S-benzyl-DL-cysteine ethyl ester hydrochloride as substrate, the D isomer did not act as a competitive inhibitor. The specificity of cystine esterase is different from that of chymotrypsin and trypsin. Chymotrypsin requires the presence of an aromatic group for activity, and the hydrolysis of L-tyrosine ethyl ester may be used as a method of assay (Balls & Jansen, 1952). Trypsin requires the presence of basic groups and rapidly hydrolyses L-lysine ethyl ester (Dixon & Webb, 1958). In the conditions described earlier, cystine esterase did not hydrolyse either of these esters to any detectable extent.

SUMMARY

1. An enzyme occurring in human placenta hydrolyses esters of cystine and *S*-benzylcysteine more rapidly than it does those of other amino acids. The enzyme has been partially purified by ammonium sulphate precipitation and chromatography on a calcium phosphate column.

The skilled technical assistance of Mrs M. Gordon and Mr J. Caird is gratefully acknowledged.

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Studies in the Biochemistry of Micro-organisms

108. METABOLITES OF *ALTERNARIA TENUIS* AUCT.: THE BIOSYNTHESIS OF TENUAZONIC ACID*

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Alternaria tenuis Auct., when grown in surface culture on Czapek–Dox medium, has been shown to produce several new metabolites. From the mycelium, Raistrick, Stickings & Thomas (1953) obtained alternariol and alternariol methyl ether, and showed the structure of alternariol to be 3:4′:5-trihydroxy-6′-methyldibenzo-α-pyrone. From the culture filtrates of various strains, Rosett, Sankhala, Stickings, Taylor & Thomas (1957) isolated several new substances of unknown structure. One of these, tenuazonic acid, was later shown by Stickings (1959) to be 3-acetyl-5-*sec.*-butyltetramic acid (I). Thomas (1959, 1961) has recently shown by tracer studies that the carbon skeleton of alternariol is derived from acetate, and has pointed out that it could be obtained by head-to-tail condensation of acetate units, with no necessity for oxidation or reduction steps. Stickings (1959) suggested that tenuazonic acid might be synthesized in the fungus from one molecule of L-isoleucine and two molecules of acetate, also without any oxidation or reduction steps, and we now report evidence which supports this proposal. A preliminary account of part of this work has been given (Stickings & Townsend, 1960).

A strain of *A. tenuis*, catalogue no. 628, was chosen for this investigation, since it had already been shown (Rosett *et al.* 1957) to give a good yield of tenuazonic acid but none of the other products. In order to decide the most suitable time for addition of tracer acetate, the growth of *A. tenuis* was studied over a period of 6 weeks. It was shown that, in undisturbed surface culture, the concentration of tenuazonic acid increased rapidly from a low value on the fifth day to a maximum on the thirteenth to the fourteenth days. Other aspects of the growth and production curves are discussed later.

Sodium [1-^{14}C]acetate was therefore added on the fifth day to growing cultures, and these were

* Part 107: Birkinshaw & Samant (1960).

harvested on the thirteenth day. The tenuazonic acid isolated was found to contain 4·5% of the added radioactivity, and was therefore degraded stepwise to ascertain the activity associated with each of the ten carbon atoms of the molecule. The degradation scheme is shown in Fig. 1: the carbon atoms are labelled arbitrarily to facilitate discussion. The acid hydrolysis product, 3-amino-4-methylhexan-2-one, was treated in the reaction solution with alkaline iodine, when iodoform was produced in good yield; the isoleucine formed was not isolated, but its presence was shown by paper chromatography and conversion in good yield into 2-methylbutanal on boiling with aqueous nin-

