the conversion of prothrombin to thrombin, and Ware, Guest & Seegers (1947a) have isolated such a factor by fractionation of prothrombin prepared by $\text{Mg}(\text{OH})_2$ adsorption. They suggest that this factor is not identical with any of Quick's prothrombins.

There seems to be no doubt that Quick's prothrombin A and Factor V are identical. The factor is concerned with the conversion of prothrombin to thrombin, however, and is not itself a component of prothrombin. The factor is partly adsorbed by $\text{Mg}(\text{OH})_2$, and prothrombin prepared by this method always contains some Factor V. (Prothrombin which is absolutely free of Factor V is not converted to thrombin by thrombokinase and Ca alone (Owren, 1944, 1947b, p. 186).) Factor V is precipitated by the ammonium sulphate fractionation used by Ware *et al.* (1947a) for isolating the accelerator factor from prothrombin preparations. The relatively slight activity of the material isolated (shortening of the conversion time to 3, 7 and 10 min.) indicates that the amount of Factor V is low.

The mode of action described by Ware *et al.* (1947b) for their accelerator factor, however, conforms with the previously described action of Factor V (Owren, 1944, 1945, 1947b), and there is no reason to believe that they are different substances.

The recorded properties of Factor V and experiments on a quantitative basis point to the fact that prothrombin A, Seegers's accelerator factor and Fantl & Nance's factor are identical with Factor V.

SUMMARY

1. A new method for the preparation of 'Factor V' is described, and its properties are outlined.

2. Experiments are submitted indicating that the prothrombin A of Quick (1943, 1946) and the accelerator factor of Fantl & Nance (1946) and of Ware *et al.* (1947a, b) are identical with Factor V.

The author is indebted to the Medical Research Council for a personal grant during the tenure of which part of the work described was carried out.

REFERENCES

- Banfi, R. F., Bay, R. & Tanturi, C. A. (1945). *J. Lab. clin. Med.* **30**, 512.
 Fantl, P. & Nance, M. (1946). *Nature, Lond.*, **158**, 708.
 Kekwick, R. A., Mackay, M. E. & Record, B. R. (1946). *Nature, Lond.*, **157**, 629.
 Link, K. P. (1945). *Fed. Proc.* **4**, 176.
 Loomis, E. C. & Seegers, W. H. (1947). *Amer. J. Physiol.* **148**, 563.
 Munro, F. L., Hart, E. R., Munro, M. P. & Walking, A. A. (1945). *Amer. J. Physiol.* **145**, 206.
 Munro, F. L. & Munro, M. P. (1947). *Amer. J. Physiol.* **149**, 95.
 Oneal, W. J. & Lam, C. R. (1945). *Amer. J. med. Sci.* **210**, 181.
 Owren, P. A. (1944). *Årb. norske VidenskAkad.* p. 21.
 Owren, P. A. (1945). *Nord. Med.* **28**, 2630.
 Owren, P. A. (1946). *Proc. Scand. Congr. intern. Med.* Gothenburg, 28 June.
 Owren, P. A. (1947a). *Lancet*, **1**, 446.
 Owren, P. A. (1947b). *Acta med. scand.*, Suppl. p. 194.
 Quick, A. J. (1943). *Amer. J. Physiol.* **140**, 212.
 Quick, A. J. (1946). *Proc. Soc. exp. Biol., N.Y.*, **62**, 249.
 Ware, A. G., Guest, M. M. & Seegers, W. H. (1947a). *J. biol. Chem.* **169**, 231.
 Ware, A. G., Guest, M. M. & Seegers, W. H. (1947b). *Science*, **106**, 41.
 Zondek, B. & Finkelstein, M. (1945). *Proc. Soc. exp. Biol., N.Y.*, **60**, 374.

The Detection of Creatine and Creatinine by Partition Chromatography

By G. A. MAW, *Department of Biochemistry, University College, London*

(Received 10 November 1947)

Methods commonly used for the identification and quantitative estimation of creatinine, based on the Jaffé reaction, suffer from the lack of specificity of this reaction. Hunter (1928) has listed 40 compounds which give a positive creatinine reaction with alkaline sodium picrate. The use of 3:5-dinitrobenzoic acid (Benedict & Behre, 1936), although it provides a slightly more specific reaction, introduces the serious complications of colour fading and sensitivity of the coloured product to the presence

of other substances. The picric acid method of estimation is reliable, however, if precautions are taken to exclude the interference of other chromogenic substances, or when combined with specific enzymic destruction of creatinine by *Corynebacterium ureafaciens* (Miller & Dubos, 1937; Krebs & Eggleston, 1939).

