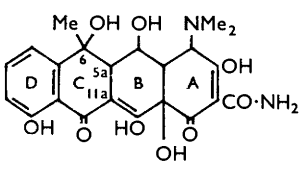
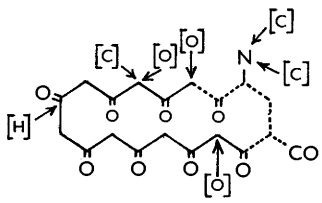
80. *Studies in Relation to Biosynthesis. Part XXVIII.* Oxytetracycline (Terramycin).*

By A. J. Birch, J. F. Snell, and (Mrs.) P. J. Thomson.

Degradation of oxytetracycline biosynthesised in the presence of $^{14}CH_3 \cdot CO_2H$ and of [Me-^{14}C]methionine suggests that the molecule contains *C*-Me and NMe_2 derived from the latter source, and that the main skeleton is largely, but not entirely, built up by the head-to-tail linkage of acetic acid units.

Inspection of the formulæ of tetracycline derivatives, *e.g.*, oxytetracycline (Terramycin) (I), reveals a typical oxygenation pattern for a compound derived from acetic acid.[1,2] There are clearly involved modifications of the main scheme, as indicated in (II), including the removal and addition of oxygen atoms and the introduction of C_1 units, types of reaction which have already been discussed.[3] The first experimental support for an origin of this type involved the incorporation of isotopically labelled acetic acid and partial degradation of the product by Snell *et al.*[4]





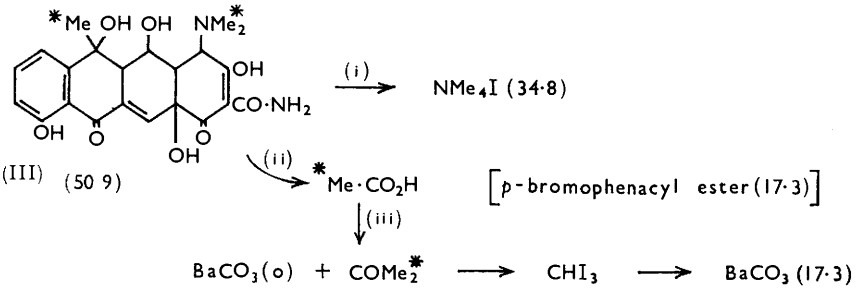
The general scheme is supported by the occurrence of derivatives lacking the 6-methyl

* Part XXVII, preceding paper. For a preliminary report of Part XXVIII see Snell, Birch, and Thomson, *J. Amer. Chem. Soc.*, 1960, **82**, 2402.

[1] Birch and Donovan, *Austral. J. Chem.*, 1953, **6**, 360.
[2] Robinson, "Structural Relations of Natural Products," Oxford, 1955.
[3] Birch, *Fortschr. Chem. org. Naturstoffe*, 1957, **14**, 186.
[4] Snell, Wagner, and Hochstein, Proc. Internat. Conf. Peaceful uses of Atomic Energy, 1955, **12**, 431; cf. also Miller, McCormick, and Doerschuk, *Science*, 1956, **123**, 1030.

group, the biochemical *C*-methylation apparently being omitted,[5] and of a 5a,11a-dehydro-derivative [6] as a normal precursor, the double bond being the remnant of an aldol ring-closure which produces the junction of rings B and C.

We now report more detailed studies on the degradation of oxytetracycline produced on media containing either $^{14}CH_3 \cdot CO_2H$ or [Me-^{14}C]methionine. The degradations have been previously described.[7]



(i) MeI ; (ii) CrO_3 ; (iii) pyrolysis of Li salt.

Incorporation of [Me-^{14}C]*Methionine.*—The expected labelling pattern is shown in formula (III) [numerical values are relative molar activities $\times 10^{-3}$ (r.m.a.)], the activities being expressed throughout in our usual manner.[8] Our previous work indicates that little activity from the C_1 pool is incorporated into acetic acid-derived molecules. The results of the degradations above are quantitatively in agreement with theory. The only major labelling is found on the three methyl groups and apparently to the same extent on each.

