SUMMARY

Two branched-chain fatty acids, (+)-12-methyltetradecanoic acid and 13-methyltetradecanoic, formerly isolated from mutton fat, have been shown to be present in butterfat.

We thank Professor E. Stenhagen of Uppsala University, Sweden, for a synthetic sample of pure 13-methyltetradecanoic acid, and Mr M. Fieldes of the Soil Bureau, Department of Scientific and Industrial Research, Wellington, New Zealand, for the X-ray measurements.

REFERENCES

Arosenius, K. E., Ställberg, G., Stenhagen, E. & Tägtström-Eketorp, B. (1949). *Ark. Kemi Min. Geol.* **26** A, no. 19.
Cason, J., Sumrell, G., Allen, C. F., Gillies, G. A., Elberg, S. (1953). *J. biol. Chem.* **205**, 435.
Cason, J. & Winans, W. R. (1950). *J. org. Chem.* **15**, 139.
Crombie, L. & Harper, S. H. (1950). *Chem. & Ind.* p. 757.
Francis, F., Piper, S. H. & Malkin, T. (1930). *Proc. Roy. Soc.* A, **128**, 214.
Ginger, L. G. (1944). *J. biol. Chem.* **156**, 453.
Hansen, R. P. & Shorland, F. B. (1951*a*). *Biochem. J.* **50**, 207.
Hansen, R. P. & Shorland, F. B. (1951*b*). *Biochem. J.* **50**, 358.
Hansen, R. P. & Shorland, F. B. (1953). *Biochem. J.* **55**, 662.
Hansen, R. P., Shorland, F. B. & Cooke, N. J. (1951). *Chem. & Ind.* p. 839.
Hansen, R. P., Shorland, F. B. & Cooke, N. J. (1952*a*). *Biochem. J.* **50**, 581.
Hansen, R. P., Shorland, F. B. & Cooke, N. J. (1952*b*). *Biochem. J.* **52**, 203.
Hansen, R. P., Shorland, F. B. & Cooke, N. J. (1953). *Biochem. J.* **53**, 374.
Klyne, W. (1953). *Biochem. J.* **53**, 378.
Kuhn, R. & Roth, H. (1933). *Ber. dtsch. chem. Ges.* **66**, 1274.
Meyer, J. D. & Reid, E. E. (1933). *J. Amer. chem. Soc.* **55**, 1574.
Morice, I. M. & Shorland, F. B. (1952). *Chem. & Ind.* p. 1267.
Nunn, J. R. (1951). *J. chem. Soc.* p. 1740.
Shorland, F. B. (1952). *J. appl. Chem.* **2**, 438.
Slagle, F. B. & Ott, E. (1933). *J. Amer. chem. Soc.* **55**, 4396.
Velick, S. F. (1947). *J. Amer. chem. Soc.* **69**, 2317.
Weitkamp, A. W. (1945). *J. Amer. chem. Soc.* **67**, 447.

Rapid Determination of Salicylate in Biological Fluids

By P. TRINDER
Biochemistry Department, Royal Infirmary, Sunderland, Co. Durham

(*Received* 14 *November* 1953)

The purple colour given by salicylate ion with ferric salts in weakly acid solution and the blue colour given with the Folin-Ciocalteu reagent in strongly alkaline solution have been most used for the determination of salicylates in serum. Of recent methods employing ferric salts, that of Tarnoky & Brews (1950) is the most convenient. The disadvantages of the method are that the determination occupies 45 min., requires the use of a mechanical shaker and gives a recovery of salicylate added to serum, of only 91–96 %. Moreover oxalated plasma, whole blood or urine cannot be used, and the final coloured solution is not always optically clear. The method of Smith & Talbot (1950), using the Folin-Ciocalteu reagent, has certain advantages. Only 0·2 ml. of sample is required, the determination occupies only 15 min., and either serum or oxalated plasma may be used. However, objections to this method are that 'blank' values on the serum of patients not taking salicylates are high and variable (the average blank being equivalent to 7·5 mg. of salicylate ion/100 ml. of serum, with a range of 4·5–9·5 mg./100 ml.) and that blank values for whole blood and for urine, are very high (about 25 mg./100 ml. for whole blood and 100 mg./100 ml. for urine). The blank values on serum by the method of Tarnoky & Brews (1950), are stated by the authors to be negligible.

A method which would combine some of the advantages of both methods and which would also be applicable to the analysis of whole blood and urine, would be most useful. The experiments described below were conducted with the object of devising such a method.

