The Intracellular Mode of Action of the Sulphonamide Derivatives. Some Condensation Products of Reductone

By E. A. BELL, W. COCKER AND R. A. Q. O'MEARA

University Chemical Laboratory and School of Pathology, Trinity College, Dublin

(Received 28 March 1949)

Colebrook & Kenny (1936), Colebrook, Buttle & O'Meara (1936) and, later, others showed that some time must elapse after bacteria are inoculated into media containing sulphonamide before an inhibitory effect is produced. Multiplication of the organism occurs in the interval. Subsequently, Wolff & Julius (1939) concluded that sulphonamides act on bacteria only when they are multiplying, and O'Meara (1942) mentioned that the lethal action of sulphonamides on bacteria is immediately exhibited when the organisms are in the logarithmic phase of growth. This was later confirmed and fully expounded by O'Meara, McNally & Nelson (1947), who pointed out that in the logarithmic phase of growth bacteria are engaged in the utilization of those compounds, present in the culture medium, which are most suited to energy and growth requirements. Glucose is one of the substances. O'Meara, McNally & Nelson (1944) had previously noted that during the logarithmic phase of growth the medium develops strongly reducing properties, and suggested that the reducing substance might be either ascorbic acid, dihydroxyacetone or reductone,

all of which could be produced from glucose. In the earlier publication, O'Meara *et al.* (1944) showed that the reducing substance gave a positive 'enediol' [$\text{—C}(\text{OH})\text{:C}(\text{OH})\text{—}$] test with *o*-dinitrobenzene and sodium hydroxide (Fearon & Kawerau, 1943). Ascorbic acid is known to be produced in certain bacterial cultures (Büsing & Peters, 1940), but this can usually be detected as its dehydro derivative (Fearon & Kawerau, 1943) and the reducing substance produced by the pathogens failed to give the test for dehydroascorbic acid. Reductone is produced when glucose is heated with alkali (von Euler & Klusmann, 1933; von Euler & Martius, 1933) and, since the culture media are alkaline and gave the enediol reaction after heating, O'Meara *et al.* (1947) considered that reductone was the most likely substance to be formed in such media.

O'Meara *et al.* (1947) showed that reductone is readily condensed with *p*-aminobenzoic acid, sulphapyridine, sulphathiazole and sulphanilamide. The reductone used was a crude solution from the

hydrolysis of glucose with sodium hydroxide, nevertheless solid products were obtained in all cases except in the reductone-sulphanilamide condensation, where the product was obviously readily soluble in water. The products were hydrolysed in alkaline solution with varying degrees of rapidity, yielding the starting materials. Condensation of the above-mentioned amines with ascorbic acid and dihydroxyacetone gave coloured solutions only.

In view of the known biological significance of *p*-aminobenzoic acid, and the fact that the compound formed by union of reductone with *p*-aminobenzoic acid is readily hydrolysed within the biological range of pH, O'Meara *et al.* (1944, 1947) considered that the function of *p*-aminobenzoic acid in cellular metabolism is to stabilize and temporarily conserve, for use of the cell, the extremely active substance reductone which can act as a source of energy for the growing cell. Since reductone is readily oxidized (compare von Euler & Martius, 1933) it must be preserved in a stabilized form, but in such a way as to be available for the requirements of the growing cell. It was, in fact, shown by O'Meara *et al.* (1947) that *Streptococcus pyogenes* can utilize the reductone-*p*-aminobenzoic acid condensation product and maintain growth on it, but *p*-aminobenzoic acid itself fails to support growth. In extension of the above, it was logical for O'Meara *et al.* (1947) to consider that sulphonamides interfere with this source of energy by combination with reductone to form compounds not available for utilization by bacteria. They showed that *Strep. pyogenes* was unable to utilize the sulphapyridine and sulphathiazole derivatives of reductone under conditions identical with those in which the *p*-aminobenzoic acid derivative was utilized by these organisms.

