuazonic acid is in fact very similar to that of the α-acyltetronic acids, a number of which were isolated by Clutterbuck, Haworth, Raistrick, Smith & Stacey (1934) from strains of *Penicillium charlesii*. These

Table 1. *Ultraviolet-absorption maxima of tenuazonic acid, synthetic α-acetyltetramic acids, carlosic acid and α-acetyltetronic acid in various solvents*

Measurements were made in 1 cm. cells at concentrations of 15–20 mg./l.

	210–220 mμ		230–240 mμ		260–280 mμ	
Tenuazonic acid in	$\lambda_{max.}$	log ε	$\lambda_{max.}$	log ε	$\lambda_{max.}$	log ε
n-Hexane	214	3·69	—	—	274	4·06
Ethanol	217	3·72	—	—	277	4·13
Water	—	—	239	4·05	279	4·16
0·09 N-NaOH	—	—	240	4·07	279	4·17
0·09 N-HCl	220	3·80	—	—	277	4·10
α-Acetyl-γ-methyltetramic acid in ethanol*	—	—	240	3·72	280	4·04
α-Acetyltetramic acid in ethanol*	—	—	239	3·98	277	4·13
Carlosic acid† in						
Water	—	—	232	4·16	265	4·22
0·09 N-HCl	—	—	232	4·09	265	4·18
N-HCl	211	3·75	232	3·77	269	4·14
α-Acetyltetronic acid in						
0·025 N-H_2SO_4‡	—	—	230	4·18	265	4·18
0·5 N-HCl	211	3·85	230	3·95	261	4·07
2 N-HCl	211	3·94	—	—	260	4·01

* Lacey (1954).
† (−)-α-*n*-Butyryl-γ-carboxymethyltetronic acid (Clutterbuck, Raistrick & Reuter, 1935*b*).
‡ Herbert & Hirst (1935).

Table 2. *Main infrared-absorption maxima of tenuazonic acid and synthetic α-acetyltetramic acids in the regions* 3500–3000 *cm.*$^{-1}$ *and* 2000–1500 *cm.*$^{-1}$

Figures for α-acetyl-γ-methyltetramic acid and α-acetyltetramic acid are derived from curves (kindly supplied by Dr R. N. Lacey) which were recorded on a Hilger H. 800 double-beam instrument.

Tenuazonic acid		α-Acetyl-γ-methyl-tetramic acid	α-Acetyl-tetramic acid
Capillary layer (cm.$^{-1}$)	CCl_4 soln. (cm.$^{-1}$)	Nujol mull (cm.$^{-1}$)	Nujol mull (cm.$^{-1}$)
3296	3236	3380	3240
3112	3098	3074	3112
*	1735	1709	1729
*	1705 (shoulder)	—	—
*	1674	1680	1667
*	1630	1615	1620

* Absorption too strong for accurate measurement.

α-acyltetronic acids are strong acids, most of which yield similar ferric colours, copper derivatives and mono-dinitrophenylhydrazones.

Lacey (1954) has described the preparation of α-acetyltetramic acids by the reaction of diketen with amino acid esters, followed by ring closure. The properties of α-acetyltetramic acid, m.p. 155°, and α-acetyl-γ-methyltetramic acid, m.p. 115°, resemble those of tenuazonic acid, including a red ferric colour, strong acidity and stability of the ring system to boiling alkali. The absorption maxima are also similar (see Tables 1 and 2). In the ultraviolet region, the two synthetic compounds have absorption maxima at 239–240 mμ and 277–280 mμ in ethanol. Tenuazonic acid shows two maxima in absolute ethanol, at 217 and 277 mμ, and in *n*-hexane, at 214 and 274 mμ. However, in water there is no absorption peak at 217 mμ but there is one at 239 mμ. The spectrum in aqueous 0·09N-sodium hydroxide is very similar to that in water, but in 0·09N-hydrochloric acid the lower maximum is again shifted to 220 mμ. It would appear that the absorption at 239–240 mμ is due to an ionic form, whereas that at 214–220 mμ is due to a non-ionic structure. The shift to 220 mμ in 0·09N-hydrochloric acid is surprising in view of the findings of Herbert & Hirst (1935), who showed that a number of α-acyltetronic acids absorbed at about 230 and 265 mμ in water, the maxima being essentially unchanged either in alkali or in 0·025N-sulphuric acid. However, it has now been shown for carlosic acid and α-acetyltetronic acid that in stronger acid the intensity of the maximum at about 230 mμ is reduced and a new maximum appears at 211 mμ. The p*K* value of α-acetyltetronic acid is about 1·8 (Baker, Grice & Jansen, 1943); more concentrated acid is therefore required to suppress the ionization, than with tenuazonic acid. The infrared-absorption curves of the synthetic compounds are sufficiently close to that of tenuazonic acid to be consistent with its formulation as an α-acetyltetramic acid (Table 2).

Simple γ-alkyltetramic acids do not appear to have been described. Benary (1911) obtained tetramic acid, m.p. 211°, from α-carboxytetramic acid. It gave a blood-red colour with ferric chloride, and an intense violet with sodium nitrite (a reaction characteristic also of tetronic acids unsubstituted in the α-position). The deacetyl compound from tenuazonic acid reacts with sodium nitrite to give a purple–red but gives only a very slight brown ferric colour, though this deepens somewhat on standing. It is difficult to see why introduction of a *sec*-butyl group should reduce the ferric reaction so markedly. The deacetyl compound gives a slight positive iodoform reaction, despite the absence of a $CH_3 \cdot CO$ group. However, β-diketones are known to give a positive reaction in this test (Fuson & Tullock, 1934).