Incorporation of [Me-^{14}C]*Acetic Acid.*—The expected pattern of incorporation of labelled atoms from [Me-^{14}C]acetic acid into a molecule of which the main skeleton of rings B, C, and D is wholly derived from acetic acid (apart from the introduced methyl groups) is shown in formula (IV). The value of label " c " will depend on the extent of randomisation of the label in the acetic acid due to involvement in the tricarboxylic acid cycle. Normally, in moulds, this is not more than 5—10% according to our experience. The extent of the labelling " a " will depend on the extent of conversion of acetic acid into labelled C_1 units, and this in our experience is usually rather small, although not negligible. The degradations shown (with some intermediate stages omitted) were carried out in order to determine the labelling pattern (relative molar activities shown).

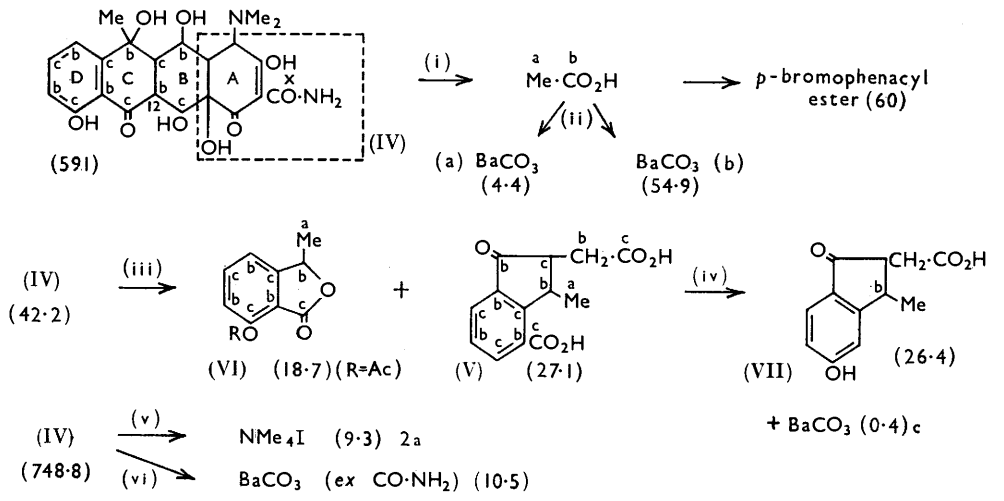
The results are set out in Table 1. For comparison all the relative molar activities ($\times 10^{-3}$) have been converted to a standard dilution (ii).

The following equations connect the various r.m.a. contributions:

Oxytetracycline (IV), 3a + 6b + 6c + x = 42·2
Terracinoic acid (V), a + 6b + 6c = 27·1 (27·3)
Acetoxymethylphthalide (VI; R = Ac), a + 4b + 4c = 18·7 (18·3)
Tetramethylammonium iodide, 2a = 0·5 (0·6)
Acetic acid (Kuhn–Roth), a + b = 4·3 (4·4)
$BaCO_3$ from $C_{(6)}$, b = 4·0 (4·1)
$BaCO_3$ from *C*–Me, a = 0·3 (0·3)
$BaCO_3$ from $CO \cdot NH_2$, b? = 0·6

[5] Webb, Broschard, Cosslich, Stein, and Wolf, *J. Amer. Chem. Soc.*, 1957, **70**, 4564.
[6] McCormick, Sjolander, Miller, Hirsch, Arnold, and Doerschuk, *J. Amer. Chem. Soc.*, 1958, **80**, 6460.
[7] Hochstein, Stephens, Conover, Regna, Pasternack, Gordon, Pilgrim, Brunings, and Woodward, *J. Amer. Chem. Soc.*, 1953, **75**, 5455.
[8] Birch, Massy-Westropp, Rickards, and Smith, *Proc. Chem. Soc.*, 1957, 98; *J.*, 1958, **360**.

The results from various combinations of these values are self-consistent; the r.m.a. contributions which give the best fit are a = 0·3, b = 4·1, c = 0·4. The values calculated on this basis are shown in parentheses above.



(i) CrO_3; (ii) pyrolysis of Li salt; (iii) alkaline hydrolysis; (iv) heat; (v) MeI; (vi) acid hydrolysis.

The r.m.a. of the carbamoyl group (0·6) we regard as being probably outside the experimental error for c = 0·4; this group may, therefore, not represent a "randomised" acetic acid carboxyl group. It is, however, possible that the carbon dioxide from this group is contaminated with more highly active substance from a more profound breakdown

TABLE 1.

