A Specific Method for the Determination of Free Acetate in Blood and Tissues

By F. LUNDQUIST, ULLA FUGMANN AND H. RASMUSSEN
University Institute of Forensic Medicine, Copenhagen, Denmark

(Received 3 February 1961)

The concentration of acetate in most mammalian blood and tissues is extremely low and quantitative determination has not been possible with the methods used, for instance, in microbiology. Various suggestions have been made to employ enzymic procedures for the quantitative analysis of acetate. Korff (1954) examined the combination of the distillation-diffusion step described by Bartley (1953) with an enzyme system from rabbit-heart muscle. The enzymic reactions involved were activation of acetate to acetyl-coenzyme A and condensation of this substance with oxaloacetate, produced through dehydrogenation of malate. This last reaction caused the formation of reduced diphosphopyridine nucleotide, which was measured spectrophotometrically. The enzyme preparation was a rather crude one and contained lactic dehydrogenase, which causes pyruvate to interfere in the determination. Sodium ions inhibit the process strongly and the enzyme is not stable for long periods. Korff's method was designed to measure 3–25 μg . of acetate. Rose (1955) has utilized an acetokinase from *Escherichia coli*. The acetyl phosphate formed in the reaction was measured as the ferric hydroxamate complex. Amounts of acetate in the range 30–150 μg . were determined.

The method of Soodak & Lipman (1948) and Soodak (1957) employs an enzyme preparation from pigeon liver catalysing the acetylation of sulphanilamide through the reactions:

of which the first is probably a two-stage reaction. The remaining sulphanilamide is determined colorimetrically.

The reaction is inhibited by alkali-metal ions and the method cannot therefore be used directly for the determination of acetate in biological material, where the acetate concentration is low compared with that of inorganic substances. Also acetoacetate may interfere seriously by giving rise to acetyl-coenzyme A. If, however, the principle used in Soodak's method is combined with a diffusion or

distillation step to isolate the volatile acids, and at the same time destroy acetoacetate, a method should result which is both sensitive and specific. A preliminary account of work on this problem has been published (Fugmann, Lundquist & Rasmussen, 1958). A short description of the final method has also appeared (Lundquist, 1961).

Diffusion in Conway units was decided upon as the method most likely to give good results with the small amounts of acetate involved, but the vacuum procedure of Bartley (1953) could probably also be modified satisfactorily for microscale work. The use of anhydrous sodium sulphate as reagent to increase the vapour pressure of acetic acid was necessary for the completion of the diffusion within a reasonable time, as recommended by Conway (1957). It was, however, essential to cool the sodium sulphate and the fluid to be analysed, and to incorporate a small amount of the decahydrate of sodium sulphate into the mixture, in order to get rapid crystallization. The importance of low temperature was noticed by Serlin & Cotzias (1955), who also found it advisable to add citrate buffer at about pH 3 to the solution subjected to diffusion.

The procedure of Serlin & Cotzias was, however, not directly applicable as it proved necessary to prevent traces of sodium sulphate from entering the receiving compartment. This was done by the use of separate Perspex dishes placed in the centre part of the diffusion unit. After the completion of this step the Perspex dishes were removed and the contents evaporated to dryness in preparation for the enzymic analysis.

EXPERIMENTAL

Preparation of pigeon-liver enzyme. The enzyme was prepared essentially as described by Soodak (1957). Livers from 12–15 animals were used for each batch of enzyme. It was observed that material from young pigeons gave the highest yield of enzyme. The final dialysed solution (about 100 ml.) was distributed in vials and kept frozen at -20° . The frozen enzyme keeps well and has been used successfully after storage for one and a half years.

Materials. It is important to use chemicals free from acetate. This is especially important for Na_2SO_4 , which is used in large quantities. Among many preparations tried

anhydrous Na_2SO_4 , p.a. (Merck), was the only one sufficiently pure. Other chemicals used were obtained from the following sources. ATP, disodium salt, CoA, 75% pure, and tris from Sigma Chemical Co., St Louis, Mo., U.S.A. Citric acid monohydrate and HClO_4 were Merck analytical reagents. Other chemicals used were analytical-grade products.

Procedure for blood plasma or serum

The analytical procedure consists of four steps: deproteinization, diffusion in Conway units, enzymic reaction and determination of sulphanilamide.