Although α-acyltetronic acids react smoothly with bromine (Clutterbuck *et al.* 1935*a*), bromination of α-acetyltetramic acids is complex (Lacey, 1954). The behaviour of tenuazonic acid itself towards bromine has not been studied, but the deacetyl compound reacts immediately with bromine water to give a high yield of a dibromo compound $C_8H_{11}O_2NBr_2$, which is insoluble in aqueous sodium bicarbonate but dissolves on treatment with sodium hydroxide. On acidification a new crystalline compound, $C_8H_{13}O_3NBr_2$, soluble in aqueous sodium bicarbonate, is precipitated. The structure of these dibromo compounds has not yet been elucidated. The dibromo derivative of (−)-γ-methyltetronic acid obtained by Clutterbuck *et al.* (1935*a*) also existed in a hydrated form.

The orientation about the two asymmetric carbon atoms in tenuazonic acid should be identical with the orientation about the corresponding atoms in the L-isoleucine obtained after ozonolysis and hydrolysis, since the acid hydrolysis of the amide link would not be expected to introduce any inversion. It is to be expected, however, that, while the asymmetric centre in the side chain would be stable, epimerization would take place readily at position 5 in the ring in both tenuazonic acid and its deacetyl derivative, owing to the α-carbonyl group. No doubt this is the explanation of the formation of the crystalline *iso*tenuazonic acid either on long keeping or, more rapidly, in boiling alkali (Rosett *et al.* 1957). It was therefore anticipated that ozonolysis of *iso*tenuazonic acid in alkali, followed by hydrolysis, under the same conditions as those used for tenuazonic acid, would yield D-*allo*isoleucine. In fact, the product obtained had practically zero specific rotation, but yielded the (+)-2-methylbutanal 2:4-dinitrophenylhydrazone in the same way as L-isoleucine: it was therefore a mixture of roughly equal parts of D-*allo*isoleucine and L-isoleucine.

It follows that the crystalline '*iso*tenuazonic acid' must be a mixture of diastereoisomers, which forms a crystalline copper salt of about the same relative composition. Accordingly, these two products have been re-examined, and by working up crystallization residues a copper salt of considerably higher dextrorotation has been obtained. However, this is probably not yet optically pure, and all attempts to separate the true *iso*tenuazonic acid or its salts or derivatives from tenuazonic acid by chromatographic methods have so far failed. The properties of the true *iso*tenuazonic acid and its derivatives are therefore unknown. The semicarbazone obtained from the crystalline acid may well be a mixture. It was recorded in the earlier paper that reaction with 2:4-dinitrophenylhydrazine yielded a mixture from which some tenuazonic acid 2:4-dinitrophenylhydrazone was isolated; it

was assumed at that time that epimerization had occurred during formation of the derivative, but this explanation is clearly no longer necessary.

The deacetyl compound is also a mixture. When ozonized in aqueous barium hydroxide and subjected to acid hydrolysis, it gave a mixture containing L-isoleucine and D-*allo*isoleucine in roughly equal quantities. The deacetyl compound therefore consists of approximately equal amounts of deacetyltenuazonic acid and deacetyl*iso*tenuazonic acid; the mixture crystallizes like a pure compound and no attempt has been made to separate the diastereoisomers. The same mixture is obtained by dilute acid hydrolysis of the crystalline '*iso*-tenuazonic acid'.

The hydrolysis product, 3-amino-4-methylhexan-2-one (III), also contains this labile asymmetric centre. The orientation of the particular isomer characterized as the benzenesulphonyl derivative, m.p. 123·5–125·0°, is unknown and it may also be a mixture; there are clear indications of the presence of two isomers in the reaction product.

So far as is known, tenuazonic acid is the first example of a substituted tetramic acid as a natural product. The tetronic acid derivatives produced by *P. charlesii* and other moulds (Clutterbuck *et al.* 1934, 1935*a*, *b*; Clutterbuck, Raistrick & Reuter, 1935*c*; Birkinshaw & Raistrick, 1936; Bracken & Raistrick, 1947) form the closest analogy, although the substituents at both 3- and 5-positions in tenuazonic acid are different from those in any of the natural tetronic acids so far described.

The ready breakdown to L-isoleucine, the naturally occurring form of this amino acid, suggests that tenuazonic acid is biosynthesized from L-isoleucine and 2 molecules of acetate, by elimination of 2 molecules of water. This follows closely the laboratory synthesis of α-acetyltetramic acids by Lacey (1954) mentioned above. Preliminary results indicate that tenuazonic acid can be synthesized in the laboratory in this way. The mode of biosynthesis of isoleucine is not fully known, but it is not directly formed from acetate. Ehrensvärd (1958) has evidence that, in two of the acyltetronic acids from *P. charlesii*, the α-side chain and carbon atoms 2 and 3 are similarly derived from acetate, whereas the remainder of the molecule arises by a different route.

EXPERIMENTAL

All melting points are corrected, except where otherwise stated. Microanalyses were by Weiler and Strauss (Oxford), Schoeller (Kronach, Germany) and Mr F. H. Oliver (Parke, Davis and Co. Ltd., Hounslow, Middlesex). Infrared spectra were determined by Miss E. M. Tanner of Parke, Davis and Co. Ltd. (except where otherwise stated), on a Grubb–Parsons double-beam instrument. Ultraviolet spectra were determined on a Hilger and Watts Uvispek spectrophotometer.

