SUMMARY

1. On the basis of its action on a number of conjugated hexuronic acids and other compounds, it is concluded that mouse-liver glucuronidase is specific for β -D-glucopyranuronides, including β -D-glucopyranuronic acid-1-phosphate.

2. Derivatives of *laevomenthol* caused slight activation of glucuronidase.

3. Mouse-liver preparations did not catalyse the condensation of *o*-aminophenol with α - or β -glucuronic acid-1-phosphate.

The authors are indebted for various materials to the donors mentioned in the text, and to Dr P. G. Walker for assistance with many of the experiments on glucuronide synthesis.

REFERENCES

- Barker, S. A., Bourne, E. J. & Stacey, M. (1951). *Chem. & Ind.* p. 970.
 Dutton, G. J. & Storey, I. D. E. (1951). *Biochem. J.* **48**, xxix.
 Fernandez-Garcia, R., Amoros, L., Blay, H., Santiago, E., Soltero-Diaz, H. & Colon, A. A. (1950). *El Crisol, Puerto Rico*, **4**, 40. Cited in *Chem. Abstr.* (1951), **45**, 555.
 Graham, A. F. (1946). *Biochem. J.* **40**, 603.
 Karunairatnam, M. C. (1950). Ph.D. Thesis, Edinburgh.
 Karunairatnam, M. C., Kerr, L. M. H. & Levvy, G. A. (1949). *Biochem. J.* **45**, 496.
 Karunairatnam, M. C. & Levvy, G. A. (1949). *Biochem. J.* **44**, 599.
 Kerr, L. M. H., Campbell, J. G. & Levvy, G. A. (1949). *Biochem. J.* **44**, 487.
 Kerr, L. M. H., Graham, A. F. & Levvy, G. A. (1948). *Biochem. J.* **42**, 191.
 Levvy, G. A. (1946). *Biochem. J.* **40**, 396.
 Levvy, G. A. (1952). *Biochem. J.* **52**, 464.
 Levvy, G. A. & Storey, I. D. E. (1949). *Biochem. J.* **44**, 295.
 Marsh, C. A. (1952). *J. chem. Soc.* p. 1578.
 Masamune, H. (1934). *J. Biochem., Tokyo*, **19**, 353.
 Mehlretter, C. L., Alexander, B. H., Mellies, R. L. & Rist, C. F. (1951). *J. Amer. chem. Soc.* **73**, 2424.
 Meyer, K., Linker, A. & Rapport, M. M. (1951). *J. biol. Chem.* **192**, 275.
 Smith, F. (1951). *J. chem. Soc.* p. 2646.
 Storey, I. D. E. (1950). *Biochem. J.* **47**, 212.
 Talalay, P., Fishman, W. H. & Huggins, C. (1946). *J. biol. Chem.* **166**, 757.
 Walker, P. G. & Levvy, G. A. (1951). *Biochem. J.* **49**, 620.
 Yackel, E. C. & Kenyon, W. O. (1942). *J. Amer. chem. Soc.* **64**, 121.

The Mechanism of the Action of Uricase

BY RONALD BENTLEY* AND A. NEUBERGER

National Institute for Medical Research, Mill Hill, London, N.W. 7

(Received 23 May 1952)

The enzyme uricase was considered formerly to oxidize uric acid with quantitative formation of allantoin, carbon dioxide and hydrogen peroxide (Wiechowski, 1907; Batelli & Stern, 1909; Keilin & Hartree, 1936a; Holmberg, 1939; Davidson, 1942), as shown in the following equation:

However, it was shown by Felix, Scheel & Schuler (1929) that under some conditions oxygen uptake was more rapid than carbon dioxide production, and Schuler (1932) found that at pH 8.9 the yield of carbon dioxide was only 17.8% of that expected from the oxygen consumption. These findings suggested that the first product of the

enzymic oxidation was not allantoin, and the pH changes during oxidation indicated that the primary product was a substance of greater acid strength than uric acid. Indications of the structure of this first product were obtained in experiments in which uric acid was oxidized with permanganate. Fischer &

Ach (1899) had found that oxidation of 1-methyl- and of 7-methyl-uric acid yields the same product, 3-methylallantoin, whilst 3-methyl- and 9-methyl-uric acid give 1-methylallantoin. Behrend (1904) pointed out that these findings can be explained by assuming that the first product of oxidation of uric acid by permanganate is a symmetrical compound, and he suggested that it possessed the structure I. This substance which Behrend was not able to isolate was named hydroxyacetylene diurein