The estimation of creatine in the presence of creatinine by the picric acid or 3:5-dinitrobenzoic acid methods has the added disadvantage of being

a method of difference. This is emphasized in the identification of small amounts of creatine in the presence of creatinine in circumstances where the Barritt diacetyl reaction for creatine (Eggleton, Elsdon & Gough, 1943) cannot be used and the Jaffé test only can be applied, since the detection must necessarily involve a quantitative determination.

The technique of partition chromatography on paper, for the study of amino-acids (Consden, Gordon & Martin, 1944), may be applied to the examination of inorganic and organic urinary constituents, including creatinine (Dent, 1947). The movements of these substances on paper chromatograms are sufficiently specific to permit their recognition. This principle has been utilized in the qualitative identification of creatine and creatinine on the basis of differences in their physicochemical rather than chemical properties, upon which present analytical procedures rely. Creatinine may be detected in biological fluids in amounts down to 1 μ g. Further, creatine may be detected in the presence of creatinine without a quantitative analysis.

METHODS

The experimental procedure employed was essentially that of Dent (1946), temperature control throughout experiments being maintained by placing the apparatus in a water thermostat.

For the detection of creatinine, enough of the test solution to contain 5–10 μ g. creatinine was pipetted in portions on to paper strips, and the water dried off. The solvent was allowed to travel 25–30 cm. down the strip, the time taken for the solvents collidine and *sec.*-butanol being c. 24 hr. After the strip had been dried at 100° for 30 min., creatinine was located by spraying first with 2*N*-NaOH, then with saturated picric acid from an atomizer. The resulting orange band was generally c. 10 mm. in width. In determining R_f values (Consden *et al.* 1944) it was found necessary to mark the position of the band soon after its appearance, since considerable or complete fading of the colour usually took place in sunlight.

For the detection of creatine, the procedure was similar except that the strip was heated at 110° for 3 hr. to convert any creatine present to creatinine. Subsequent spraying of the strip with NaOH and picric acid indicated the presence of a creatine band, detected as creatinine. An alternative method, described by Maw (1947) for the conversion of creatine to creatinine by spraying the strip with mineral acid and heating to dryness at 100°, has not proved entirely satisfactory, owing to the strips becoming brittle and occasionally charred. The direct heating method has the advantage of simplicity, with the desirable minimum of manipulation.

RESULTS

Constancy of the factors influencing the movement of a compound on a chromatogram, listed by Consden *et al.* (1944), was maintained during runs. Comparison of the R_f values of bands obtained from test material was made with those of creatine and

creatinine added to the test material. All R_f values quoted represent the average of 6–12 determinations, deviations from mean values being at the most $\pm 5\%$ and generally not more than $\pm 2\%$.

R_f values of creatine and creatinine. In order to obtain a separation between creatine and creatinine, several solvents were tried, *sec.*-butanol and collidine giving satisfactory results (Table 1). Creatine and creatinine showed no movement in *n*-pentanol, whereas in phenol creatinine moved as fast as the solvent itself. In *n*-butanol the R_f value of creatine is too low to be of practical use.

Table 1. R_f values of creatine and creatinine in various solvents at 17°

(20 μ l. of 0.1% aqueous solution used.)

Compound	Solvent		
	<i>n</i> -Butanol	<i>sec.</i> -Butanol	Collidine
Creatine	0.04	0.18	0.19
Creatinine	0.20	0.33	0.46

Table 2. Effect of temperature on R_f values of creatine and creatinine

(20 μ l. of 0.1% aqueous solution used.)

Temperature	Solvent: <i>sec.</i> -butanol		Solvent: collidine	
	Creatine	Creatinine	Creatine	Creatinine
17°	0.18	0.33	0.19	0.46
20°	—	—	0.14	—
25°	0.17	0.29	0.11	0.40

A rise in temperature produced a general decrease in R_f values (Table 2). This is in part due to the influence of temperature on the composition of the solvent and water phases, and the consequent distribution of the compounds between the phases. The effect of extraneous salts may be explained similarly. The effect is more marked with collidine as solvent, the composition of the solvent phase changing from 41.7 to 62.8% collidine over the temperature range 10–30°, in contrast to the change for the *sec.*-butanol-water system of 61.7 to 65.0% *sec.*-butanol. Without temperature control, variations of 40% between values have been obtained.