Tenuazonic acid

The following properties are in addition to those already described (Rosett *et al.* 1957).

Iodoform reaction. Tenuazonic acid (a few milligrams) was treated with 10N-NaOH, and just sufficient water was added to give a clear solution. Dropwise addition of a 10% solution of iodine in aq. 20% KI gave an immediate yellow precipitate and the characteristic smell of iodoform.

Sodium nitrite test. Tenuazonic acid was triturated with aq. $NaNO_2$. It dissolved to a yellow solution, but no red or violet developed even on long standing.

Approximate p*K value.* Titration of tenuazonic acid (0·196 g.) in methanol (10 ml.) and water (20 ml.) required 9·96 ml. of 0·1N-NaOH (Calc. for $C_{10}H_{15}O_3N$: 9·95 ml.). pH at half-neutralization (4·98 ml.) was 3·35.

Hydrolysis of tenuazonic acid by 2N-hydrochloric acid and formation of 3-amino-4-methylhexan-2-one

A mixture of tenuazonic acid (0·205 g.) and 2N-HCl (50 ml.) was boiled for 5·5 hr. in a stream of N_2. Water slowly distilled and was condensed, and boiled water was added at the same rate to the reaction flask. The stream of N_2 was carried through a bubbler containing Brady's reagent (0·32% of 2:4-dinitrophenylhydrazine in 2N-HCl), then through bubblers containing 0·2N-$Ba(OH)_2$, which were changed at intervals and titrated with 0·1N-HCl. Most of the CO_2 was evolved in the first 2 hr. [Found, after 5·5 hr.: 25·1 ml. of 0·1N-HCl. Calc. for 1 mol. of CO_2: 20·8 ml. The high result may be due to HCl spray (see below)]. The distillate was titrated at 50 ml. intervals; after 2·5 hr., the 50 ml. portions gave a fairly constant mean titre of 1·35 ml. of 0·1N-NaOH, and gave a positive chloride test. The titrated distillate was therefore treated with Ag_2SO_4 (0·5 g. in 100 ml. of water), filtered, treated with 2N-H_2SO_4 (1 ml.) and re-evaporated on the water pump for volatile-acid determination [Found: 8·95 ml. of 0·1N-NaOH (negative test for sulphate). Calc. for 1 equiv.: 10·4 ml.]. Only a slight precipitate appeared in the Brady's reagent.

The reaction solution was evaporated to dryness in a warm-water bath on the water pump. A bubbler containing Brady's reagent was interposed between receiver and pump, but no precipitate developed in it. The residual gum (0·16 g.) would not crystallize readily. A portion (0·14 g.) was dissolved in 2N-NaOH (6 ml.) and treated dropwise with benzenesulphonyl chloride (0·5 ml.) with shaking. The clear solution was acidified, cooled and filtered to give the crude benzenesulphonyl derivative, m.p. 84–110° (0·193 g.; 79% yield). Recrystallization from 75% methanol and cooling for a short time at room temperature yielded a fraction of m.p. 122–123° (0·07 g.); after further recrystallizations, this *benzenesulphonyl derivative of 3-amino-4-methylhexan-2-one* separated as colourless prisms, m.p. 123·5–125·0°; $[\alpha]_{5461}^{22} - 99 \pm 2°$ in methanol (*c*, 1·0) (Found: C, 57·95; H, 7·1; N, 5·1; S, 11·6. $C_{13}H_{19}O_3NS$ requires C, 57·9; H, 7·1; N, 5·2; S, 11·9%). The substance is soluble in cold 2N-NaOH. If this solution is treated with excess of Brady's reagent, yellow needles are produced. The iodoform test, carried out as described above, gives a strong positive result. Attempts to purify the residues from the

crystallizations were unsuccessful. Various fractions of m.p. between 84° and 90° were obtained, but did not recrystallize to constant m.p. One fraction, m.p. 84–85°, gave $[\alpha]_{5461}^{22} - 18 \pm 2°$ in methanol (*c*, 0·67) (see also below).

Identification of volatile acid as acetic acid. The titration solution was converted into the *p*-bromophenacyl derivative in the usual way. The crude derivative (m.p. 81–84°, 63% yield) was recrystallized from light petroleum (b.p. 60–80°). The crystals, m.p. 83·5–85·5°, were sublimed at 60–70° *in vacuo* to give a colourless sublimate, m.p. 85·0–86·5° alone or mixed with authentic *p*-bromophenacyl acetate; in each case the melt reset on cooling, and remelted at the same temperature (for an analysis, see next section).

Hydrolysis of tenuazonic acid and crystalline 'iso-tenuazonic acid' by 0·1 N-hydrochloric acid and formation of deacetyl compound

Tenuazonic acid (0·41 g.) and 0·1 N-H_2SO_4 (50 ml.) were boiled under reflux for 4·25 hr. in a stream of N_2; the effluent gas was passed through Brady's reagent, then through $Ba(OH)_2$, as described above. Carbon dioxide was evolved slowly throughout the experiment (Found: 4·40 ml. of 0·1 N-HCl. Calc. for 1 mol. of CO_2: 41·6 ml.). The precipitate in the Brady's reagent was negligible. Part of the hydrolysis solution was evaporated on the water pump for determination of volatile acid (Found: 7·9 ml. of 0·1 N-NaOH. Calc. for 1 equiv.: 8·4 ml.). Extraction of the remaining solution with ether or ethyl acetate yielded a largely crystalline product, which after washing with ether melted at 105–120°. After one recrystallization from ethyl acetate the *deacetyl compound* separated as colourless prisms, m.p. 117·5–119·0°, unchanged on further recrystallization; $[\alpha]_{5461}^{20} + 16 \pm 1°$ in methanol (*c*, 1·00) [Found: C, 62·2; H, 8·4; N, 8·8; equiv. by titration (phenolphthalein), 160. $C_8H_{13}O_2N$ requires C, 61·9; H, 8·4; N, 9·0%; mol.wt., 155].