* Present address: Department of Biochemistry, Columbia University, College of Physicians and Surgeons, New York.

carboxylic acid (HDC).^{*} Later, Schuler & Reindel (1932) obtained HDC, in the form of a trisilver salt, by chemical oxidation of uric acid; these authors also observed that the free acid was unstable and was rapidly converted to allantoin and carbon dioxide. Finally, Schuler & Reindel (1933) succeeded in obtaining a silver salt with similar properties by oxidation of uric acid by uricase. It was concluded that the primary product of enzymic oxidation of uric acid was the symmetrical HDC (I).

Recently, the nature of the primary product of the enzymic oxidation was reinvestigated by Klemperer (1945) and by Praetorius (1948). Klemperer confirmed the earlier observations that with many buffer systems carbon dioxide production was smaller than calculated from the rate of disappearance of uric acid. From the amounts of nitrogen liberated under different conditions on treatment with nitrous acid, he concluded that the primary product is an unknown and unstable substance which gives rise to either HDC (I), uroxic acid (II) or allantoin. Praetorius (1948) measured the changes in absorption of light which occurred during the oxidation of uric acid by uricase, using different buffer systems. He concluded that the primary, very unstable, product has maximum absorption in the long-wave ultraviolet (300–330 m μ). This first product could then be converted either to a second product with a maximum of absorption at about 260 m μ , giving rise to allantoin or be transformed to HDC and further to uroxic acid. Further evidence for the existence of a symmetrical intermediate in the chemical oxidation of uric acid and also in the oxidation occurring in the whole animal was provided by the use of ¹⁵N-labelled uric acid (Brown, Roll & Cavalieri, 1947; Cavalieri & Brown, 1948).

In the work reported in this paper, the action of uricase has been studied in solutions containing H₂¹⁸O (gas phase ¹⁸O₂) and with systems in which ¹⁸O₂ was present in the gas phase. Further, [6-¹⁴C]

uric acid has been synthesized and used to study the source of the carbon dioxide evolved in the enzymic reaction.

EXPERIMENTAL

Materials

Uricase. Two preparations were used in these experiments: (a) a preparation made from pig-liver powder as described by Davidson (1942). Purification included dialysis against distilled water, extraction with phosphate buffer, pH 7.4, and solution in 0.1 M-borate, the final purification with (NH₄)₂SO₄ and acetone precipitation being omitted. The enzyme solution was a pale straw-coloured solution and when tested by Davidson's method had a specific activity of 32.8 μ l./ml./min. It was free from catalase activity. (b) A second preparation was obtained through the courtesy of Dr E. Leone, Molteno Institute, Cambridge. This preparation was obtained by a method as yet unpublished and differed from other uricase preparations in not precipitating on dialysis against distilled water. It gave an O₂ uptake with uric acid of 270 μ l./ml./hr.; dry wt., 2.5 mg./ml.

Catalase. This was made from horse red cells by the method of Bonnichsen (1947). The haematin content of the catalase solution was determined according to Keilin & Hartree (1936b). The solutions used were between 10⁻⁷ and 10⁻⁸ M.

Synthesis of [6-¹⁴C] uric acid

The first stages of the preparation of uric acid labelled in the desired position were based on the condensation of ethyl [carboxyl-¹⁴C]cyanoacetate and urea to [4-¹⁴C]6-aminouracil (III) (Traube, 1923) and the conversion of the latter by successive nitrosation and reduction to [4-¹⁴C]5:6-diaminouracil (IV) (Traube, 1900). The experimental procedure actually used was the simplified method of Bogert & Davidson (1933). The diamino compound (IV) was condensed with urea (Johnson & Johns, 1914) to give [6-¹⁴C] uric acid which was further purified by established methods and characterized by elementary analysis, absorption spectrum and by oxidation with uricase. The location of the labelled carbon atom is unambiguous as shown by the reactions employed (the labelled atom is indicated by an asterisk):

[carboxyl-¹⁴C] Bromoacetic acid (162 mg.; 26 μ c.), kindly supplied by Dr J. R. Catch, Radiochemical Centre, Amsterdam, was dissolved in water together with non-isotopic bromoacetic acid (14.34 g.). The solution was filtered and made up to 100 ml. To 95 ml. of this solution was added