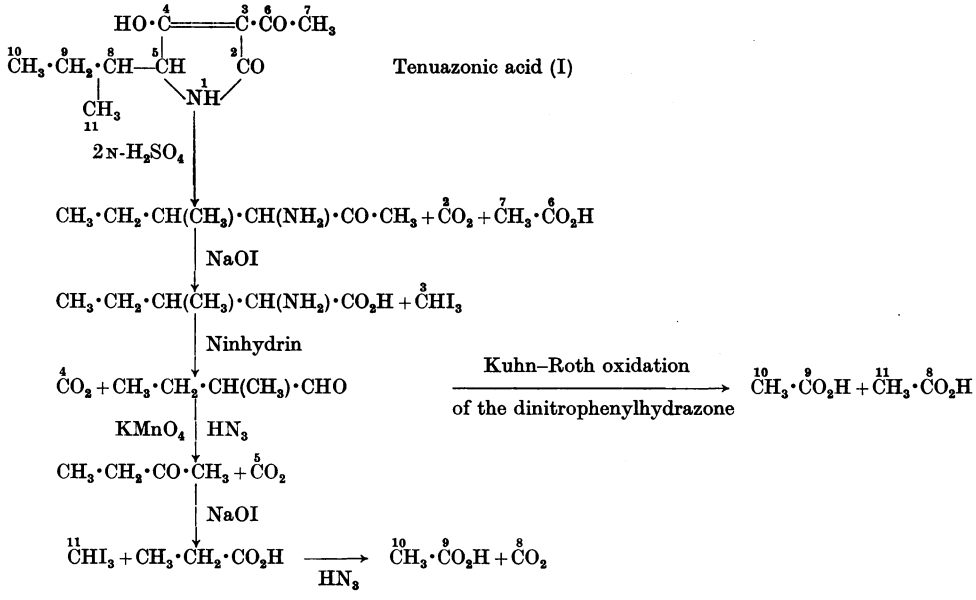

Fig. 1. Degradation scheme for tenuazonic acid. The numbering of the carbon atoms in the side chains is arbitrary. Acetic acid was degraded by the Schmidt reaction, or by the method of Popják (1955), to give each carbon atom as CO_2. Iodoform was also oxidized to CO_2.

Table 1. *Radioactivity of degradation products of tenuazonic acid*

The tenuazonic acid was produced by growing *A. tenuis* on Czapek–Dox medium with the addition of tracer sodium [1-^{14}C]acetate. Series I and II refer to two different lots of tenuazonic acid produced in this way. The degradation scheme and numbering are shown in Fig. 1.

		Series I			Series II		
Degradation product	Carbon atoms derived from tenuazonic acid	Activity of degradation product (μc/mole)	Activity of carbon atoms of tenuazonic acid (μc/mole of tenuazonic acid)	Fraction of total activity of tenuazonic acid (%)	Activity of degradation product (μc/mole)	Activity of carbon atoms of tenuazonic acid (μc/mole of tenuazonic acid)	Fraction of total activity of tenuazonic acid (%)
Barium carbonate	C-2	410	410	45·9	1162	1162	46·9
Barium carbonate	C-6	428	428	48·0	1197	1197	48·3
Barium carbonate	C-4	15·9	15·9	1·8	51·9	51·9	2·1
2-Methylbutanal 2:4-dinitrophenylhydrazone	C-5, -8, -9, -10 and -11	19·9	19·9	2·2	52·7	52·7	2·1
Barium carbonate	C-10, C-11	—	—	—	25·6	51·2	2·1
Barium carbonate	C-10	—	—	—	52·7	52·7	2·1
Barium carbonate	C-3, -5, -7, -8, -9 and -11 (all separately)	0	0	0	0	0	0
Total	C-2 to C-11	—	873·8	97·9	—	2463	99·4
Copper tenuazonate	C-2 to C-11	1785	893	100·0	4953	2477	100·0

hydrin. The 2-methylbutanal was degraded by the Schmidt and iodoform reactions (Strassman, Thomas, Locke & Weinhouse, 1956) and the activities of C-10 and C-11 were confirmed by a Kuhn–Roth oxidation.