In a second experiment, tenuazonic acid (2·12 g.) and 0·1 N-HCl (50 ml.) were refluxed for 4·75 hr. and the solution was extracted as described above. The ether washings were shown to consist largely of a mixture of starting material and *iso*tenuazonic acid (0·23 g.), and gave more deacetyl compound after further hydrolysis. The evolution of CO_2 suggested that some 3-amino-4-methylhexan-2-one was being formed. A portion of the extracted reaction solution was therefore treated with 2 N-NaOH and benzenesulphonyl chloride as described above. The yield of crude benzenesulphonyl derivative was 9%, m.p. 80–85°; $[\alpha]_{5461}^{22} - 23°$ in methanol (*c*, 1·00). This material (0·16 g.) was recrystallized from 75% methanol, yielding colourless prisms (0·03 g.), m.p. 121–124°; $[\alpha]_{5461}^{21} - 97 \pm 2°$ in methanol (*c*, 1·00). From the filtrate were obtained colourless needles (0·105 g.), m.p. 83–95°; $[\alpha]_{5461}^{22}$ 0 ± 1° in methanol (*c*, 1·00). Thus 11% of the starting material was not hydrolysed and another 9% was converted into the amine. The yield of crude deacetyl compound (0·83 g.) represents a further 50%; the purified material, m.p. 118–120°, weighed 0·68 g.; various crops of lower m.p. were obtained, and, in view of the results of ozonolysis described below, may contain the two isomers in different proportions.

The deacetyl compound gives only a slight brown with $FeCl_3$ in aqueous or ethanolic solution. On standing for some days this slowly intensifies to a moderate brown. The compound is soluble in water and methanol, but scarcely soluble in light petroleum and ether. An aqueous solution treated with Brady's reagent soon clouds and deposits yellow needles. If the deacetyl compound is dissolved in cold aq. $NaNO_2$, a pink colour develops after a few minutes, deepening to a purple–red on standing. It gives a positive, but not strong, iodoform reaction.

Identification of volatile acid. The titration solutions were evaporated to dryness and converted into the *p*-bromophenacyl derivative as described above; m.p. and mixed m.p. with *p*-bromophenacyl acetate, 86–87° (Found: C, 46·7; H, 3·5; Br, 31·15. Calc. for $C_{10}H_9O_3Br$: C, 46·7; H, 3·5; Br, 31·1%).

Hydrolysis of crystalline 'isotenuazonic acid'. The crystalline mixture gave essentially the same results as pure tenuazonic acid. After 3·5 hr., 9·5% of 1 mol. of CO_2 had been evolved. Evaporation yielded 79% of 1 equiv. of volatile acid, identified as acetic acid in the manner already described. The yield of recrystallized deacetyl compound was 34%, m.p. 117·5–119·0°, unchanged on mixing with the material from tenuazonic acid; $[\alpha]_{5461}^{21} + 15·5 \pm 1°$ in methanol (*c*, 1·00).

2:4-*Dinitrophenylhydrazone of deacetyl compound.* The deacetyl compound (21 mg.) was dissolved in ethanol (1 ml.) and Brady's reagent (15 ml.) was added. After standing overnight, the yellow crystals were collected (43 mg., m.p. 203·5–205·5°) and recrystallized from ethanol. The 2:4-*dinitrophenylhydrazone of the deacetyl compound* separated as fine yellow needles, m.p. 203·5–204·5° (Found, on material dried at 100° *in vacuo*: C, 50·4; H, 5·2; N, 20·4. $C_{14}H_{17}O_5N_5$ requires C, 50·2; H, 5·1; N, 20·9%). A dilute ethanolic solution gives a red with a drop of 2 N-NaOH.

Ozonolysis of tenuazonic acid and formation of L-*isoleucine*

Preliminary experiments showed that tenuazonic acid was rapidly ozonized in $CHCl_3$ solution, no ferric reaction being detectable after 25 min. The product, on boiling with 2 N-HCl, yielded a mixture containing oily volatile ketonic material, ammonium salts (detected by the Nessler test) and an amino acid which behaved chromatographically like DL-isoleucine. Ozonolysis in 0·1 N-NaOH was complete in 1 hr. and appeared to be less complex. Rough quantitative tests indicated that 25% of the N was converted into NH_3 and 30% into the amino acid.