Compound	Dilution (i)	Dilution (ii)	Dilution (iii)	Conversion to (ii)
Oxytetracycline	591	42·2 (441 *)	748·8 *	42·2
Terracinoic acid (V)		27·1		27·1
7-Acetoxy-3-methylphthalide (VI)		18·7		18·7
Decarboxyterracinoic acid (VII)		26·4		26·4
$BaCO_3$ from C-11 (c)		0·4 (3·3 *)		0·3
Acetic acid (C-6 + *C*-Me)	60·0			4·3
$BaCO_3$ from $C_{(6)}$ (b)	54·9			4·0
$BaCO_3$ from *C*-Me (a)	4·4			0·3
NMe_4I (2a)			9·3 *	0·5
$BaCO_3$ from $CO{\cdot}NH_2$			10·5 *	0·6

* Measured on a proportional counter which had a greater sensitivity than the counter used for the other results.

of the molecule. Calculated on the basis of the r.m.a. figures above, x = 14·3, which is close to the value of 13·5 calculated from 3b + 3c; *i.e.*, an origin of ring A from three acetic acid units is possible. It is unfortunate that no degradations are available which give fragments containing ring A in other than minute yields. Earlier results [4] are not so consistent with this origin of ring A: these are given in Table 2, the r.m.a. being in this case decompositions per minute per micromole. From these figures, a = 0·65, and b + c (*i.e.*, a complete incorporated acetic acid unit) has the value 8·56 (calculated from terracinoic acid) and 8·34 (calculated from hydroxymethylphthalide). However, b + c calculated from the r.m.a. of the succinic acid is 7·5, if this substance is in fact entirely derived as postulated [4] from the 5,5a,11a,12-carbon atoms. The r.m.a. contribution of

TABLE 2.

Compound	R.m.a.	Compound	R.m.a.
Oxytetracycline	73	Succinic anhydride	15
Terracinoic acid	52	7-Hydroxy-3-methylphthalide,H_2O	34
Succinic acid (from alkali-fusion) ...	15	Dimethylamine,HCl	1·3

the same carbon atoms should be given by the r.m.a. of terracinoic acid minus that of hydroxymethylphthalide, which gives a value of 17, in agreement with the value 8·5 for b + c above. It is likely, therefore, that the succinic acid produced by alkali fusion does not arise entirely from the postulated source.

If b + c = 8·5, then x = 20, which is decidely low although of the correct order of magnitude to afford 3b + 3c = 25·5 which is to be expected for complete acetate origin. It is probable, therefore, that ring A has a rather lower activity than the rest of the molecule, and incorporation of the acetic acid may proceed by a different route. Other work[9] shows that in fact it is probably derived from glutamic acid.

EXPERIMENTAL

Radioactive Assay.—Specimens were mainly assayed for radioactivity with an end-window counter as infinitely thick solid samples of 1 cm.^{2} or, in the case of the dilution (ii), of 0·3 cm.^{2} cross-sectional area. Counting rates were corrected for background and dead time of instrument; the counts per 100 sec were determined by recording the time required for 10^4 counts to be aggregated. Hence the statistical counting error is not greater than 3%. Specimens of low activity were counted with a methane-flow proportional counter of greater sensitivity and related to the other specimens by the ratio of the counts for Terramycin under the different conditions. The results are given as the relative molar activities, *i.e.*, counts per 100 sec. × molecular weight. Since these figures are relative they have been multiplied by 10^{-3} to avoid large numbers. All compounds were crystallised to constant activity.

Counting equipment consisted of an EKCO automatic scaler of type N350D, in conjunction with an EKCO probe unit of type N558 and an EHM2S Geiger tube for the end-window counter, or with an EKCO power unit N570, A.E.R.E continuous proportional flow counter of type 1364A and an EKCO amplifier unit of type N568B, for the proportional counter.

Degradation of Oxytetracycline derived from [Me-^{14}C]*Methionine.*—The active oxytetracycline, derived from [Me-^{14}C]methionine feeding experiments, was diluted with 5 parts of inactive material and recrystallised from aqueous *NN*-dimethylformamide (Found: r.m.a. 50·9).