^{*} As pointed out to us by Dr R. S. Cahn, HDC should be called 5-hydroxy-3:7-diketo-2:4:6:8-tetra-azabicyclo [3:3:0] octane-1-carboxylic acid.

sufficient 2N-KCN to bring the pH to approx. 8.5. Further KCN was added over a period of 3 hr. to maintain the pH at this value. Altogether 85 ml. 2N-KCN were required. N-HCl (50 ml.) was now added and the solution concentrated to dryness under reduced pressure. The solid was dissolved in ethanol (200 ml.) containing conc. H_2SO_4 (1 ml.) and the solution boiled under reflux for 3 hr. and then concentrated under reduced pressure. The residue was neutralized with 10% (w/v) $\text{Na}_2\text{CO}_3 \cdot 10\text{H}_2\text{O}$. This solution was unfortunately left at a slightly alkaline pH for about 18 hr., partial saponification resulting. However, 1.19 g. ethyl [carboxyl- ^{14}C] cyanoacetate distilling sharply at 97° (16 mm.) were recovered. This was combined with 9.55 g. of redistilled unlabelled cyanoacetate and converted to [4- ^{14}C]5:6-diaminouracil described by Bogert & Davidson (1933). Yield was 8 g.

[4- ^{14}C]5:6-Diaminouracil (4 g.) and urea (4 g.) were intimately mixed by grinding in a mortar and the mixture heated at 160° (bath temperature) for 1 hr. The mixture, which first liquefied and then solidified again, was dissolved in 0.5N-KOH and the filtered solution acidified with conc. HCl. The amorphous and impure uric acid was filtered off and redissolved in 0.5N-piperidine. An excess of solid NH_4Cl was added and ammonium urate filtered off after standing at 2° for 24 hr. The salt was converted to uric acid by digestion with 2N-HCl at 70° . Analysis indicated that this material was still somewhat impure. It was therefore dissolved in conc. H_2SO_4 and the solution filtered through glass wool. On careful addition of water crystalline uric acid slowly precipitated. The material was recrystallized from a large volume of boiling water. (Found: C, 35.4; H, 2.8; N, 33.0. Calc. for $\text{C}_5\text{H}_4\text{O}_6\text{N}_4$: C, 35.7; H, 2.4; N, 33.3%). The ultraviolet absorption was measured on a solution containing 10 mg. uric acid/l. 0.001N-LiOH (pH approx. 11.0) using a Beckman spectrophotometer model DU; ϵ_{max} was 12 300 at 294 m μ . and ϵ_{min} was 2950 at 260 m μ . At pH 2.15 ϵ_{max} was 10 900 at 284 m μ . These values agree well with those given by Stimson & Reuter (1943). On addition of uricase the ultraviolet absorption above 270 m μ . disappeared completely within 5 min. On measuring the oxygen absorption with uricase in a Warburg apparatus theoretical values were obtained. The specific radioactivity of the uric acid was 1.64×10^{-3} $\mu\text{C.}/\text{m-mole}$.

Isotope experiments

Reactions using isotopic oxygen were carried out in a closed system consisting of a 50 and a 25 ml. flask (both with B19 ground-glass joints), connected by a suitable adaptor; this adaptor carried a vacuum stopcock through which the apparatus could be evacuated.

Reaction in $^{18}\text{O}_2$. A sample of the enzyme prepared according to Davidson (1942) (10 ml. in 0.1M-borate, pH 10) was pipetted into the 25 ml. flask; the larger flask contained 0.2M-borate (pH 10; 5 ml.) and a solution of uric acid in LiOH, prepared as described by Davidson (20 ml. containing 0.11 g. uric acid). The ground joints and stopcock were well greased and the contents of the flasks frozen in an ethanol- CO_2 bath. The system was evacuated, the stopcock closed, and the solutions allowed to thaw and de-gas. After freezing again, evacuation was completed. The reaction system was attached to an electrolysis apparatus (Bentley, 1949) in which $^{18}\text{O}_2$ was generated. The liquids were kept frozen during the electrolysis which was continued until approximately 15 ml. of $^{18}\text{O}_2$ (at N.T.P.) had been liberated.