Tenuazonic acid (0·79 g.) was dissolved in cold 0·16 N-$Ba(OH)_2$ (100 ml.) and ozonized O_2 was passed through for 2 hr. Some white solid was deposited (probably $BaCO_3$—see below). To this suspension was added a volume of 2 N-H_2SO_4 equivalent to 100 ml. of 0·16 N-$Ba(OH)_2$, and the mixture was then evaporated to dryness at 16 mm. Hg in a warm-water bath; two further small volumes of water were then added and distilled. The distillates were titrated with 0·1 N-NaOH (Found: 30·4 ml. of 0·1 N-NaOH. Calc. for 1 equiv.: 40·0 ml.). A quantity of 2 N-H_2SO_4 equivalent to 200 ml. of 0·16 N-$Ba(OH)_2$ was then added to the residue, and the solution was heated for 8·5 hr. on a steam bath. After cooling, the solution was extracted continuously for 10 hr. with ether. Evaporation of the ether left a mainly crystalline residue (0·54 g.) consisting largely of oxalic acid (see below). The reaction solution was now treated with 200 ml. of 0·16 N-$Ba(OH)_2$. A small portion was filtered and tested for Ba^{2+} and SO_4^{2-} ions in the usual way, and slight adjustments were made until both reactions were negligible. Finally, the $BaSO_4$ was removed by filtration

through fluted Whatman no. 50 paper, and the filtrate evaporated to dryness. The mainly solid residue (0·40 g.) was heated with absolute ethanol (4 ml.), most of the solid remaining undissolved. The mixture was cooled to 0–5° and filtered, leaving a nearly colourless solid (0·255 g.), which was sublimed at 170–180° in high vacuum on to a cold finger. [It has been shown by Gross & Grodsky (1955) that optically active amino acids, including L-isoleucine, sublime quantitatively and rapidly under these conditions with no racemization.] The colourless sublimate (0·22 g.; 42% of the theoretical yield of isoleucine) was crystallized twice from 80% ethanol, from which it separated as colourless plates with a silvery sheen, m.p. 284–285° (decomp.; sealed tube); $[\alpha]_D^{23}+14\pm1°$, $[\alpha]_{5461}^{23}+16\pm1°$ in water (*c*, 1·00); $[\alpha]_D^{22}+54\pm1°$, $[\alpha]_{5461}^{22}+63\pm1°$ in acetic acid (*c*, 1·98) (Found: C, 54·5; H, 9·9; N, 11·0. Calc. for $C_6H_{13}O_2N$: C, 54·9; H, 10·0; N, 10·7%). A specimen of L-isoleucine (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) had $[\alpha]_D^{23}+13\pm1°$, $[\alpha]_{5461}^{23}+14\pm1°$ in water (*c*, 1·02); $[\alpha]_D^{23}+53\pm1°$, $[\alpha]_{5461}^{23}+63\pm1°$ in acetic acid (*c*, 1·99). Until recently, there has been no satisfactory method of separating the four isomers of isoleucine, but the work of Greenstein, Levintow, Baker & White (1951) and Greenstein, Birnbaum & Otey (1953), based on specific enzymic hydrolysis of acetyl derivatives, has shown that L-isoleucine has $[\alpha]_D^{25}+13·5°$ in water (*c*, 1–5), +49° in acetic acid (*c*, 0·5–2·0); whereas for L-*allo*isoleucine, $[\alpha]_D^{25}$ is +18° in water (*c*, 1–5), +42·5° in acetic acid (*c*, 0·5–2·0). Using the Moore & Stein method (1951) of separation of amino acid mixtures, van Dam-Bakker (1958) showed that *allo*isoleucine could be separated from isoleucine, and that certain commercial preparations of isoleucine contained large amounts of *allo*isoleucine; however, she found that the L-isoleucine supplied by Nutritional Biochemicals Corp. was free from the *allo* isomer.

In another experiment, the rotation of the sublimate was determined before crystallization from aq. ethanol. The figures obtained {$[\alpha]_D^{20}+13\pm1°$ in water (*c*, 1·005), $+52\pm2°$ in acetic acid (*c*, 2·00)} indicate that the amino acid is optically homogeneous.

The amino acid was chromatographed at various stages of its purification in propan-1-ol–0·045M-sodium pyrophosphate buffer, pH 8·4 (4:1, v/v), on no. 1 Whatman paper previously soaked in the same buffer and dried (communication from Dr R. Thomas of this Department). After development, the paper was dipped in ninhydrin solution (0·1% in $CHCl_3$) and heated at 100°. In all cases, the major component was indistinguishable from DL-isoleucine, R_F 0·49–0·51 (descending). There was a slight impurity with R_F 0·35–0·37, and even smaller amounts with R_F 0·23 and 0·06. These were not completely eliminated by the sublimations and crystallization described.

Reaction of the amino acid with ninhydrin. Final proof of the structure of the amino acid was obtained by using the reaction with ninhydrin (Fones, 1954). The recrystallized material (40 mg.) was dissolved in water (15 ml.) and heated in a stream of steam. Ninhydrin (0·25 g. in 5 ml. of water) was added rapidly and the steam-distillation was continued until the distillate no longer clouded with Brady's reagent. Excess of Brady's reagent was added to the distillate and when precipitation was complete the yellow solid was filtered, washed and dried *in vacuo* over P_2O_5: wt. 56 mg., m.p. 131·0–132·5°; $[\alpha]_D^{22}+34\pm3°$ in $CHCl_3$ (*c*, 0·68). Recrystallization from ethanol yielded the (+)-2:4-dinitrophenylhydrazone of 2-methylbutanal as orange rectangular plates, m.p. 135–136°; $[\alpha]_D^{22}+33\pm3°$ in $CHCl_3$ (*c*, 0·74). One further crystallization raised the m.p. to 135·5–136·5°; $[\alpha]_D^{20}+30\pm2°$ in acetic acid (*c*, 1·00) (Found: C, 49·55; H, 5·3; N, 20·7. Calc. for $C_{11}H_{14}O_4N_4$: C, 49·6; H, 5·3; N, 21·0%). Fones (1954) gives m.p. 135–137°; $[\alpha]_D$ +30° in acetic acid (*c*, 1), +36° in $CHCl_3$ (*c*, 0·73). When the reaction was carried out with the sublimed amino acid, before recrystallization, the rotation was $[\alpha]_D^{22}+36\pm5°$ in $CHCl_3$ (*c*, 0·7).