Reductone possesses a highly active aldehydic group which readily reacts with electron-donating groups (compare von Euler & Martius, 1933) and reaction may take place by end attack on the charged mesomeric form of the enol

The condensation products of reductone and *p*-aminobenzoic acid and the various sulphonamides

are highly coloured, and it is probable that they are of the type

According to the theory propounded by O'Meara *et al.* (1947), the effectiveness of the sulphonamides in interfering with the growth of bacteria is linked with the stability of the Schiff bases mentioned above. Those which are most readily hydrolysed will be the least effective in inhibition of growth and vice versa, and it is very likely that the ease of hydrolysis of the Schiff bases in the biological range of pH will be dependent upon the solubility of these compounds under these conditions. Before a final conclusion can be reached, the relative solubilities of the compounds must be determined, but it is known that sulphanilamide gives a condensation product with reductone which is very readily soluble in water at neutrality and difficult to isolate, and that sulphanilamide is relatively ineffective as growth inhibitor.

In accordance with the views expressed on the importance of reductone, it seems likely that in mixtures of *p*-aminobenzoic acid and a sulphonamide with reductone there will be competition for the aldehydic group by the amino groups. This can probably be represented by the following equation, where only the enolic forms are given,

The position of equilibrium in the above reaction will depend, amongst other factors, upon (a) the affinity of the aromatic amino groups for the aldehydic group, i.e. upon the relative electron-donating qualities of these groups, and (b) upon the concentration of the amino compounds. According to Bell & Roblin (1942) and Albert & Goldacre (1942) the basic dissociation constant of the aromatic amino group in the sulphonamides differs only slightly from 2.6×10^{-12} , which is the basic dissociation constant of *p*-aminobenzoic acid. Maphenide (marfanil, *p*-aminomethylbenzenesulphonamide) is in another category. Here the amino group is aliphatic and the compound correspondingly more basic. In general, it is probable that the concentrations of the amino compounds are important in deciding the position of equilibrium. In the bacterial cell other factors might have prominence such as degree of adsorption of the various compounds on some surface.

In the present investigation our primary object has been the preparation and examination of the products of condensation of reductone with *p*-aminobenzoic acid and various sulphonamides. We have

obtained solid products in all cases, but purification, by crystallization, has not always been possible owing to the low solubility of the products in most organic solvents. The reductone-sulphanilic acid condensation product is very readily soluble in water, and we have not been able to isolate this compound although its formation undoubtedly occurs, since the colour of the mixed solutions rapidly becomes a deep red.

Although our investigations are not complete we feel that an interim survey is necessary since Angier, Stokstad, Mowat, Hutchings, Boothe, Waller, Semb, SubbaRow, Cosulich, Fahrenbach, Hultquist, Kuh, Northey, Seeger, Sickels & Smith (1948) have recently published details of the preparation and properties of the reductone *p*-aminobenzoic acid condensation product and some of its derivatives, and Forrest & Walker (1948) have also indicated the scope of their work similar to that which we had contemplated (cf. Bell, Cocker & O'Meara, 1948), and that which has been in hand since September 1947 (cf. O'Meara *et al.* 1947).

EXPERIMENTAL

The preparation of reductone. Reductone is best prepared by the method of von Euler & Martius (1933) in which glucose is treated with NaOH and the reductone is precipitated as its Pb salt. No improvement in yield was obtained when fructose was employed. The decomposition of the Pb salt with H_2S was, however, performed with shaking in a previously evacuated flask attached to a source of H_2S . This gave a better product than that obtained when H_2S was bubbled through a suspension of the Pb salt (cf. Kuchlin & Böeseken, 1928). The yield of reductone, m.p. 200° , was about 7% of theory. The oxidation of glycerol by FeSO_4 and H_2O_2 (den Otter, 1937) and the oxidation of dihydroxyacetone with copper acetate in ultraviolet light (Kuchlin & Böeseken, 1928) gave disappointing results.

Condensation of reductone with amino compounds. The earlier condensations of reductone with the various amino compounds were performed in aqueous sodium acetate, but it was later found that the condensations could be readily performed in aqueous acetone with or without the addition of a few drops of acetic acid. A better product was thus obtained.