The results are shown in Table 1, which also includes the results of an earlier incomplete series of degradations on a different sample of tenuazonic acid. It will be seen that the two series agree very closely, and that the bulk of the radioactivity (94–95%) is concentrated at C-2 and C-6 as expected. The remaining activity is shared equally between C-4 and C-10, and the corresponding atoms in isoleucine were found by Strassman *et al.* (1956) to be active when *Torulopsis utilis* was grown on a medium containing [1-^{14}C]acetate. They found 47 and 46% of the activity in the carboxyl and terminal methyl carbon atoms respectively. These results were part of the evidence in support of the biosynthesis of isoleucine from acetaldehyde (derived from pyruvate) and α-oxobutyrate.

It therefore seems probable that tenuazonic acid is synthesized in *A. tenuis* from two molecules of acetate and one of isoleucine, the latter being biosynthesized by a path identical with or very similar to that by which it is formed in *Torulopsis utilis*. The source of the labelling in the isoleucine is thought to be α-oxobutyrate, derived from oxaloacetate. Acetate would be incorporated into oxaloacetate by means of the citric acid cycle, but net production of oxaloacetate can only arise from other processes, such as the carboxylation of pyruvate. Pyruvate would be unlabelled, so also would the carbon dioxide arising from it during acetate formation. Some labelled carbon dioxide would arise during the citric acid-cycle reactions, and this, together with the operation of the cycle itself, could give rise to the labelling of the carboxyl-carbon atoms of oxaloacetate. It is clear, however, that this oxaloacetate would be expected to be very much less heavily labelled than the acetate, and this is in accord with the 20:1 ratio of labelling between C-2 and C-6 on the one hand, and C-4 and C-10 on the other.

This tentative biosynthetic path is outlined in Fig. 2.

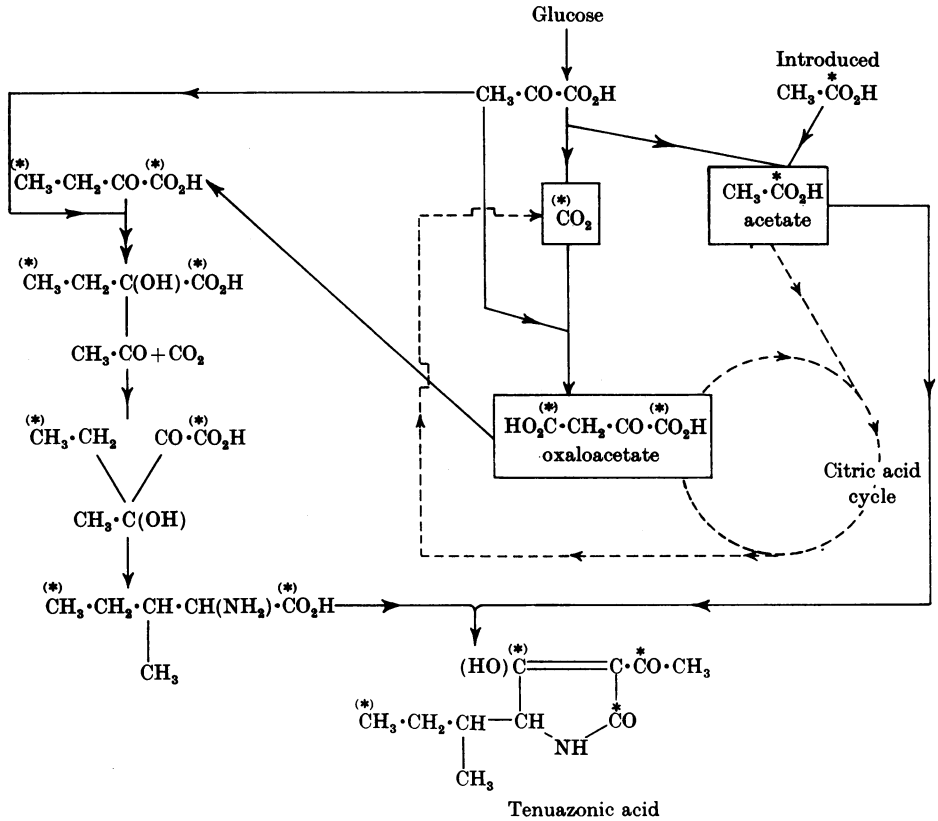

Fig. 2. Proposed biosynthetic scheme for tenuazonic acid, showing the labelling derived from [1-^{14}C]acetate. The intermediates in the biosynthesis of isoleucine are some of those proposed by Strassman *et al.* (1956). *, Major labelling; (*), minor labelling; ——→, main biosynthetic path; - - - -→, source of minor labelling.