METHOD

It was decided to utilize the purple colour given by salicylates with ferric salts. To eliminate any inhibition of this colour by phosphates or oxalates, a reagent was prepared containing a high concentration of ferric nitrate. In order to precipitate the protein in the serum, mercuric chloride and hydrochloric acid were incorporated into the solution. A solution containing 4 g. of ferric nitrate, $Fe(NO_3)_3$, $9H_2O$, 4 g. of mercuric chloride and 12 ml. of N-HCl/100 ml., was found to precipitate the serum protein and give a purple colour with serum salicylate. The intensity of the purple colour became weaker if more hydrochloric acid was used in the reagent. If the final strength of the acid was much less than 0·12 N, the colour given by serum salicylate was not the same tint as that given by aqueous solutions of salicylate. The effect of the concentration of hydrochloric acid on the colour is shown in Table 1.

Table 1. *Effect of hydrochloric acid concentration on the optical density of the ferric nitrate: salicylic acid colour*

1 ml. of solution containing 0·4 mg. of salicylic acid, plus 5 ml. of colour reagent.
Filter Ilford 624. Cells 10 mm.

Final strength of HCl (N)	0·04	0·08	0·12	0·16	0·20
Optical density	0·75	0·715	0·66	0·605	0·54

Table 2. *Blank values on biological fluids*

No. of specimens analysed	Type of fluid	Salicylic acid found (mg./100 ml.) Maximum	Minimum	Mean
35	Serum	0·9	0·15	0·51 (S.D. ±0·17)
10	Plasma	1·1	0·6	0·8
10	Whole blood	1·8	1·2	1·4
10	C.s.f.	0·6	0·3	0·4
10	Urine	4·5	1·1	2·2

Table 3. *Recovery of salicylic acid added to biological fluids*

Salicylic acid added (mg./100 ml.)	Salicylic acid found (mg./100 ml.) Serum	Whole blood	Urine	Recovery (%) Serum	Whole blood	Urine
0	0·75	1·8	1·1	—	—	—
10	10·8	—	10·9	100·5	—	98
20	20·5	—	20·5	99	—	97
30	30·8	—	30·2	100	—	97
40	40·3	42·0	41·1	99	100·5	100
50	50·5	—	—	99·5	—	—

Reagents

Colour reagent. With the aid of heat, 40 g. of A.R. mercuric chloride are dissolved in 850 ml. of water. The solution is cooled and 120 ml. of N-HCl and 40 g. of ferric nitrate, $Fe(NO_3)_3$, $9H_2O$, are added. When all the ferric nitrate has dissolved, the volume of the solution is made to 1 l. with water. This solution is stable indefinitely.

Stock salicylate solution. 580 mg. of sodium salicylate, $C_7H_5O_3Na$, are dissolved in sufficient water to make 250 ml. of solution. A few drops of $CHCl_3$ are added as a preservative. This solution contains 200 mg. of salicylic acid/100 ml.

Standard salicylate solution. 20 ml. of the stock solution are added to sufficient water to make 100 ml. of solution. A few drops of $CHCl_3$ are added as a preservative. This solution contains 40 mg. of salicylic acid/100 ml. Both stock and standard solutions keep for at least 6 months if stored in a refrigerator.

Procedure

For cerebrospinal fluid (c.s.f.) plasma or whole blood. Wintrobe's anticoagulant (Wintrobe & Landsberg, 1935; cf. also in this paper p. 303) is used for plasma and whole blood. 1 ml. of fluid is placed in a cylindrical centrifuge tube and 5 ml. of colour reagent are added; the tube is shaken during the addition. The contents of the tube are shaken for a few seconds to ensure that the protein precipitate is finely dispersed. The tube is centrifuged at 2000 *g* for 2 min. and the supernatant fluid, which should be optically clear, is transferred to a test tube. A photoelectric colorimeter is set at full-scale deflexion (optical density, 0) with a blank prepared by mixing 1 ml. of water with 5 ml. of colour reagent. The optical density of the coloured unknown is read, using 10 mm. cells and the Ilford 624 green filter. If this filter is not available, the Ilford green 404 or Chance OG1 filters are suitable. If a spectrophotometer is used the wavelength should be set at 540 mμ. The optical density of the purple colour is constant for at least 60 min. The salicylate content of the sample is read from a graph prepared by treating 1 ml. quantities of sodium salicylate solutions containing the equivalent of 0·1, 0·2, 0·3, 0·4 and 0·5 mg. of salicylic acid, with 5 ml. of colour reagent and plotting the optical densities of the resultant coloured solutions. The quantities shown above correspond to blood salicylic acid levels of 10–50 mg./100 ml. and the colours obey Beer's law over this range. By this method the optical densities obtained on analysing solutions containing the equivalent of 12–40 mg. of salicylic acid/100 ml., lie between 0·2 and 0·7, the range in which the relative error is least (Archibald, 1950). If the optical density of the unknown is greater than 0·7 the analysis is repeated, using a smaller sample diluted to 1 ml. with water.