Analysis of compounds formed. The identity of the compounds was established by analysis and by colorimetric estimation of the *p*-aminobenzoic acid or sulphonamide in the compound. This was performed by hydrolysis with dilute alkali, and the resultant amino compound was condensed with *p*-dimethylaminobenzaldehyde (Werner, 1939, 1944), the colour so obtained being examined on the Spekker absorptiometer. It was possible approximately to estimate the reductone-*p*-aminobenzoic acid condensation product by titration with alkali, but the values of the equivalent obtained were variable. When the compound was left in contact with 0.05N-NaOH, the equivalent was found to be 57–60 in contrast to 110–120 which was obtained by rapid solution of the compound in the alkali, followed by back titration. The lower values of the equivalent are probably due to the oxidation of the reductone released by hydrolysis.

A number of the condensation products with reductone have been found to be hemihydrated, monohydrated or dihydrated. The reductone-sulphaguanidine condensation product is dihydrated and this is interesting since sulphaguanidine itself is monohydrated. Dehydro-L-ascorbic acid has recently been shown by Kenyon & Munro (1948) to be hydrated. This compound has a structure not unlike the keto form of the condensation products of reductone.

Reactions of reductone condensation products

The reductone-*p*-aminobenzoic acid condensation product reacts readily with phenylhydrazine (O'Meara *et al.* 1947) to give an osazone. This osazone has been prepared and analysed. It possesses a *p*-aminobenzoic acid residue.

Woods (1940) showed that *p*-aminobenzoic acid can inhibit the action of sulphonamide derivatives, its power of inhibition being greatest with sulphanilamide and least with sulphathiazole. In view of these results, we tried the effect of *p*-aminobenzoic acid on the reductone-sulphathiazole condensation product. When these compounds are mixed in hot aqueous sodium acetate which has a pH similar to that found under biological conditions, the sulphathiazole residue is quickly displaced and the reductone-*p*-aminobenzoic acid condensation product results, whereas the reverse process has not been found to take place under similar conditions. A corresponding result was obtained when the reductone-sulphaguanidine condensation product was treated with *p*-aminobenzoic acid. On the other hand, when the reductone-*p*-aminobenzoic acid condensation product is treated with maphenide (marfanil, *p*-aminomethylbenzenesulphonamide) hydrochloride in the presence of excess sodium acetate the *p*-aminobenzoic acid is rapidly replaced by maphenide, the reductone derivative of the latter being formed.

Reductone-p-aminobenzoic acid condensation product, p-2':3'-dihydroxyprop-2'-enylideneaminobenzoic acid. A solution of reductone (0.4 g.) in water (5 ml.) was shaken with charcoal and filtered. It was then added, with shaking, to a filtered solution of *p*-aminobenzoic acid (0.7 g.) in a mixture of water (10 ml.) and acetone (10 ml.). The clear solution soon became dark red and orange needles were quickly deposited. After 3 hr. the compound was collected, washed with acetone, water and finally acetone, until the filtrate no longer gave a reaction with *p*-dimethylaminobenzaldehyde. The product (0.45 g.) crystallized from dilute acetic acid as orange-yellow needles, m.p. 254°. (Found: C, 53.15; H, 5.2; N, 6.1. Calc. for $C_{10}H_9O_4N.H_2O$: C, 53.3; H, 4.9; N, 6.2%.) It gave absorption maxima in ethanol at 284 mμ. (log ε, 4.21) and 355.5 mμ. (log ε, 2.74). On drying on the water bath, the orange-yellow needles became red with loss of 1 mol. of water.

Titration. The compound (0.057 g.) in 24.9 ml. 0.05N-NaOH was allowed to stand for 15 min. at room temperature, when the solution had become colourless. It was then

titrated with 0.05N- H_2SO_4 using phenolphthalein as indicator, when 14.3 ml. of acid were required for neutralization. This gives an equivalent of 108 and mol. wt. of 216, assuming that the reductone is completely enolized. $C_{10}H_9O_4N.H_2O$ requires mol. wt. 225 (compare the titration of reductone by Norrish & Griffiths, 1928); other estimations gave values of 240 and 228. When the compound was left in contact with 0.05N-NaOH for 24 hr. before titration, values of 62, 57 and 57 were found in three estimations.