The stopcock was closed, the reaction system detached from the vacuum line and the contents of the flasks thawed and mixed. The apparatus was shaken gently for 1 hr. in a bath at 37° , the flasks being submerged. The liquid was then all collected in the large flask and frozen in ethanol- CO_2 . The residual gas was examined in the mass spectrometer. It contained only a trace of CO_2 . Oxygen was plentiful and had atom % excess $^{18}\text{O} = 0.584$. Air was admitted, a second 25 ml. flask containing catalase solution (0.5 ml.) was attached and the H_2O_2 formed in the reaction was decomposed *in vacuo* using the technique previously described (Bentley & Neuberger, 1949). The oxygen produced had atom % excess $^{18}\text{O} = 0.571$.

The system was again brought to atmospheric pressure and a flask containing conc. H_2SO_4 was attached. The solutions were mixed *in vacuo* in the usual way: an abundant amount of CO_2 was produced which did not contain any excess ^{18}O above normal.

Reaction in H_2^{18}O . Uricase solution ('Davidson', 20 ml.) was treated with an equal volume of ice-cold, saturated $(\text{NH}_4)_2\text{SO}_4$. The precipitate was centrifuged in the high-speed centrifuge at 0° and redissolved in H_2^{18}O (4 ml., atom % excess = 1.20). To this solution were added $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ (300 mg.), uric acid (50 mg.) and LiOH $\cdot \text{H}_2\text{O}$ (12.5 mg.). The mixture was shaken in a bath at 38° for 3 hr. (atmosphere of normal O_2). The O_2 uptake was approximately 5 ml. at N.T.P. The contents of the flask were then decomposed successively with catalase and conc. H_2SO_4 as previously described. O_2 from catalase decomposition: 0.000 atom % excess ^{18}O . CO_2 from H_2SO_4 decomposition: 1.18 atom % excess ^{18}O .

To check whether CO_2 would exchange under these experimental conditions, uric acid (25 mg.), LiOH $\cdot \text{H}_2\text{O}$ (6.5 mg.), $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ (150 mg.), and NaHCO_3 (5 mg.) in H_2^{18}O (2 ml., atom % excess = 1.20) were shaken under the same conditions. The CO_2 obtained after treatment with conc. H_2SO_4 had 1.13 atom % excess ^{18}O .

Experiment with [6- ^{14}C] uric acid. Uricase solution ('Leone's preparation', 23 ml.) was dialysed overnight against tap water at 0° and then against fresh distilled water at 0° for 2 hr. $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ (0.95 g.) was added to the dialysed enzyme solution, followed by a solution of [6- ^{14}C] uric acid (75 mg., 1.64×10^{-3} $\mu\text{C.}/\text{m-mole}$) and LiOH $\cdot \text{H}_2\text{O}$ (20 mg.) in boiled-out water (5 ml.). The mixture was shaken for 2 hr. at 38° in an O_2 atmosphere. Conc. H_2SO_4 (3 ml.) was then run into the flask and the solution aspirated in a stream of CO_2 -free N_2 . CO_2 in the emergent gas was precipitated as BaCO_3 ; two successive precipitates were collected. They were centrifuged, washed with boiled-out distilled water and ethanol, and dried *in vacuo* over P_2O_5 . For counting, they were resuspended in absolute ethanol and filtered using the apparatus and perforated disks described by Popják (1950). Sample A (2.5 mg.) contained 1.34×10^{-3} $\mu\text{C.}/\text{m-mole}$; sample B (7.4 mg.) contained 1.28×10^{-3} $\mu\text{C.}/\text{m-mole}$ (both values are corrected to infinite thickness).

DISCUSSION

The experiments with labelled gaseous oxygen and oxygen-labelled water prove conclusively that uricase catalyses the transfer of hydrogen, or more probably of electrons, from uric acid to the oxygen of the gas phase. The prosthetic group of uricase is

not known, but the general mechanism of the action of this enzyme resembles that of the flavin enzyme, notatin, which has been the subject of an earlier communication (Bentley & Neuberger, 1949). Electron transfer may occur in two distinct steps as has been postulated for all biological oxidations by Michaelis (1946). If this is the case, the electron acceptor O_2 is converted in the first step into the free radical $O_2^{\cdot -}$ or, if a proton is taken up at the same time, into the free radical $HO_2^{\cdot}$. In the second step these radicals will be converted into hydrogen peroxide or one of its ions, HO_2^- or O_2^{2-} .