L-Isoleucine (Nutritional Biochemicals Corp.) was subjected to the same reaction. The crude derivative had m.p. 129–132°; $[\alpha]_D^{23}+30\pm2°$ in $CHCl_3$ (*c*, 0·72). One recrystallization raised the m.p. to 135–136°; $[\alpha]_D^{23}+32\pm2°$ in $CHCl_3$ (*c*, 0·73). After one more recrystallization the derivative separated as orange plates, m.p. 135·5–136·5° either alone or mixed with the derivative obtained above; $[\alpha]_D^{21}+33\pm2°$ in $CHCl_3$ (*c*, 0·74), $+31\pm2°$ in acetic acid (*c*, 1·00).

Birkinshaw (1952) obtained this same 2:4-dinitrophenylhydrazone after ozonolysis of a hydrolysis product of sclerotiorin. A sample (kindly supplied by Professor J. H. Birkinshaw) did not depress the m.p. of the derivative described above.

The formation of an optically active aldehyde by this reaction eliminates the other possible structural isomers of formula $C_6H_{13}O_2N$. The specific rotation of the amino acid itself limits the choice to L-isoleucine and L-*allo*isoleucine, and the rotation of the above-mentioned 2:4-dinitrophenylhydrazone proves that the amino acid is L-isoleucine substantially unmixed with any other isomer.

Identification of volatile acid. The titration solution was converted into the *p*-bromophenacyl derivative as already described. The sublimed derivative melted at 85·5–86·5°, either alone or mixed with authentic *p*-bromophenacyl acetate.

Identification of non-volatile acid after hydrolysis. The mainly crystalline residue (0·54 g.) was treated with dry ether and filtered to give white prisms (0·25 g.; 50% of the theoretical yield), m.p. 100–102°; on slowly raising the temperature the melt reset, and remelted at 184–186° with vigorous effervescence and sublimation. A mixture with oxalic acid dihydrate (m.p. 100·5–102·0°, remelt 188–189°) melted at 100·5–102·0°, remelt 186–187°. A portion was titrated [Found: equivalent, 65·8. Calc. for $(CO_2H)_2,2H_2O$: 63·0]; the titration solution was then acidified with 2N-acetic acid and treated with excess of N-$CaCl_2$. The precipitated calcium salt was filtered and washed with water, dissolved in hot 2N-H_2SO_4, filtered and titrated with 0·1N-$KMnO_4$ [Found: equivalent, 63·5. Calc. for $(CO_2H)_2$, $2H_2O$: 63·0].

The ethereal filtrate from the crystalline oxalic acid was evaporated, dissolved in water and precipitated with $CaCl_2$. Titration of the calcium oxalate indicated that a further 13% of the theoretical yield of oxalic acid was present.

The identity of the oxalic acid was further confirmed by conversion of a portion into oxanilide, m.p. 255–257°, by boiling with aniline. Oxanilide prepared from authentic oxalic acid gave m.p. 256–258°, and a mixture melted at 256·0–257·5°. With an uncorrected thermometer the m.p. of oxanilide was 247–249°; the calculated correction for emergent stem was +8°. Most workers give the m.p. of this compound in the range 247–249° (e.g. Miller & Dittman, 1956; Roedig, Becker, Fugmann & Schoedel, 1955). The only higher figures found were 254° (Bornwater, 1912) and 252–253° (Figee, 1915). It is not stated whether these figures are corrected for emergent stem.

*Ozonolysis of crystalline 'iso*tenuazonic acid'

'*iso*Tenuazonic acid', m.p. 60·0–62·5° (1·30 g.), was dissolved in 0·16 N-$Ba(OH)_2$ and ozonized and hydrolysed in the manner described above. The volatile acid was distilled (Found: 37·4 ml. of 0·1 N-NaOH. Calc. for 1 equiv.: 66·0 ml.). The non-volatile acid fraction (0·565 g.) gave white crystals (0·191 g.) identified as oxalic acid. The amino acid fraction, after washing with ethanol, weighed 0·53 g. (49% yield); $[\alpha]_D^{20}$ 0 ± 1° in water (*c*, 0·99), unchanged on sublimation. The product was chromatographically indistinguishable from the product from tenuazonic acid. Reaction with ninhydrin followed by Brady's reagent, as already described, yielded a crude derivative, $[\alpha]_D^{23}$ + 32 ± 2° in $CHCl_3$ (*c*, 0·72). After two recrystallizations from ethanol, the compound melted at 135·5–136·0°; $[\alpha]_D^{23}$ + 33 ± 2° in $CHCl_3$ (*c*, 0·72). A mixture with the (+)-2:4-dinitrophenylhydrazone of 2-methylbutanal, obtained from authentic L-isoleucine, melted at 135·5–136·5°.