(a) *Tetramethylammonium iodide.* A mixture of anhydrous oxytetracycline (460 mg.) and redistilled methyl iodide (2 ml.) in acetone (20 ml.) was warmed until a clear solution was obtained and then left at room temperature for 48 hr. The precipitate (65 mg.) was collected and recrystallised from aqueous alcohol (Found: r.m.a. 34·8. Calc. for 2C: r.m.a. 34·0).

(b) *Kuhn–Roth oxidation.* Kuhn–Roth oxidation of oxytetracycline (992 mg.) gave acetic acid (107 mg., 89%), isolated by titration of the steam-distillate with 0·1N-lithium hydroxide. A small proportion of the acetic acid was converted into the *p*-bromophenacyl ester (Found: r.m.a. 17·3. Calc. for 1C: r.m.a. 17·0) and the remainder pyrolysed[10] to yield barium carbonate derived from the carboxyl group of the acetic acid (Found: r.m.a. 0) and acetone. The acetone was converted into iodoform, which was then oxidised to give barium carbonate (Found: r.m.a. 17·3. Calc. for 1C: r.m.a. 17·0).

Degradation of Oxytetracycline derived from $^{14}CH_3{\cdot}CO_2H$.—The radioactive material was diluted with inactive oxytetracycline in three different proportions.

Dilution (i). Radioactive tetracycline (1·1 g.) was mixed with inactive material (1·0 g.) and recrystallised from aqueous dimethylformamide (Found: r.m.a. 591).

Kuhn–Roth oxidation and further reactions were carried out as described previously, to give *p*-bromophenacyl acetate (Found: r.m.a. 60·0) and barium carbonate, derived from the methyl group (Found: r.m.a. 4·4) and from the carboxyl group of acetic acid (Found: r.m.a. 54·9).

Dilution (ii). Radioactive oxytetracycline (1 g.) was mixed with inactive material (8 g.) and recrystallised from aqueous dimethylformamide (Found: r.m.a. 42·2).

[9] Snell, unpublished work.
[10] Cornforth, Hunter, and Popjak, *Biochem. J.*, 1953, **54**, 597.

(*a*) This oxytetracycline (20 g.) was treated with hot sodium hydroxide solution and granulated zinc as described by Hochstein *et al.*[7] The terracinoic acid (4·2 g.) recrystallised from acetone–light petroleum as needles, m. p. 231—232° (Found: r.m.a. 27·1). The 7-hydroxy-3-methylphthalide was sublimed at 140°/0·1 mm., to give a nearly colourless white solid on exposure to air (105 mg.). This was converted into the acetate which recrystallised from benzene–ligroin as needles, m. p. 89—90° (Found: r.m.a. 18·7).

(*b*) Terracinoic acid (264 mg.) was refluxed in 85% phosphoric acid (4 ml.) under nitrogen for 4 hr., and the issuing gases were passed through saturated barium hydroxide solution contained in 15 ml. centrifuge tubes. The dark solution was diluted with water and extracted with ether, and the residue on evaporation of the ether was recrystallised from water to give colourless prisms, m. p. 167—168° (Found: r.m.a. 26·4). The barium carbonate was collected by centrifugation, washed with hot water, and dried (155 mg.) (Found: r.m.a. 0·4). Because the activity was so low, the material was recounted by means of a methane-flow proportional counter with a 1 cm. planchette and a proportionality factor calculated from the relative molar activity of oxytetracycline measured under these conditions (Found: oxytetracycline, r.m.a. 441; barium carbonate, r.m.a. 3·3).

Dilution (iii).—The remaining samples of this radioactive oxytetracycline were recrystallised from aqueous *NN*-dimethylformamide (Found: r.m.a. 748·8).

(*a*) Tetramethylammonium iodide was prepared from the anhydrous oxytetracycline (460 mg.) as described above (Found: r.m.a. 9·3).

(*b*) Oxytetracycline (248 mg.) was heated with 12N-sulphuric acid (30 ml.) under nitrogen at 95° for 3 days. The effluent gases were washed with water and passed into barium hydroxide solution. After 3 days, little more carbon dioxide was evolved. The barium carbonate (85 mg.) was collected by centrifugation, washed with hot water, and dried (Found: r.m.a. 10·5).

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