The finding that the carbon dioxide which arises in the oxidation of uric acid by uricase is derived from the carbon atom in position 6 of the uric acid molecule is not unexpected and does not by itself indicate how allantoin is formed from the primary oxidation product. However, the results obtained in the present work, together with the many observations made by other workers, suggest a probable mechanism for this rather complex reaction. Owing to the exchange reaction between carbon dioxide and water which took place under the experimental conditions, it was impossible to determine the source of the oxygen atoms of carbon dioxide.

Uric acid is a dibasic acid with pK_1 5.4 and pK_2 10.6 (Johnson, 1952) and the actual substrate of uricase is therefore almost certainly either the mono-anion or, in view of the rather alkaline pH optimum (9.25; Keilin & Hartree, 1936*a*) of the enzyme, the di-anion. Which of the four hydrogen atoms of uric acid dissociate as protons in the first and second steps of the ionization is not certain; but Biltz & Herrmann (1921) deduced from a comparison of the ionization constants of various methyluric acids, that the imino-hydrogen atom in position 3 was the most acidic and that the hydrogen in position 9 had a slightly weaker tendency to dissociate. Later, Biltz (1936*a, b*) suggested that the order of acidities of the hydrogen atoms was $9 > 3 > 7 > 1$. Thus the mono-anion may be represented by one of the tautomeric formulae V or VI:

For each tautomeric form there are several resonance structures, some of which are derived from similar structures of the undissociated uric acid

molecule. Resonance in the ion, however, is greater than in the acid, and the negative charge in the former is not localized on the nitrogen atom, but will be spread over adjacent carbon and oxygen atoms. One set of structures which is likely to make an important contribution contains the negative charge on the carbon atom in position 5; these structures, one of which is shown (VII), will be favoured by the polar character of the adjacent amide bond in positions 7 and 8

It is suggested that the actual substrate of uricase is a mono-anion represented largely by a structure such as VII or a di-anion carrying a second charge in another unspecified position. The enzyme is then assumed to catalyse either a simultaneous or successive transfer of two electrons to oxygen, giving as primary product a carbonium ion such as VIII. It is proposed that this transfer of electrons is the only step catalysed by the enzyme and that the further changes observed are purely chemical reactions arising from the reactivity of the intermediates, especially that of the carbonium ion structure.

In a carbonium ion such as VIII carbon atom 5 is strongly electrophilic and will have a tendency to interact with the nitrogen atom in position 1 which is the only nucleophilic centre with which, for geometrical reasons, a bond can be formed. The resulting structure IX is unstable, largely owing to strain of the three-membered ring, and reaction with a hydroxyl ion will give X.

Rearrangements involving intermediate formation of carbonium ions are common in organic chemistry, but a particularly close analogy to the proposed series of reactions is afforded by the rearrangements of bicyclic α -amino ketones during Clemmensen reduction (Leonard & Wildman, 1949; Leonard & Ruyle, 1949). In these reactions a bond between a positively charged carbon and a tertiary nitrogen atom is formed, resulting in a transformation of a system containing two fused six-membered rings to one containing a five- and a seven-membered ring. The acid X, which may be the primary product

of Praetorius (1948), is likely to have some aromatic character owing to the presence of a double bond and the resonance of the amide bonds, and thus will show absorption of light in the ultraviolet range. Although the compound X is not symmetrical, the double bond is likely to shift readily to the other ring since the adjacent imino-hydrogen atom will be very mobile. It is further suggested that compound X may be transformed in three ways. It may give rise, by opening of one ring, addition of the elements of water, and loss of carbon dioxide, to allantoin, possibly involving another unstable intermediate; both rings may be opened with the addition of the elements of two water molecules giving uroxanic acid (II), or hydration may occur at the 3:4 double bond to give the symmetrical HDC (I).