Colorimetric estimation of purity of the reductone-p-aminobenzoic acid condensation product. Ten ml. of each of a series of aqueous solutions containing 3–15 mg./l. of *p*-aminobenzoic acid were added to 2 ml. portions of a solution of Ehrlich reagent prepared by dissolving *p*-dimethylaminobenzaldehyde (3 g.) in a mixture of conc. H_2SO_4 (7 ml.) and water (100 ml.). The intensity of coloration in each case was measured by means of a Spekker absorptiometer using a blue filter. Concentration of *p*-aminobenzoic acid was plotted against extinction on a graph. Reductone-*p*-aminobenzoic acid condensation product (15 mg.) in 10 ml. 0.1N-NaOH was warmed for a few minutes and then diluted to 1 l.: 10 ml. of this solution were treated with 2 ml. of the Ehrlich reagent and the extinction was again determined. The *p*-aminobenzoic acid content determined from the calibration graph was 58.0%. $C_{10}H_9O_4N.H_2O$ requires 60.9 and $C_{10}H_9O_4N$ 66.1%. Repeat estimations gave values varying from 57 to 58%.

Reductone-sulphathiazole condensation product. 2-(p'-2':3'-Dihydroxyprop-2'-enylideneaminobenzenesulphonamido)thiazole. Sulphathiazole (2.0 g.) in acetone (30 ml.) containing a few drops of 80% acetic acid was added to reductone (0.5 g.) in dilute acetone (30 ml.). After several hours the required compound was obtained as yellow needles, which, after washing with water, gave m.p. 232° (decomp.) with some pre-softening. (Found: C, 43.0; H, 4.0. $C_{12}H_{11}O_4N_2S_2.H_2O$ requires C, 43.1; H, 3.6%.) This compound lost water when heated at 100° in a vacuum, after which the sulphathiazole residue was estimated by Ehrlich reagent. (Found: sulphathiazole, 80.0. $C_{12}H_{11}O_4N_2S_2$ requires sulphathiazole, 78.5%.)

Reductone-sulphapyridine condensation product. 2-(p'-2':3'-Dihydroxyprop-2'-enylideneaminobenzenesulphonamido)pyridine. This compound was obtained as an orange amorphous powder which did not melt sharply but started to darken and decompose at 194°. (Found: C, 50.6; H, 4.5. $C_{14}H_{13}O_4N_3S.H_2O$ requires C, 49.9; H, 4.4%.)

Reductone-sulphamezathine condensation product. 4:6-Dimethyl-2-(p'-2':3'-dihydroxyprop-2'-enylideneaminobenzenesulphonamido)pyrimidine. This compound was an amorphous green powder, m.p. 200–240° (decomp.). (Found: C, 48.2; H, 4.9; N, 14.8. $C_{15}H_{14}O_4N_4S.H_2O$ requires C, 49.2; H, 4.9; N, 15.3%.)

Reductone-sulphamethylthiazole condensation product. 4-Methyl-2-(p'-2':3'-dihydroxyprop-2'-enylideneaminobenzenesulphonamido)thiazole. This compound consisted of a yellow-orange, amorphous powder which shrivels and decomposes at 166–168°. (Found: C, 45.2; H, 4.3. $C_{13}H_{13}O_4N_2S_2.H_2O$ requires C, 44.8; H, 4.0%.)

Reductone-sulphaguanidine condensation product. p-(2':3'-Dihydroxyprop-2'-enylideneaminobenzenesulphonyl)guanidine. This compound formed yellow needles, m.p. 152°, which crystallized from water. (Found: C, 37.5; H, 5.1. $C_{10}H_{12}O_4N_4S.2H_2O$ requires C, 37.5; H, 5.0%.)