Deproteinization. Deproteinization is best performed with HClO_4 . Metaphosphoric acid (Merck, in sticks) has also been used successfully. Blood plasma is mixed thoroughly with 1 vol. of 5% (w/v) HClO_4 and centrifuged after 10 min. The clear supernatant is neutralized and buffered at pH 3 by the addition of 0.1 vol. of a solution containing 2.3N-NaOH and 0.5M-citric acid [NaOH (9.4 g.) and citric acid (10.5 g.) are dissolved in 100 ml. of water]. This reagent is kept in a well-closed plastic bottle.

Diffusion. Standard Conway units no. 1 are used. Anhydrous Na_2SO_4 is ground in a mortar with about 5% of crystalline $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$. The powder (5 or 10 g.) is distributed in the outer ring of the units. If more than 1 ml. of fluid is used 10 g. of Na_2SO_4 is employed. It is convenient to cover the centre compartment by a plastic cone, which fits into the well while the salt is poured on. The powder is lightly compressed by means of a Perspex ring fitting into the outer chamber. The units are now placed at 4° for 1 hr. before addition of the sample to be analysed, or, alternatively, the whole procedure may be carried out in a cold room. In the centre compartment is placed a plastic dish (external diameter, 32 mm.; internal diameter, 29–30 mm.; internal height, 4 mm.; external height, 5 mm.) made from Perspex rod, 35 mm. in diameter. With a Carlsberg constriction pipette, 0.033N-KOH (600 μl .) is measured into the dish. Cold, neutralized HClO_4 filtrate (0.4–2 ml., depending on the concentration of acetate) is distributed evenly on the surface of the Na_2SO_4 layer. For sealing the units a mixture of liquid (3 parts) and solid (1 part) paraffin is applied to the rim of the unit by a glass syringe.

The units are replaced at 4° for about 1 hr. to accelerate the crystallization, and thereafter for 20–24 hr. at room temperature (below 30°). The plastic dishes are removed and placed at 90° for 30 min. Hereby all the water is removed and any acetoacetate that may be present is destroyed.

Enzymic reaction. (i) Reagents. A coenzyme-sulphanilamide mixture is prepared by dissolving ATP (300 mg. of the disodium salt) and CoA (5 mg.) in 5 ml. of water. Then 25 mM-sulphanilamide (3 ml.), M-potassium citrate (2 ml.) and 0.1M-MgCl₂ (0.5 ml.) are added, and the mixture is distributed in vials and frozen at –20°. It keeps for at least 1 month. Immediately before use a buffer-coenzyme reagent is made up consisting of coenzyme-sulphanilamide mixture (2 vol.), M-tris-HCl buffer, pH 8.1 (6 vol.), 0.5M-cysteine-HCl (1 vol.) and 0.1M-MgCl₂ (1 vol.). This mixture is kept in an ice bath.

(ii) Procedure. Into the dried plastic dishes is measured (by Carlsberg pipettes) 0.1N-HCl (200 μl .) followed by the buffer-coenzyme reagent (250 μl .). The contents are thoroughly mixed to dissolve all KOH and acetate, and

400 μl . is transferred to a small (7 ml.) glass-stoppered centrifuge tube. Enzyme (200 μl ., or according to the activity, see Fig. 1) is added, a stream of N_2 is led through the tube for 20 sec., and the stopper replaced. The tip of the N_2 tube should be held just above the surface of the liquid. The tubes are incubated for 1 hr. at 37° and, after cooling, cold 5% (w/v) trichloroacetic acid (2 ml.) is added. The tubes are centrifuged after 10 min.

Sulphanilamide determination. The clear supernatant (1 ml.) is transferred into 0.9N-HCl (9 ml.) in a test tube. The determination is made according to Bratton & Marshall (1939) through consecutive addition of 0.1% NaNO_2 (1 ml.), 0.5% sulphamic acid (1 ml.) and 0.1% *N*-naphthylethylenediamine dihydrochloride (1 ml.). The red colour is measured at 540 m μ .

Blank and standard analyses. These are carried through the whole procedure. 5% HClO_4 (1 vol.) is neutralized with NaOH and made up to 2 vol. Citrate buffer, pH 3 (0.5M, 0.2 vol.), is added. This 'blank solution' is employed directly for the diffusion step in the blank analyses. Suitable standards are made from the blank solution by addition of a stock solution containing 100 μl . (105 μg .) of acetic acid/100 ml. (17.5 mM). Standards containing 10 and 20 μg . are generally employed.