This sequence of reactions explains satisfactorily most of the findings of other workers and is in accord with the results presented here. In the first place it is reasonable to assume that an enzyme catalyses one step in a reaction; the results obtained with ^{18}O clearly indicate that this step is a transfer of two electrons and this must yield a carbonium ion, if the mono-anion is the substrate, or a dipolar molecule of similar structure, if the di-anion were the substrate. The rearrangement of a six- or a five-membered ring invokes a mechanism accepted in the theory of organic reactions. The results of Felix *et al.* (1929) and those of Schuler (1932), Klemperer (1945) and Praetorius (1948) clearly indicate that the first product of uricase oxidation is not allantoin, but a compound still containing the same number of carbon atoms as uric acid. That this product is not HDC is suggested by the ultraviolet absorption data of Praetorius (1948) and also by the fact that this unstable product yields allantoin apparently more readily than HDC. It is therefore suggested that this substance has the structure X and that it can be converted to allantoin without preliminary hydration to HDC. The tautomeric shift of the double bond mentioned above explains the finding of Brown *et al.* (1947) that on feeding [1:3- ^{15}N] uric acid to rats, the urinary allantoin is uniformly labelled both in the hydantoin and urea moieties; presumably the only reaction involved in this *in vivo* experiment was oxidation by uricase. An analogous mechanism involving X would also explain the similar uniform isotope distribution in allantoin obtained by the alkaline oxidation of [1:3- ^{15}N] uric acid (Cavalieri & Brown, 1948).

The concept that the primary product of oxidation has a carbonium ion structure is likely to apply to many or most biological oxidations. In the special case of the oxidation of the urate ion by uricase the reaction is not complicated by the presence of a hydrogen atom on the reactive carbon centre; oxidation simply consists of a transfer of electrons. In most oxidations this electron transfer

is accompanied by a proton transfer; thus the oxidation of an aldehyde to the corresponding acid may be written thus:

This proposed mechanism also provides a reasonable explanation of the linkage of oxidation with formation of a labile phosphate bond of high energy, since the carbonium ion may react with a phosphate ion, instead of a hydroxyl ion:

In the reaction with water the high energy of the carbonium ion is dissipated as heat, whilst in the reaction with phosphate it is transformed into phosphate-bond energy. It is assumed that this reaction does not take place with free phosphate in solution, but with an ion which is adsorbed on the enzyme protein and is placed in a favourable geometrical position with regard to the substrate which is also combined with the enzyme.

A similar mechanism can also be suggested for the linkage between oxidative decarboxylation and phosphorylation:

Such enzyme complexes containing labile phosphate bonds may transfer their phosphate to adenosinediphosphate or other substances.

SUMMARY

1. By using water and gaseous oxygen labelled with ^{18}O it is shown that the oxygen atoms of the hydrogen peroxide formed in the oxidation of uric acid by uricase are derived from molecular oxygen. It is concluded that uricase catalyses the transfer of two electrons from a urate ion to oxygen.

2. Uric acid labelled with ^{14}C in the 6 position was synthesized. It is shown that the carbon dioxide formed in the enzymic oxidation is derived from the labelled carbon atom.

3. On the basis of the results reported here and the work of others a mechanism of enzymic oxidation of uric acid is proposed which assumes that the enzyme acts on the anion transforming it into a carbonium ion with loss of two electrons. The further changes which are probably non-enzymic consist mainly of an electrophilic rearrangement characteristic of carbonium ion structures.

4. The possible role of carbonium ions as intermediates in biological oxidations and coupled phosphorylations is discussed.

The authors wish to thank Mrs F. Higginson for assistance in the chemical work and Mr G. Dickenson for help with the mass spectrometer.