Reductone-soluseptazine condensation product. Reductone-p-(sodium-α-sulphoethyl)aminobenzenesulphonamide condensa-

tion product. Reductone-p-aminobenzenesulphonamide condensation product. *p*-(2':3'-Dihydroxyprop-2'-enylideneamino)benzenesulphonamide. Reductone was found to displace the substituting groups in the N⁴ position in the first two compounds. As a consequence all three gave the same condensation product with reductone. The yellow condensation product was crystallized from hot water as orange radiating needles which darkened, shrank and finally decomposed at 170–172°. The compounds derived from each of the three sources did not depress the melting point of one another. (In two analyses found: C, 42.0; H, 4.4. C, 40.8; H, 4.6. C₉H₁₀O₄N₂S.H₂O requires C, 41.5; H, 4.6%.)

Reductone-diaminodiphenylsulphone condensation product. *Bis-p*-(2':3'-dihydroxyprop-2'-enylideneamino)phenylsulphone. This compound consisted of yellow micropisms which started to decompose at 156°, but gave no definite m.p. (Found: C, 50.7; H, 4.8. C₁₈H₁₆O₆N₂S.2H₂O requires C, 50.9; H, 4.7%.)

Oszone of the reductone-p-aminobenzoic acid condensation product. The solid obtained when a mixture of the condensation product (0.5 g.), phenylhydrazine hydrochloride (1.0 g.) and sodium acetate (1.0 g.) in acetic acid was warmed on the water bath for 0.5 hr. was collected and the osazone crystallized from ethanol as yellow needles, m.p. 188–189°. (Found: C, 68.3; H, 5.3; N, 17.0. C₂₂H₁₈O₂N₆ requires C, 68.6; H, 4.9; N, 18.1%.) The *p*-aminobenzoic acid content was also estimated by hydrolysis with HCl. Phenylhydrazine was distilled in steam and the residue was treated with Ehrlich reagent and the depth of colour estimated as above. (Found: *p*-aminobenzoic acid, 36.0. C₂₂H₁₈O₂N₆ requires 35.6%.)

Action of p-aminobenzoic acid on reductone-sulphathiazole condensation product. A mixture of the reductone-sulphathiazole condensation product (0.5 g.), *p*-aminobenzoic acid (0.5 g.), sodium acetate (2 g.) and water (5 ml.) was boiled for a few minutes and filtered whilst hot. On cooling, a yellow precipitate separated and the solution exhibited the green fluorescence characteristic of the reductone-*p*-aminobenzoic acid condensation product. The precipitate was thoroughly washed with hot water to remove *p*-aminobenzoic acid and sulphathiazole. On drying, the yellow solid became orange and gave m.p. 250°, undepressed by authentic reductone-*p*-aminobenzoic acid condensation product.

Reductone-maphenide(marfanil) condensation product. *p*-(2':3'-Dihydroxyprop-2'-enylideneaminomethyl)benzenesulphonamide. A mixture of reductone-*p*-aminobenzoic acid condensation product, maphenide hydrochloride (0.5 g.) sodium acetate (2 g.) and water (5 ml.) was boiled for 2–3 min. with stirring. The hot mixture was filtered and cooled, when a yellow-green solid separated. It crystallized from ethanol as pale green needles, m.p. 183–184°. (Found: C, 46.9; H, 4.8. C₁₀H₁₂O₄N₂S requires C, 46.9; H, 4.7%.) On hydrolysis of this compound and diazotization of the product, a solution was obtained which did not couple with β-naphthol. Reductone-*p*-aminobenzoic acid condensation product, gives, by similar treatment, the expected azo compound.

DISCUSSION

The results here recorded provide chemical support for the theory advanced by O'Meara *et al.* (1944, 1947) that the sulphonamides act by uniting with reductone during the active phase of normal bacterial metabolism. According to the view of these authors,

p-aminobenzoic acid is the normal cell metabolite which unites with reductone and stabilizes it prior to further utilization by the cell. If the place normally taken by *p*-aminobenzoic acid be occupied by a sulphonamide, cellular metabolism ceases.