Assay of enzyme. The enzyme is tested by making a number of analyses (without the diffusion step) on the same acetate solution with increasing amounts of enzyme as shown in Fig. 1. Blank analyses containing only enzyme but no acetate are included as the enzyme may contain a small amount of acetate (generally, however, less than 2 μg ./ml.).

Analysis of other materials

For analysis of tissues a suitable HClO_4 extract may be employed in the same way as blood plasma (see Table 4). With urine the pH is adjusted to 3 and 0.1 vol. of 0.5M-citrate buffer, pH 3, is added. Diffusion and further treatment is identical in all cases.

Fig. 1. Assay of pigeon-liver enzyme: change in extinction at 540 m μ as a function of the amount of enzyme. All figures are corrected for the blank values obtained with the same amount of enzyme but without addition of acetate. The incubation mixture contained: coenzyme-buffer solution (250 μl .), acetate solution (10 μg . in 100 μl .), and enzyme + water (300 μl .). With this enzyme preparation 200 μl . will be optimum for acetate analysis, but satisfactory results can be obtained with less than this amount.

RESULTS

The composition of the incubation mixture employed by Soodak was found not to be the optimum. Especially the magnesium chloride concentration was too low, but increases of the concentrations of ATP, cysteine, sulphanilamide and tris buffer were also found desirable. As a result of these changes the colour corresponding to 1 $\mu\text{g.}$ of acetate is increased considerably (50–100 %). In direct experiments (without diffusion) the colour obtained corresponds to about 85 % of the theoretical yield (i.e. when all acetate is converted into acetylsulphanilamide).

Below 15–20 $\mu\text{g.}$ of acetate, somewhat dependent on the enzyme preparation, proportionality is found between colour and amount of acetate both for aqueous acetate solutions analysed directly and for serum analysed by the complete procedure.

Diffusion process. The recovery of acetate from aqueous solutions containing citrate buffer is nearly quantitative after diffusion for 24 hr. as seen in Table 1, which also demonstrates that the volume of the liquid added to the Conway units should preferably not exceed 2 ml. In general diffusion for 24 hr. is sufficient also with deproteinized serum and other material, but for concen-

trated urine the recovery is often low (Table 2). It is therefore advisable to dilute concentrated urine samples with water before diffusion or to use a 48-hr. diffusion period.

Recovery and accuracy. Table 3 shows the recovery of acetate added to serum, urine and liver homogenate. In a series of analyses of the same serum sample the s.d. of the final reading corresponded to 0.22 $\mu\text{g.}$ of acetate ($n = 10$). As the s.d. on the blank value is of the same magnitude the overall s.d. is $0.22\sqrt{2} = 0.3 \mu\text{g./ml.}$ Direct analyses (without diffusion) of serum gave s.d. = 0.15 $\mu\text{g.}$ ($n = 19$). The absolute error is independent of the amount of acetate within the limits given.

Table 1. *Influence of volume of analysis and diffusion time on acetate recovery in aqueous solutions*

All analyses contained 10 $\mu\text{g.}$ of acetate in 0.05 M-citrate, pH 3. Results are given as percentage of the colour obtained by direct analysis with omission of the diffusion step.

Na ₂ SO ₄ in Conway unit (g.)	Volume analysed (ml.)	Recovery of acetate (%)	
		Diffusion for 24 hr.	Diffusion for 48 hr.
5	1	98	105
10	2	97	98
10	3	82	82

Table 2. *Analysis of urine*

The Conway units contained 5 g. of Na₂SO₄. Neutralized urine (1 ml.) buffered at pH 3 was used as described in the text. The results are based on simultaneously analysed aqueous acetate solutions.

Material	Acetate added ($\mu\text{g./ml.}$)	Diffusion for 24 hr.		Diffusion for 48 hr.	
		Concn. of acetate ($\mu\text{g./ml.}$)	Recovery (%)	Concn. of acetate ($\mu\text{g./ml.}$)	Recovery (%)
Conc. urine*	None	8	—	10	—
	20	23	75	29	95
Conc. urine*	None	1.6	—	3.0	—
	13	12.3	82	15.0	92
Dil. urine†	None	2.9	—	2.7	—
	20	22.7	100	22.3	98

* Morning urine. † Urine flow approx. 3 ml./min.