For urine. The urine is diluted with water so that it contains between 10 and 40 mg. of salicylic acid/100 ml. The diluted urine is analysed as for serum. After obtaining the optical density of the unknown, a blank reading is obtained by setting the instrument with water and reading the optical density of a solution prepared by mixing 1 ml. of diluted urine with 5 ml. of colour reagent and 0·1 ml. of syrupy phosphoric acid (sp.gr. 1·75), using the same cells and filter as before. Urine solutions often do not require centrifuging; if centrifuging is necessary both unknown and urine blank are centrifuged.

Urine salicylic acid mg./100 ml. = (mg. salicylic acid/100 ml. in diluted unknown − mg. salicylic acid/100 ml. in diluted urine blank) × dilution factor.

RESULTS

Successive c.s.f., plasma, whole blood, serum and urine samples, obtained from patients who were not taking salicylates, were analysed by the proposed method. The results in Table 2 show that the 'blank' values (in mg. salicylic acid/100 ml.), were less than 1·1 for serum, c.s.f., and plasma; less than 2·0 for whole blood and less than 4·5 for urine. Recovery experiments were performed by analysing pooled serum, whole blood and urine, to which known amounts of sodium salicylate had been added. The results given in Table 3 indicate that the recoveries were almost quantitative. The recovery figures were not affected by the addition of 100 mg. of phosphate ion, 20 mg. of bilirubin, 25 mg. of phenol, 10 000 i.u. of heparin, 1000 mg. of glucose or 1000 mg. of urea, per 100 ml. of serum. The addition of 250 mg. of Wintrobe's anticoagulant (150 mg. of ammonium oxalate, $(COONH_4)_2$, H_2O, plus 100 mg. potassium oxalate, $(COOK)_2$, H_2O/100 ml. of serum) increased the results by 0·3 mg. of salicylic acid/100 ml. The addition of 50 mg. of ethyl acetoacetate/100 ml. of serum, increased the results by 1 mg. of salicylic acid/100 ml. The effect of other keto acids on the recovery figures was not studied.

DISCUSSION

Although a rapid method is worth developing in the interests of economy, the aim should not be to sacrifice accuracy for speed. In the present method a single determination occupies only 5 min., but the method is at least as accurate as other methods. The recovery figures are good and the blank values are low even for whole blood, which cannot be analysed by other methods. If an instrument, such as the Spekker Absorptiometer, is available, which can give readings with small volumes of fluid, the method can be used on samples of blood obtained by finger prick, using 0·2 ml. of blood and 1 ml. of colour reagent. This modification would be especially useful for the determination of salicylate in the blood of children. The results obtained using urine are not so reliable as those obtained using serum, owing to the somewhat high blank values for normal urine. In practice the concentration of salicylate in urine, even after small doses of salicylates, is so high (100 mg. or more of salicylic acid/100 ml. of urine) that the relative error is small.

SUMMARY

1. A rapid method for the determination of salicylate in biological fluids is presented, based on a reagent containing ferric nitrate, mercuric chloride and hydrochloric acid, which precipitates the proteins and simultaneously reacts with salicylic acid to give a purple colour.

2. The recovery of sodium salicylate added to biological fluids is quantitative.

3. The blank values on normal serum and plasma samples are less than 1·1 mg. of salicylic acid/100 ml.

4. The effect of some possible interfering substances is considered.

5. A single determination occupies 5 min.

REFERENCES

Archibald, R. M. (1950). *Analyt. Chem.* **22**, 639.

Smith, M. J. H. & Talbot, J. M. (1950). *Brit. J. exp. Path.* **31**, 65.

Tarnoky, A. L. & Brews, V. A. L. (1950). *J. clin. Path.* **3**, 289.

Wintrobe, M. M. & Landsberg, J. W. (1935). *Amer. J. med. Sci.* **189**, 202.

The Inhibition of Trypsin and Chymotrypsin by Certain Organic Phosphorus Esters

By B. A. KILBY and G. YOUATT

Department of Biochemistry, 9 Hyde Terrace, Leeds 2

(*Received* 13 *January* 1954)

Certain organophosphorus compounds have recently acquired a rapidly increasing importance as insecticides, and as they also show high mammalian toxicity, it appears desirable to have as much information as possible about their mode of action. A full knowledge of this will involve not only a knowledge of which essential enzyme systems are inhibited in living organisms, but also an understanding of the chemical process of inhibition. A very powerful anticholinesterase activity is one of the characteristic properties of the phosphorus compounds in this group, but other enzymes, such as human milk and liver esterase, citrus acetylesterase, chymotrypsin and trypsin are also inhibited. It is now generally agreed that the toxicity towards vertebrates is closely associated with the