We have found that under biological conditions of pH, *p*-aminobenzoic acid and all the sulphonamides tested readily form compounds with reductone. These compounds have been isolated and their constitution has been established. It is apparent that the aldehydic group of reductone undergoes condensation with the free primary aromatic amino group of *p*-aminobenzoic acid and the sulphonamides. It is interesting to note that reductone readily condenses with soluseptasine and *p*-(sodium-α-sulphoethyl)-aminobenzene sulphonamide to give, in each case, the reductone-sulphanilamide condensation product. Northey (1940) has expressed the view that 'unless the substituting group in the N⁴ position is hydrolysed, reduced or otherwise removed *in vivo* it appears that the derivative will have little if any activity'. The chemical evidence here provided supports this view. Particularly interesting are the interactions of reductone-sulphonamide condensation products with *p*-aminobenzoic acid and of the reductone-*p*-aminobenzoic acid condensation product with maphenide (marfanil). Since, within the biological range of pH, *p*-aminobenzoic acid readily replaces the sulphonamides from their combinations with reductone, a chemical explanation is provided of the inhibition of the bacteriostatic action of the sulphonamides by *p*-aminobenzoic acid demonstrated by Woods (1940). Similarly, the replacement of *p*-aminobenzoic acid, from its combination with reductone, by maphenide, provides a chemical explanation of the facts that *p*-aminobenzoic acid does not antagonize maphenide (Schreuss, 1942; Goldacre, 1944) and that maphenide, unlike the sulphonamides, does not show a time lag in coming into operation (Jensen, Schmith & Brand, 1942). Moreover, these observations enable the mode of action of maphenide to be brought into line with that of the sulphonamides. Maphenide is a relatively strong base, and high concentrations are possible because of its high solubility in water. Both factors will operate so as to facilitate the replacement of *p*-aminobenzoic acid by maphenide in cellular metabolism.

We believe that the reductone-*p*-aminobenzoic acid condensation product system is likely to prove very important in biological synthesis. Reference to biochemical processes, possibly involving reductone and the reductone-*p*-aminobenzoic acid condensation product, has been made elsewhere (Bell, Cocker & O'Meara, 1948), and the synthesis of pteric acid and its 7-isomer by Forrest & Walker (1948) and of folic acid and its related compounds by Angier *et al.* (1948), lend colour to this view.

SUMMARY

1. Condensation products of reductone have been prepared with *p*-aminobenzoic acid, 2-*p*-aminobenzenesulphonamidothiazole (sulphathiazole), 2-*p*-aminobenzenesulphonamidopyridine (sulphapyridine), 4:6-dimethyl-2-(*p*-aminobenzenesulphonamido)pyrimidine (sulphamezathine), *p*-aminomethylbenzenesulphonamide (maphenide, marfanil), 4-methyl-2-(*p*-aminobenzenesulphonamido)thiazole (sulphamethylthiazole), *p*-aminobenzenesulphonylguanidine, 4:4'-diaminodiphenylsulphone, *p*-(disodium- γ -phenyl- α -disulphopropyl)aminobenzenesulphonamide (soluseptasine)

p-(sodium- α -sulphoethyl)aminobenzenesulphonamide ($\text{Me} \cdot \text{CH}(\text{SO}_3\text{Na})\text{NHC}_6\text{H}_4\text{SO}_2\text{NH}_2$) and *p*-aminobenzenesulphonamide. The condensation pro-

ducts of reductone with the three last compounds were identical.

2. It has been shown that the condensation product of reductone and sulphathiazole is decomposed by *p*-aminobenzoic acid. The resultant product is identical with that obtained from reductone and *p*-aminobenzoic acid (*p*-2':3'-dihydroxy-prop-2'-enylideneaminobenzoic acid), but the reverse process is apparently not possible under similar conditions. Maphenide, however, displaces *p*-aminobenzoic acid from *p*-2':3'-dihydroxyprop-2'-enylideneaminobenzoic acid.

3. A theory of the mode of action of sulphonamide derivatives is discussed.

One of us (E. A. B.) was in receipt of a grant from the Sarah Purser Medical Research Fund.

We desire to express our thanks to Imperial Chemical Industries Ltd. and the Medical Research Council of Ireland for financial support.