Table 3. *Recovery of acetate added to blood serum, urine and liver suspension*

The material was analysed in accordance with the description given in the text. The liver suspension was prepared by homogenization of rabbit liver in 4 vol. of water. In all experiments 1 ml. of deproteinized filtrate or buffered urine was used. The Conway units contained 5 g. of Na₂SO₄ in the outer ring. The diffusion time was 24 hr.

Material	No. of analyses	Acetate added ($\mu\text{g./ml.}$)	Acetate concn. measured ($\mu\text{g./ml.}$)	Recovery (%)
Blood serum	4	None	0.6	—
	10	40.3	41.5 (s.d. 0.6)	101.5
Urine	6	None	2.8	—
	4	20	22.6 (22–23)	99
Liver suspension	4	None	4.4	—
	8	40.3	45.6 (43–49)	102

Specificity. Table 4 shows the results of experiments with a number of substances likely to interfere in the acetate analysis. Among the fatty acids examined only propionic acid reacted, but to a very small extent. In fact a small contamination by acetate cannot be excluded even though twice-recrystallized calcium propionate was used. Acetoacetate reacts with the pigeon-liver enzyme, which contains an acetoacetate-activating enzyme system. Through the procedure described (diffusion and heating) acetoacetate is completely destroyed, as

Table 4. *Specificity of the sulphanilamide-acetylating enzyme system*

Aqueous solutions of the sodium salts (Ca salt in the case of propionic acid) of the acids examined (200 μ l.) were incubated with buffer-coenzyme mixture (250 μ l.) and enzyme (200 μ l.) for 1 hr., and the remaining sulphanilamide was determined as described in the text.

Substance	Colour as percentage of the colour obtained with equivalent amounts of acetate	
	Soodak (1957)	Present work
Formate	0.0	—
Propionate	0.7	1.4
Butyrate	1.3	0.1
Valerate	0.4	0.0
Pyruvate	0.0	—
Ethanol	0.0	0.0
β -Hydroxy- β -methylglutarate	—	0.0
Acetoacetate		
2.5 mg./ml.	—	90
5.0 mg./ml.	—	85
10 mg./ml.	—	84

Fig. 2. Influence of KCl (○), NaCl (▽) and CaCl₂ (●) on the acetylation of sulphanilamide. Values are given as percentage of the change in extinction obtained in control experiments without added salts.

shown in experiments with additions of up to 100 mg. of acetoacetate/100 ml. of plasma.

Inhibition. Fig. 2 shows the influence of some inorganic cations on the enzymic acetylation. Sodium and potassium inhibit to the same degree. Although the inhibition in these experiments is not very large, it is evident that the determination of small concentrations of acetate in material containing inorganic salts can only be performed after removal of the salts, e.g. through the diffusion step described.

Determination of acetate directly in blood plasma or serum. Under certain conditions it may be permissible to use plasma directly without any separation of protein or non-volatile matter. Table 5 shows recovery of acetate added to plasma and analysed directly.

DISCUSSION

Soodak (1957) has observed that the pigeon-liver preparation contains enzymes which hydrolyse acetylsalicylic acid. If the complete procedure is employed interference from this substance or other acetate esters will not be possible unless the substances in question are volatile. This possibility can probably be neglected.

Another enzyme that might interfere in the analysis if present is oxaloacetate transacetase which under the conditions employed (relatively high concentration of citrate and CoA) could cause formation of acetyl-CoA from citrate. When the procedures of Soodak (1957) are followed carefully this enzyme is absent from the final enzyme preparation.

The question arises whether any of the known forms of 'active' acetate are determined as acetate in this analysis. Acetyl-CoA, if present in the incubation mixture, would of course react with sulphanilamide, but when the complete procedure, including diffusion, is used acetyl-CoA will not interfere, as this substance is stable under the conditions used for deproteinization and diffusion (Stadtman, 1957).

Table 5. *Recovery of added acetate by direct analysis of plasma*

Blood plasma was incubated at 37° with buffer-sulphanilamide-coenzyme mixture (250 μ l.) and enzyme (200 μ l.). Sulphanilamide was determined as described in the text. Control experiments contained water instead of plasma.

Vol. of plasma or water (ml.)	Acetate added (μ g./ml.)	Extinction difference		Recovery (%)
		Water	Plasma	
0.2	None	—	0.018	—
	50	0.177	0.191	98
	90	0.297	0.320	102
0.1	None	—	0.024	—
	105	0.199	0.223	100

Acetyladenylic acid, which may be an intermediate in the activation of acetate (Berg, 1956), on the other hand is a labile substance, which is rapidly hydrolysed at pH 2. Nothing seems, however, to be known about the possible presence of acetyladenylic acid in biological material.