The present work affords no proof that the molecule of isoleucine is incorporated as a whole into tenuazonic acid; it may be that a precursor of isoleucine, rather than isoleucine itself, condenses with acetate or acetoacetate. If isoleucine is a direct precursor, the remaining stages could be via acetoacetylisoleucine, or deacetyltenuazonic acid (5-*sec.*-butyltetramic acid).

Lybing & Reio (1958) have made a similar study of the biosynthesis of carolic acid and carlosic acid by *Penicillium charlesii* G. Smith. These acids (II and III) are closely related structurally to tenuazonic acid, as are other substituted tetronic acids isolated from *P. charlesii*, *P. terrestre* Jensen, *P. cinerascens* Biourge and *P. viridicatum* Westling (Clutterbuck, Haworth, Raistrick, Smith & Stacey, 1934; Clutterbuck, Raistrick & Reuter, 1935; Birkinshaw & Raistrick, 1936; Bracken & Raistrick, 1947; Birkinshaw & Samant, 1960). Lybing & Reio (1958) found that radioactivity from [1-^{14}C]-acetate was incorporated mainly as shown in Fig. 3. The results are not as clear-cut as those we have found for tenuazonic acid, but the general picture is very similar. The degradation procedure was such as to make possible an equilibration in

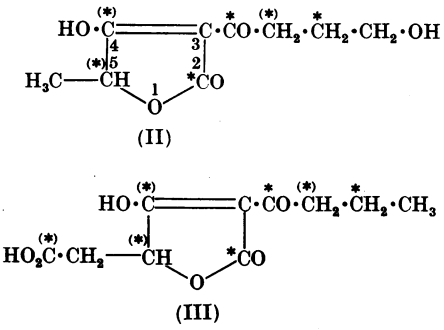

Fig. 3. Labelling pattern in carolic acid (II) and carlosic acid (III) from *P. charlesii* grown on modified Czapek–Dox medium with the addition of tracer [1-^{14}C]acetate. *, Major labelling; (*), minor labelling [based on Lybing & Reio (1958)].

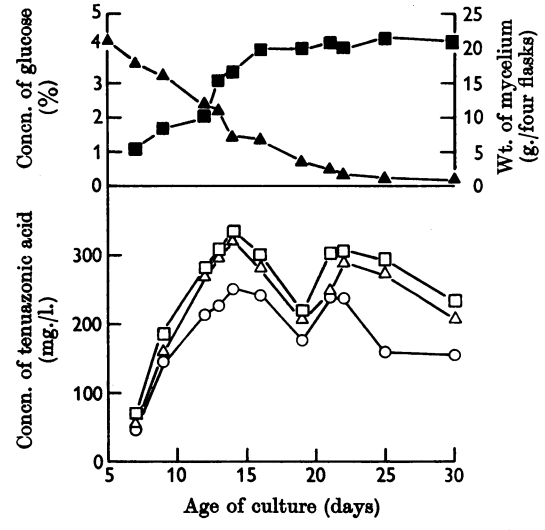

Fig. 4. Concentration of tenuazonic acid in filtrates from surface cultures of *A. tenuis*, on Czapek–Dox medium. Measurements were made on combined samples from four flasks. Calculated from: □, wt. of light petroleum extract; △, titration value of light petroleum extract; ○, wt. of copper tenuazonate isolated. ▲, Glucose (%), calculated from optical rotation; ■, total wt. of dried mycelium from four flasks.