REFERENCES

- Albert, A. & Goldacre, R. (1942). *Nature, Lond.*, **149**, 245.
 Angier, R. B., Stokstad, E. L. R., Mowat, J. H., Hutchings, B. L., Boothe, J. H., Waller, C. W., Semb, J., SubbaRow, Y., Cosulich, D. B., Fahrenbach, M. J., Hultquist, M. E., Kuh, E., Northey, E. H., Seeger, D. R., Sickels, J. P. & Smith, J. N. jun. (1948). *J. Amer. chem. Soc.* **70**, 25.
 Bell, E. A., Cocker, W. & O'Meara, R. A. Q. (1948). *Lancet*, **i**, 924.
 Bell, P. & Roblin, R. (1942). *J. Amer. chem. Soc.* **64**, 2905.
 Büsing, K. H. & Peters, F. (1940). *Biochem. Z.* **304**, 134.
 Colebrook, L., Buttle, G. A. H. & O'Meara, R. A. Q. (1936). *Lancet*, **ii**, 1323.
 Colebrook, L. & Kenny, M. (1936). *Lancet*, **i**, 1279.
 Euler, H. von & Klusmann, E. (1933). *Ark. Kemi Min. Geol.* **2**, B, 12.
 Euler, H. von & Martius, C. (1933). *Liebigs Ann.* **505**, 73.
 Fearon, W. R. & Kawerau, E. (1943). *Biochem. J.* **37**, 326.
 Forrest, H. S. & Walker, J. (1948). *Nature, Lond.*, **161**, 721.
 Goldacre, R. J. (1944). *Nature, Lond.*, **154**, 796.
 Jensen, K. A., Schmith, K. & Brand, P. (1942). *Klin. Wschr.* **21**, 1042.
 Kenyon, J. & Munro, N. (1948). *J. chem. Soc.* p. 158.
 Kuchlin, A. T. & Böeseken, J. (1928). *Rec. Trav. chim. Pays-Bas*, **47**, 1011.
 Norrish, R. G. W. & Griffiths, J. G. A. (1928). *J. chem. Soc.* p. 2829.
 Northey, E. H. (1940). *Chem. Rev.* **27**, 115.
 O'Meara, R. A. Q. (1942). *Irish J. med. Sci.* p. 1.
 O'Meara, R. A. Q., McNally, P. A. & Nelson, H. G. (1944). *Nature, Lond.*, **154**, 796.
 O'Meara, R. A. Q., McNally, P. A. & Nelson, H. G. (1947). *Lancet*, **ii**, 747.
 Otter, H. P. den (1937). *Rec. Trav. chim. Pays-Bas*, **56**, 474.
 Schreuss, H. (1942). *Klin. Wschr.* **21**, 1042.
 Werner, A. E. A. (1939). *Lancet*, **i**, 18.
 Werner, A. E. A. (1944). *Sci. Proc. R. Dublin Soc.* **23**, 214.
 Wolff, L. K. & Julius, H. W. (1939). *Ann. Inst. Pasteur*, **62**, 616.
 Woods, D. D. (1940). *Brit. J. exp. Path.* **21**, 74.

The Micro-estimation of Citric Acid

By H. WEIL-MALHERBE AND A. D. BONE, *Runwell Hospital, Wickford, Essex*

(Received 12 April 1949)

The estimation of citric acid by its conversion into pentabromoacetone (Stahre, 1895) has been greatly improved, notably by Pucher, Sherman & Vickery (1936), who introduced a sensitive colorimetric procedure. Recent modifications (Perlman, Lardy & Johnson, 1944; Krebs & Eggleston, 1944; Goldberg & Bernheim, 1944; Hunter & Leloir, 1945; Speck, Moulder & Evans, 1946; Taussky & Shorr, 1947;

Natelson, Lugovoy & Pincus, 1947, 1948; Wolcott & Boyer, 1948) have further increased the reliability and sensitivity of the method. However, the fact that modifications of the method continue to appear indicates that satisfaction is not general. Most authors agree that the addition of permanganate is a critical step which must be performed slowly and cautiously.