Ozonolysis of deacetyl compound

The deacetyl compound (155 mg., 1 m-mole) was dissolved in 0·189 N-$Ba(OH)_2$ (20 ml.) and ozonized O_3 was passed through for 2 hr. The solution was titrated with N-H_2SO_4 (with phenolphthalein): 1·42 ml. was required, indicating the presence of 2·36 equiv. of acid. N-H_2SO_4 (2·36 ml.) was now added, and the mixture was heated on a steam bath in a stream of N_2, the effluent being carried through $Ba(OH)_2$ bubblers for CO_2 estimation (Found: 0·57 ml. of N-H_2SO_4. Calc. for 1 mol. of CO_2: 2·00 ml.). Thus, apart from CO_2, 1·79 equiv. of acid was present (Calc. for oxalyl isoleucine, 2·00 equiv.). The solution was filtered and evaporated to dryness; further water was added and re-evaporated. Titration of the distillates gave, as expected, a negligible amount of volatile acid (5% of 1 equiv.). The residue was hydrolysed with N-H_2SO_4 and worked up as already described. The ether extract yielded a largely crystalline residue (130 mg.), which, when washed with ether, gave crystals of hydrated oxalic acid (60 mg.; 48% yield). The sublimed amino acid (52 mg.; 40% yield) behaved chromatographically as isoleucine; $[\alpha]_D^{23}$ − 3 ± 2° in water (*c*, 1·01).

Reaction with ninhydrin followed by Brady's reagent, as described above, yielded the crude derivative, m.p. 127–131°; $[\alpha]_D^{22}$ + 32 ± 5° in $CHCl_3$ (*c*, 0·74). After two recrystallizations from ethanol, the compound melted at 135–136°; $[\alpha]_D^{22}$ + 32 ± 2° in $CHCl_3$ (*c*, 0·73). A mixture with material derived from authentic L-isoleucine melted at 135·0–136·5°.

Bromination of deacetyl compound

The deacetyl compound (48 mg.) was dissolved in water (20 ml.) and treated dropwise with bromine water until a slight excess was present. The yellow colour was discharged with bisulphite and the crystals were filtered, m.p. 115·5–116·5° (70 mg., 72% yield). After recrystallization from water, then from light petroleum, the *dibromodeacetyl compound* separated as colourless needles, m.p. 116·5–117·5° (Found: C, 31·1; H, 3·7; Br, 50·7; N, 4·2. $C_8H_{11}O_2NBr_2$ requires C, 30·7; H, 3·5; Br, 51·1; N, 4·5%). The compound was insoluble in aq. $NaHCO_3$, but soluble in aq. NaOH. On acidification of the alkaline solution, well-formed prisms separated, m.p. 177·5–178·5°. Recrystallization from water and then aq. ethanol yielded glistening plates, m.p. 179–180° (Found: C, 29·1; H, 3·9; N, 4·9; Br, 47·9. $C_8H_{13}O_3NBr_2$ requires C, 29·0; H, 4·0; N, 4·2%; Br, 48·3). The compound is soluble in aq. $NaHCO_3$.

iso*Tenuazonic acid*

Copper tenuazonate trihydrate (0·76 g.) was converted into the free acid, and epimerized by boiling with 0·1 N-NaOH (59 ml.) for 9 hr. (see Rosett *et al.* 1957). It was then cooled and titrated to neutrality with 0·1 N-HCl, by the use of phenolphthalein. 0·1 N-Copper acetate (50 ml.) and $CHCl_3$ (50 ml.) were added, and the copper salt was extracted with further volumes of $CHCl_3$ until no further ferric colour was given by the extract. The $CHCl_3$ was evaporated and the residue dissolved in methanol (76 ml.). Measurement of rotation gave a minimum $[\alpha]_{5461}^{20}$ + 56° in methanol (*c*, 1) [cf. copper salt of '*iso*tenuazonic acid' (trihydrate), $[\alpha]_{5461}^{22}$ + 24·5° (Rosett *et al.* 1957)]. The solution was re-evaporated to dryness and immediately dissolved in $CHCl_3$ (7·6 ml.). After cooling at 0–5°, the crystals were filtered, washed and dried in air (0·58 g., equivalent to 0·43 g. of anhydrous); $[\alpha]_{5461}^{20}$ + 30 ± 1° in methanol (*c*, 1·00) for material dried at 100°. The filtrate was evaporated to dryness (0·27 g., equivalent to 0·25 g. of anhydrous); $[\alpha]_{5461}^{20}$ + 116 ± 2° in methanol (*c*, 1·00) for material dried at 100°. This residue was recrystallized from aq. methanol, yielding material of $[\alpha]_{5461}^{20}$ + 134°, but further crystallization from this solvent did not increase the rotation significantly.

In another experiment, copper tenuazonate trihydrate (1·30 g.) and 2 N-NaOH (100 ml.) were heated to boiling for 1 min., cooled and filtered to remove cupric oxide. The rotation (α_{5461}^{20} + 0·76°) was unchanged on boiling for a further 1·5 hr. The mixture was converted into the copper salt as above and crystallized from $CHCl_3$. The filtrate from the crystals was evaporated (0·35 g.), dissolved in warm $CHCl_3$ (3·5 ml.) and treated with benzene (14 ml.). The solid which separated on cooling was amorphous, but gave $[\alpha]_{5461}^{20}$ + 176 ± 3° in methanol (*c*, 1·00) when dried at 100°. Further purification by this method did not alter the rotation significantly. Recrystallization three times from aq. methanol raised it slightly; $[\alpha]_{5461}^{22}$ + 187° ± 3° in methanol (*c*, 1·00).

Some of this material ($[\alpha]_{5461}^{22}$ + 187°) was dissolved in aq. methanol and treated with excess of Brady's reagent. The precipitated solid, m.p. 145–151°, had $[\alpha]_{5461}^{23}$ + 118 ± 5° in methanol (*c*, 0·20), but attempts to crystallize it led to amorphous products.