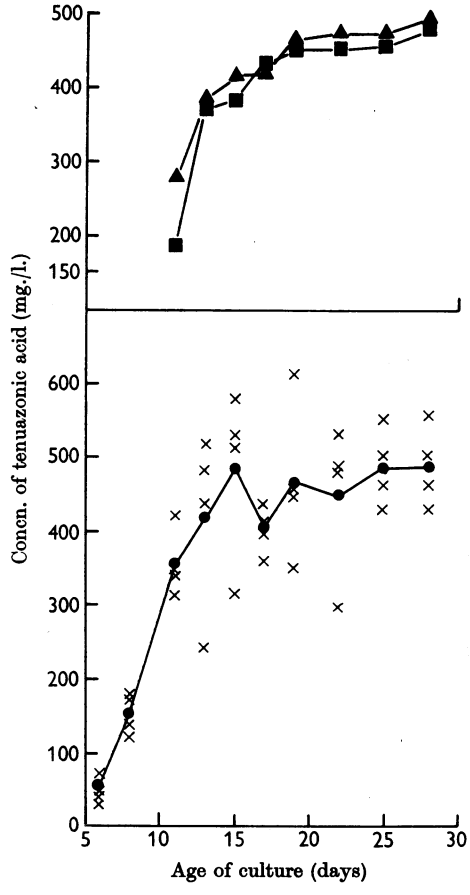

Fig. 5. Concentration of tenuazonic acid in filtrates from surface cultures of *A. tenuis*, calculated from *E* at 280 mμ. Four flasks were harvested on each day indicated: ×, separate values for the four flasks; ●, mean values. ■, Serial samples from one flask, taken with minimum disturbance of culture; ▲, serial samples from one flask, taken after mixing to ensure a representative sample. Curves for ■ and ▲ are displaced vertically to avoid confusion.

apparent activities at C-4 and C-5. Assuming that this originated mainly from C-4, the authors tentatively advance the theory that the tetronic acids arise by condensation of β-oxohexanoyl-coenzyme A and a C_4 compound of the citric acid cycle. The activity of C-4 and the carboxyl-carbon atom varied between one-quarter and one-sixth of that of the most active carbon atoms, marked with asterisks (a much higher fraction than the one-twentieth observed in tenuazonic acid). This could be due to greater citric acid-cycle action in *P. charlesii*, resulting in enhanced radioactivity in the carboxyl-carbon atoms of the C_4 acids.

Growth curves

The growth of the mould and production of tenuazonic acid were studied in five separate batches of culture flasks, by methods based on extraction procedures and u.v. absorption, which are described in detail in the Experimental section. Results for three batches are shown in Figs. 4–6. The u.v.-absorption figures gave higher values than those from the extraction procedure, but the shape of the curves was in general the same during the first 20 days.

Despite considerable variations between flasks (Fig. 5), there was in all five batches an initial rapid rise in mean concentration of tenuazonic acid to a maximum, followed by a drop to a minimum. Thereafter the behaviour was less uniform, but a fairly steady level was maintained. However, it was not possible to confirm this picture in single flasks, presumably due to the difficulty of obtaining a representative sample without disturbing the culture. The decrease in tenuazonic acid content after the initial rapid production period indicates that the metabolite can be utilized by the organism, at a time when the total glucose concentration is still quite high. On the other hand, there is no indication of substantial utilization of tenuazonic acid after most of the glucose is exhausted: thus in the first batch, the concentration of tenuazonic acid was practically unchanged from the twenty-seventh to the forty-fifth days.

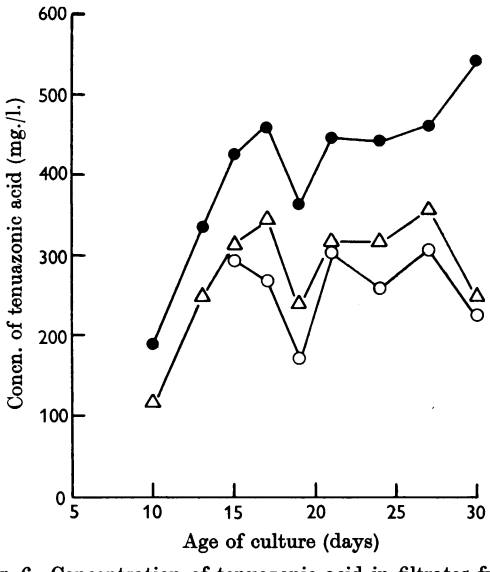

Fig. 6. Concentration of tenuazonic acid in filtrates from surface cultures of *A. tenuis*: comparison of methods of estimation. Four flasks were harvested on each day indicated. Calculated from: ●, mean value of *E* at 280 mμ; △, titration value of light petroleum extract; ○, wt. of copper tenuazonate isolated.