The method described has been used by us extensively for determination of acetate in liver suspensions (Lundquist, Fugmann, Klänning & Rasmussen, 1959) and in blood during alcohol metabolism in man, where the concentration of acetate reaches values of about 50 $\mu\text{g./ml.}$ (Fugmann *et al.* 1958; Lundquist, 1960). At concentrations of this magnitude the diffusion step may be omitted, when the concentration of acetoacetate can be shown to be small and constant.

SUMMARY

1. A specific enzymic method for the quantitative determination of acetate in the range 1–15 $\mu\text{g.}$ in a volume not exceeding 3 ml. is described.

2. The procedure involves diffusion in Conway microdiffusion units and reaction with sulphanilamide by means of a pigeon-liver enzyme preparation, which is stable at -20° for at least 1 year.

3. The method is highly specific for acetate. Interference from a number of substances examined is negligible.

4. The accuracy is about 0.3 $\mu\text{g.}$ (s.d.) independent of the concentration in the range covered.

This investigation has been partly supported through a grant from Statens almindelige Videnskabsfond.

REFERENCES

- Bartley, W. (1953). *Biochem. J.* **53**, 305.
 Berg, P. (1956). *J. biol. Chem.* **222**, 1015.
 Bratton, A. C. & Marshall, E. K., jun. (1939). *J. biol. Chem.* **128**, 537.
 Conway, E. J. (1957). *Microdiffusion Analysis*, 4th ed. London: Crosby, Lockwood and Son Ltd.
 Fugmann, U., Lundquist, F. & Rasmussen, H. (1958). *Abstr. Comm. 4th int. Congr. Biochem., Vienna*, no. 13–85, p. 175.
 Korff, R. W. von. (1954). *J. biol. Chem.* **210**, 539.
 Lundquist, F. (1960). *Acta physiol. scand.* **50**, suppl. 175, 97.
 Lundquist, F. (1961). *Methoden der enzymatischen Analyse* (in the Press). Ed. by Bergmeyer, H.-U. Heidelberg: Verlag Chemie.
 Lundquist, F., Fugmann, U., Klänning, E. & Rasmussen, H. (1959). *Biochem. J.* **72**, 409.
 Rose, I. A. (1955). In *Methods in Enzymology*, vol. 1, p. 591. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
 Serlin, I. & Cotzias, G. C. (1955). *J. biol. Chem.* **215**, 263.
 Soodak, M. (1957). In *Methods in Enzymology*, vol. 3, p. 266. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
 Soodak, M. & Lipmann, F. (1948). *Fed. Proc.* **7**, 190.
 Stadtman, E. R. (1957). In *Methods in Enzymology*, vol. 3, p. 931. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Biochem. J. (1961) **80**, 397

Studies on Vitamin A Esterase

5. A COMPARATIVE STUDY OF VITAMIN A ESTERASE AND CHOLESTEROL ESTERASE OF RAT AND CHICKEN LIVER*

By P. SESHADRI SASTRY AND J. GANGULY

Department of Biochemistry, Indian Institute of Science, Bangalore 12, India

(Received 29 December 1960)

Although esterases have received a great deal of attention for a long time, the existence of vitamin A esterase and cholesterol esterase was recognized only relatively recently; however, information about the identity and specificity of these two esterases does not seem to be available. Seshadri Sastry, Krishnamurthy & Ganguly (1957) reported the general properties of vitamin A esterase of rat liver and demonstrated by differential-inactivation studies that the common esterase (the enzyme hydrolysing short-chain esters of simple

alcohols, e.g. ethyl butyrate) and vitamin A esterase of the same tissue are not identical. All attempts at solubilization of vitamin A-esterase activity from rat liver were unsuccessful.

During the course of these investigations it was noticed that the common esterase, vitamin A-esterase and cholesterol-esterase activities of chicken-liver homogenate are distributed between the nuclear and microsomal fractions, whereas in rat-liver homogenates they are present only in the microsomal fraction (Krishnamurthy, Seshadri Sastry & Ganguly, 1958). Cases of bimodal distribution of enzymes, e.g. aryl sulphatases in the rat-

* Part 4: Mahadevan, Murthy, Krishnamurthy & Ganguly (1961).