SUMMARY

1. On mild acid hydrolysis, tenuazonic acid yields acetic acid and a deacetyl compound.

2. Hydrolysis with stronger acid produces acetic acid, carbon dioxide and a basic ketone, shown to be 3-amino-4-methylhexan-2-one.

3. Ozonolysis of tenuazonic acid, followed by acid hydrolysis, gives acetic acid, oxalic acid and L-isoleucine.

4. It is concluded that tenuazonic acid is α-acetyl-γ-*sec*-butyltetramic acid.

5. The configuration about the two asymmetric centres corresponds to that in L-isoleucine.

6. The crystalline isomer obtained from tenuazonic acid, and previously called *iso*tenuazonic acid, is shown to be a mixture of tenuazonic acid and a diastereoisomer.

7. Tenuazonic acid is believed to be the first substituted tetramic acid isolated from natural sources.

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REFERENCES

Baker, W., Grice, K. D. & Jansen, A. B. A. (1943). *J. chem. Soc.* p. 241.
Benary, E. (1911). *Ber. dtsch. chem. Ges.* **44**, 1759.
Birkinshaw, J. H. (1952). *Biochem. J.* **52**, 283.
Birkinshaw, J. H. & Chaplen, P. (1958). *Biochem. J.* **69**, 505.
Birkinshaw, J. H. & Raistrick, H. (1936). *Biochem. J.* **30**, 2194.
Bornwater, J. T. (1912). *Rec. Trav. chim. Pays-Bas*, **31**, 105.
Bracken, A. & Raistrick, H. (1947). *Biochem. J.* **41**, 569.
Clutterbuck, P. W., Haworth, W. N., Raistrick, H., Smith, G. & Stacey, M. (1934). *Biochem. J.* **28**, 94.
Clutterbuck, P. W., Raistrick, H. & Reuter, F. (1935*a*). *Biochem. J.* **29**, 300.
Clutterbuck, P. W., Raistrick, H. & Reuter, F. (1935*b*). *Biochem. J.* **29**, 871.
Clutterbuck, P. W., Raistrick, H. & Reuter, F. (1935*c*). *Biochem. J.* **29**, 1300.
Ehrensvärd, G. (1958). *Chem. Soc. Spec. Publ.* **2**, 14.
Figee, T. (1915). *Rec. Trav. chim. Pays-Bas*, **34**, 289.
Fones, W. S. (1954). *J. Amer. chem. Soc.* **76**, 1377.
Fuson, R. C. & Tullock, C. W. (1934). *J. Amer. chem. Soc.* **56**, 1638.
Greenstein, J. P., Birnbaum, S. M. & Otey, M. C. (1953). *J. biol. Chem.* **204**, 307.
Greenstein, J. P., Levintow, L., Baker, C. G. & White, J. (1951). *J. biol. Chem.* **188**, 647.
Gross, D. & Grodsky, G. (1955). *J. Amer. chem. Soc.* **77**, 1678.
Herbert, R. W. & Hirst, E. L. (1935). *Biochem. J.* **29**, 1881.
Lacey, R. N. (1954). *J. chem. Soc.* p. 850.
Miller, W. T. & Dittman, A. L. (1956). *J. Amer. chem. Soc.* **78**, 2793.
Moore, S. & Stein, W. H. (1951). *J. biol. Chem.* **192**, 663.
Raistrick, H., Stickings, C. E. & Thomas, R. (1953). *Biochem. J.* **55**, 421.
Roedig, A., Becker, H. J., Fugmann, N. & Schoedel, S. (1955). *Liebigs Ann.* **597**, 214.
Rosett, T., Sankhala, R. H., Stickings, C. E., Taylor, M. E. U. & Thomas, R. (1957). *Biochem. J.* **67**, 390.
van Dam-Bakker, A. W. I. (1958). *Nature, Lond.*, **181**, 116.

Oligosaccharide Synthesis in the Banana and its Relationship to the Transferase Activity of Invertase

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The banana fruit is normally picked when it is firm and green; ripening is then carried out under controlled conditions. It is well known that during such ripening the starch is largely converted into glucose, fructose and sucrose, and water is lost from the skin to the pulp (see Loesecke, 1949). In addition to these sugars, we have detected an oligosaccharide which increases in amount during the ripening process.

The invertase activity of banana pulp has been recognized for many years (Mierau, 1893*a*, *b*). Invertases from a variety of plant tissues have been shown *in vitro* to synthesize oligosaccharides by transglycosidation (see Edelman, 1956; Gottschalk, 1958). It therefore seemed probable that this action was responsible for the formation of banana oligosaccharide *in vivo*.

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This paper describes the separation and provisional identification of the above oligosaccharide, along with the partial purification of banana invertase and some studies of its hydrolytic and transferase activities.

MATERIALS AND METHODS

Bananas. Fruits of the variety *Musa cavendishii* (Lamb.), sent by rail from New South Wales, reached Melbourne within a few days of picking in a very firm and quite green condition. They were placed in single layers on wooden trays in a metal bin where ripening was accelerated by exposure for about 16 hr. to an atmosphere of coal gas. The trays were then transferred to a ventilated room which was maintained at 25° and ripening allowed to proceed in darkness. The first sample batch of thirty bananas was taken immediately upon arrival and before gassing; then at intervals of 4–5 days during ripening, similarly sized batches were withdrawn at random and held in a cold room