EXPERIMENTAL

Culture. The strain of *Alternaria tenuis* used, no. G.A. 628, has already been described (Rosett *et al.* 1957), together with details of cultural methods and of the isolation of tenuazonic acid. The mould was grown in the same way at 24° in surface culture in 1 l. conical flasks, each containing 350 ml. of Czapek–Dox medium.

Growth and production curves. (*a*) Extraction method. In order to determine the period of maximum tenuazonic acid production for the ^{14}C-incorporation experiments, a batch of flasks was sown and groups of four representative flasks were harvested at intervals. The following determinations were made on the combined filtrates on each occasion.

(1) Glucose concentration, determined approximately by optical rotation. (2) Mycelial weight (dry). (3) The culture filtrate was acidified to pH 2·0 and extracted by hand with light petroleum (b.p. 40–60°) until the extract no longer gave the typical orange–red ferric colour. The residue after evaporation of the solvent gave the weight of crude tenuazonic acid. (4) This residue was titrated with 0·1 N-NaOH (to phenolphthalein); the titration value represents tenuazonic acid plus acidic impurities. (5) The titration solution was extracted with chloroform several times (this extract was rejected), then treated with slightly more than 1 equiv. of 0·1 N-copper acetate and extracted again with chloroform until the extract no longer gave a ferric colour. The chloroform solution was evaporated and the residue dried at 100° (16 mm. Hg) and weighed. It contained all the tenuazonic acid as nearly pure copper salt.

The results were substantially confirmed when two further batches were followed by the same method.

Results for batch 3 are shown in Fig. 4.

(*b*) U.v. methods. The u.v.-absorption curve of the diluted culture filtrate was measured at various intervals. It was closely similar to that of pure tenuazonic acid, with interference from the medium below 250 mμ in the earlier stages of metabolism. The maximum at 279–280 mμ and the minimum at 255 mμ were observable at all times; from about the thirteenth day onwards, the second maximum at 240 mμ was also observed. The extinction at 280 mμ was therefore taken as a measure of the concentration of tenuazonic acid. Beer's law was checked by observing a series of dilutions of a sample of metabolism solution: dilutions of 1/21, 1/41, 1/61, 1/81, 1/101 gave the following values of *E* for the undiluted solution: 33·6, 33·5, 33·6, 32·8, 33·2.

The extinction (at 280 mμ) of appropriately diluted 1 ml. samples was measured in two further batches of culture flasks (nos. 4 and 5). Four representative flasks were again harvested at intervals. *E* (280 mμ) and glucose by optical rotation were measured on each flask. The values of *E* and their means, for batch 5, are shown in Fig. 5. There was no obvious correlation between values of *E* and glucose levels and these are not shown. With batch 4, portions (250 or 125 ml.) from each flask were combined and the extraction procedure described above was carried out to compare the two methods of estimation. The results are shown in Fig. 6.

In view of the higher figures obtained by the u.v. method, especially during the later stages of metabolism, the absorption curve of the extracted culture filtrate was measured. At 27 and 30 days, this gave general absorption over the range 230–290 mμ with a flat maximum at 260 mμ. The absorption of this residual material accounted for about 15% of the total absorption of the culture filtrate at 280 mμ.

Batches 4 and 5 also each included two flasks equipped with side arms and taps for sampling. In one of each pair the side arm extended to the middle of the solution, in the hope that a representative sample could be withdrawn without disturbing the cultures. The other flask was rotated gently to mix the solution before the sample was taken. Samples (1 ml.) were withdrawn from these flasks on the same days as the four-flask samples were taken, and *E* (280 mμ) was measured. The values for batch 5 are shown in Fig. 5; the curves for the single flasks in batch 4, and for a number of other single flasks, were very similar, and showed no distinct peaks.