REFERENCES

- Batelli, F. & Stern, L. (1909). *Biochem. Z.* **19**, 219.
 Behrend, R. (1904). *Liebigs Ann.* **333**, 153.
 Bentley, R. (1949). *Biochem. J.* **45**, 591.
 Bentley, R. & Neuberger, A. (1949). *Biochem. J.* **45**, 584.
 Biltz, H. (1936a). *J. prakt. Chem.* **145**, no. 2, 65.
 Biltz, H. (1936b). *Ber. dtsh. chem. Ges.* **69**, 2750.
 Biltz, H. & Herrmann, L. (1921). *Ber. dtsh. chem. Ges.* **54**, 1676.
 Bogert, M. T. & Davidson, D. (1933). *J. Amer. chem. Soc.* **55**, 1667.
 Bonnichsen, R. K. (1947). *Arch. Biochem.* **12**, 83.
 Brown, G. B., Roll, P. M. & Cavalieri, L. F. (1947). *J. biol. Chem.* **171**, 835.
 Cavalieri, L. F. & Brown, G. B. (1948). *J. Amer. chem. Soc.* **70**, 1242.
 Davidson, J. N. (1942). *Biochem. J.* **36**, 252.
 Felix, F., Scheel, F. & Schuler, W. (1929). *Hoppe-Seyl. Z.* **180**, 90.
 Fischer, E. & Ach, F. (1899). *Ber. dtsh. chem. Ges.* **32**, 2721.
 Holmberg, C. G. (1939). *Biochem. J.* **33**, 1901.
 Johnson, E. A. (1952). *Biochem. J.* **51**, 133.
 Johnson, T. B. & Johns, C. O. (1914). *J. Amer. chem. Soc.* **36**, 545.
 Keilin, D. & Hartree, E. F. (1936a). *Proc. Roy. Soc. B*, **119**, 114.
 Keilin, D. & Hartree, E. F. (1936b). *Proc. Roy. Soc. B*, **121**, 173.
 Klemperer, F. W. (1945). *J. biol. Chem.* **160**, 111.
 Leonard, N. J. & Wildman, W. C. (1949). *J. Amer. chem. Soc.* **71**, 3089.
 Leonard, N. J. & Ruyle, W. V. (1949). *J. Amer. chem. Soc.* **71**, 3094.
 Michaelis, L. (1946). *Currents in Biochemical Research*, p. 207, ed. by D. E. Green. New York: Interscience.
 Popják, G. (1950). *Biochem. J.* **46**, 560.
 Praetorius, E. (1948). *Biochim. Biophys. Acta*, **2**, 602.
 Schuler, W. (1932). *Hoppe-Seyl. Z.* **208**, 237.
 Schuler, W. & Reindel, W. (1932). *Hoppe-Seyl. Z.* **208**, 248.
 Schuler, W. & Reindel, W. (1933). *Hoppe-Seyl. Z.* **215**, 258.
 Stimson, M. M. & Reuter, M. A. (1943). *J. Amer. chem. Soc.* **65**, 153.
 Traube, W. (1900). *Ber. dtsh. chem. Ges.* **33**, 1371.
 Traube, W. (1923). *Liebigs Ann.* **432**, 266.
 Wiechowski, W. (1907). *Beitr. chem. physiol. Path.* **9**, 295.

A Combustion Technique for the Assay of Tritium, ^{13}C and ^{14}C in a Single 10 mg. Sample of Biological Material

By R. F. GLASCOCK

National Institute for Research in Dairying, University of Reading

(Received 24 March 1952)

Payne, Campbell & White (1952) have recently described their method for the quantitative combustion of 500–600 mg. samples of tritiated metabolites. This scale is dictated by the method they prescribe for the determination of tritium, namely the reaction of 100–200 mg. of water with aluminium carbide and the counting of the tritiomethane so produced in a proportional gas counter. As it is not always easy or even possible to isolate as much as 0.5 g. of a particular metabolite other workers may be interested to know that there was recently published (Glascock, 1951a, b) a method for determining tritium on the 5–10 mg. scale. Sufficient water for the determination of tritium by this method and sufficient carbon dioxide for the determination of both ^{13}C and ^{14}C are produced by the quantitative combustion of about 10 mg. of most organic substances. A method for doing this has been in routine use in this laboratory for the past two and a half years, and a description of it now seems appropriate in view of the communication by Payne *et al.* (1952). It is very much easier to burn 10 mg. quantitatively than 0.5 g. and thus obtain

from 10 to 20 ml. of carbon dioxide. This is usually more than enough for the gas counting of several samples by the method of Brown & Miller (1947) and a few ml. can readily be spared for the mass spectrometer if ^{13}C is to be determined also. Then, if the compound is one which yields relatively little carbon dioxide, either a rather larger sample is burnt or the dose of ^{14}C is adjusted in advance so that smaller samples can be counted, thus leaving more for the spectrometer. The water produced usually amounts to about 10 mg. and it is treated with an excess of *n*-butyl magnesium bromide in a previously prepared 'Grignard tube' to yield *n*-tritiobutane almost quantitatively (Glascock, 1951a, b). If, for some reason, tritiomethane were preferred it could probably be prepared on this scale by using the appropriate Grignard reagent but butane has several important advantages. Firstly, it is an excellent counter gas and can be counted with high efficiency in the Geiger region of a suitable gas counter; hence the additional electronic equipment required for proportional counting as used by Payne *et al.* is unnecessary. Secondly, unlike methane,