The method of sowing used for batches 4 and 5 was modified to ensure sowing exclusively with spores, rather than with spores and mycelium. This resulted in a slower start in growth and probably accounts for the delayed maximum as compared with batches 1–3.

Radioactivity measurements. Substances were counted (usually for $3 \times 10\,000$ counts) at 'infinite thickness' on 1·5 cm.-diam. planchets with an end-window Geiger–Müller tube. Accuracy was ±3% for 10 000 counts. Background count (5–13 counts/min.) was subtracted in all cases, but no corrections were made for back-scattering. Three different tubes were used during the work, with standard counts of about 720, 910 and 1130 counts/min., with a 2·5 cm.-diam. disk of polymethylmethacrylate of nominal activity 1 μc/g. Results are accordingly calculated as μc/mole.

Incorporation of [1-^{14}C]*acetate into tenuazonic acid.* Sodium [1-^{14}C]acetate (50 μc; about 0·2 mg. in 1 ml. of sterile water) was added to each of two flasks of batch 2 on the sixth day after inoculation. The flasks were harvested and extracted on the thirteenth day as described above, and the crude copper salt (437 mg.) was recrystallized from aqueous methanol; $[\alpha]_{20}^{5461} - 122 \pm 1°$ (*c*, 0·2 in methanol); 4538 counts/min. ≡ 8980 μc/mole (yield 257 mg.; incorporation of tracer 4·5%).

In a repeat experiment, sodium [1-^{14}C]acetate (100 μc) was added to each of two flasks; yield, 471 mg. of purified copper salt, 10 440 μc/mole.

The copper salt was diluted with inactive material for the degradation experiments.

Complete degradation of tenuazonic acid

The scheme of degradation used is shown in Fig. 1, and the radioactivity measurements are given in Table 1. Each carbon atom was obtained as CO_2, which was converted into $BaCO_3$ for assay.

Hydrolysis. In a typical experiment, copper tenuazonate trihydrate (938 mg.) was converted into the free acid and hydrolysed as described by Stickings (1959), with 2N-H_2SO_4 (300 ml.) instead of HCl and omission of the Brady's reagent bubbler. The barium carbonate was filtered and dried (649 mg.). The acetic acid was degraded by the method of Popják (1955).

Reaction of 3-amino-4-methylhexan-2-one with alkaline hypoiodite and ninhydrin degradation of the resulting isoleucine. The hydrolysis reaction mixture was neutralized (NaOH), treated with $BaCl_2$ to remove SO_4^{2-} ions and evaporated under reduced pressure to a small volume. It was then treated with 0·1N-iodine solution (150 ml.) and 10N-NaOH (20 ml.) at 0°. Iodoform was immediately precipitated in good yield and was centrifuged and oxidized to CO_2 (Popják, 1955). The supernatant was acidified and the excess of iodine destroyed by titration with 0·1N-sodium thiosulphate. It was then neutralized, evaporated and made to a volume of 250 ml. with water. A drop of this solution was chromatographed on paper as described by Stickings (1959) and showed the presence of isoleucine. The solution was boiled with ninhydrin (1·22 g.) in a stream of N_2, and yielded 2-methylbutanal, isolated as its 2:4-dinitrophenylhydrazone, m.p. 136° (671 mg.). Further confirmation of the stability of isoleucine to alkaline hypoiodite was obtained by boiling an aqueous solution of the two substances for 1 hr., after which isoleucine could still be shown chromatographically to be present at about the same concentration.

Kuhn–Roth oxidation of 2-methylbutanal 2:4-dinitrophenylhydrazone. 2-Methylbutanal 2:4-dinitrophenylhydrazone (254 mg.) was heated for 4 hr. with a chromic acid mixture [CrO_3 (12 g.), $K_2Cr_2O_7$ (3 g.), water (30 ml.) and H_3PO_4 (20 ml.)]. The volatile acid was titrated (Found: 18·85 ml. of 0·1N-NaOH. Calc. for $2CH_3 \cdot CO_2H$: 19·08 ml.). The sodium acetate was degraded by the Schmidt reaction to CO_2 and methylamine, which was oxidized with permanganate to CO_2 (Phares, 1951).

Stepwise degradation of 2-methylbutanal 2:4-dinitrophenylhydrazone. The method followed closely that given by Strassman *et al.* (1956), with the conditions for the Schmidt reaction described by Phares (1951). Yields of $BaCO_3$ (from 245 mg. of 2-methylbutanal 2:4-dinitrophenylhydrazone) were: from C-5, 179 mg.; from C-11, 172 mg.; from C-8, 190 mg.; from C-9, 164 mg.; from C-10, 120 mg.

SUMMARY

1. A strain of *Alternaria tenuis*, growing on Czapek–Dox medium, has been shown to incorporate over 4% of the ^{14}C from added sodium [1-^{14}C]-acetate into tenuazonic acid.

2. Over 94% of the radioactivity is shared equally between the lactam and side-chain carbonyl groups; the remainder is shared equally between C-4 of the ring and the ω-carbon atom of the *sec.*-butyl side chain.

3. It is suggested that tenuazonic acid is synthesized from two molecules of acetate and a molecule of L-isoleucine.

4. The relationship of this pathway to the biosynthesis of fungal tetronic acids is discussed.

5. During growth of *A. tenuis* in undisturbed surface culture, the concentration of tenuazonic acid increases rapidly during the second week, then decreases to a minimum and rises again.

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Tissue-Specific and Species-Specific Esterases

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Although electrophoresis has been used extensively to study the protein components of biological fluids, few attempts have been made to investigate soluble proteins from solid tissues by this means, mainly because classical techniques give inadequate resolution of complex mixtures. Zone electrophoresis in starch gels (Smithies, 1955) permits sharper separation and greater resolution, and in the modification introduced by Hunter & Markert (1957) can also be used for specific proteins by applying methods derived from histochemistry. For instance, by applying the method of Nachlas & Seligman (1949) these authors demonstrated several bands with esterase activity in starch-gel-electrophoresis diagrams of aqueous extracts of mouse tissues.

Electrophoresis of serum proteins on starch gels has revealed differences between species (Latner & Zaki, 1957) and even among individuals of the same species (Ashton, 1957; Smithies, 1955, 1959). By other techniques Augustinsson (1958) has demonstrated that there are considerable differences in the serum esterases of different species.

In this investigation starch-gel electrophoresis was used to compare the tissue esterases of a variety of organs in several species, in order to investigate the amount of variation among proteins with the same enzyme activity. Typical patterns were found for each tissue, and their constancy was demonstrated by their persistence in mammalian cell strains, which had been cultivated *in vitro* for long periods.

MATERIALS AND METHODS

Tissues. Animals were killed by exsanguination after stunning or after anaesthetizing with ether. The organs were immediately removed and treated as described below. Human tissues were obtained from the post-mortem room, washed thoroughly with 0·15M-NaCl immediately after removal, and frozen at −10° until required. Cell strains were grown as monolayers on glass by standard techniques (Paul, 1959). They were washed *in situ* with 'balanced salt solution' (Hanks & Wallace, 1949) and harvested by scraping from the glass with a rubber 'policeman' before the preparation of extracts. Except for the strains described below, cells were derived from recently disaggregated tissues. Cell strains used were the mouse subcutaneous (fibroblast) strain L, clone 929 (Sanford, Earle & Likely, 1948), the human cervical carcinoma cell, strain HeLa (Gey, Coffman & Kubicek, 1952), the human epithelioma cell, strain HeP 1 (Toolan, 1954) and the human foetal liver (parenchymal) cell, strain HLM (Leslie, Fulton & Sinclair, 1956).

Preparation of extracts. The method of preparation of tissue extracts was found to affect the pattern obtained.