In Table 3 are given R_f values for creatine and creatinine determined in pure solution, in rat urine and in aqueous muscle extracts. Chromatograms of rat urines gave a single band when the strips were simply dried free of solvent, and an additional slower moving band when the strips were heated at 110°. Chromatograms run with added creatine and creatinine showed that the bands obtained from urine alone were attributable to these compounds. The presence of urinary constituents produced distinct effects on the R_f values of the two compounds, the presence of creatinine in large excess, however,

having no effect on the movement of creatine. The solubility of creatine in water is known to be increased by the presence of urinary constituents and sodium salts as a result of salt formation. An effect on the partition coefficient of this substance between organic solvents and water would therefore be expected.

Table 3. R_F values of creatine and creatinine at 17°

(20 μ l. of solution used.)

	Solvent		
	<i>n</i> -Butanol	<i>sec.</i> -Butanol	Collidine
Creatine:			
0.1% Solution	0.04	0.18	0.19
0.025% Solution containing 0.5% creatinine	—	0.18	—
Rat urine (band I)	—	0.17	0.13
0.1% Creatine added to rat urine	c. 0	0.16	0.13
5% Aqueous muscle extract	—	0.16	—
Creatinine:			
0.1% Solution	0.20	0.33	0.46
Rat urine (band II)	0.17	0.29	0.57
0.1% Creatinine added to rat urine	0.17	0.28	0.57

The bands obtained with rat urine were considerably broader than those from the pure solutions, and were much less sharply defined at the edges. Apart from the amount of material used, the width of bands is determined by the presence of salts which tend to distort the bands, either by salt formation with the compound being chromatographed, or by the production of local alterations in solvent and water-phase composition on the strip. As might be anticipated from its amphoteric nature, creatine is the more affected in this way.

R_F values have been determined on pure solutions containing 4–100 μ g. of creatinine. Over this range the values were not affected by the amount of compound used. Creatine and creatinine were easily detectable in amounts down to 1 μ g.

R_F values of other 'Jaffé-positive' substances. The majority of substances other than creatinine giving the Jaffé reaction may be recognized by further chemical tests, but the detection of creatinine at the same time presents numerous difficulties. These are to a large extent overcome when test solutions are examined by partition chromatography. 'Jaffé-positive' substances such as acetone, acetaldehyde, glyoxal, etc., are eliminated by volatilization during the drying of the paper strips. 'Jaffé-positive' inorganic salts, e.g. ferrous sulphate, showed little or no movement in the presence of organic solvents, and were detectable only in relatively large amounts. Pyruvic and acetoacetic acids gave R_F values close

to those of creatine, but quite distinct from those of creatinine (Table 4). Since these substances give a direct Jaffé reaction, there is little likelihood of confusion with creatine. Pyruvic acid did not give bands when present in amounts much less than 100 μ g. The test was more sensitive for acetoacetic acid, but the resulting bands faded rapidly.

Table 4. R_F values of some 'Jaffé-positive' substances in pure solution at 17°

(20 μ l. of solution used.)

Compound	Solvent	
	<i>sec.</i> -Butanol	Collidine
Pyruvic acid (0.5%)	0.18	—
Acetoacetic acid (0.1%)	0.21	—
Glycocyamidine (0.1%)	0.30	0.43
		(0.37 at 25°)

Glycocyamidine was not converted to glycocyamidine on paper strips after heating for several hours at 110°. Glycocyamidine, however, gave R_F values in collidine and *sec.*-butanol close to those of creatinine. No separation between creatinine and glycocyamidine on chromatograms has been obtained with the solvents used, mixtures of the two compounds giving rise to one broad band. The bands obtained from equivalent amounts of the separate compounds were considerably different in their rates of colour development with alkaline sodium picrate. The creatinine band gave an intense and almost maximal colour within a few seconds. The glycocyamidine band gave little or no colour in this time, and required at least 10 min. for strong colour development. The various alkyl and acyl derivatives of creatine and creatinine have not been tested.

DISCUSSION

In the identification of creatine and creatinine on the basis of partition chromatography, emphasis is transferred from the lack of specificity of the Jaffé reaction to the similarity in chromatographic behaviour of 'Jaffé-positive' substances present in the test material to that of the pure compounds. It has been shown that one compound most likely to interfere with the detection of creatinine is glycocyamidine, although in the amounts used the two compounds have very different rates of colour formation, which might be used to distinguish the major component of a mixture of both compounds.

The R_F values of creatine and creatinine are sufficiently far apart to give clear-cut separation of the two compounds, and to enable creatine, in the presence of its anhydride, to be identified in mixtures in which the creatine content is 1% of the creatinine present or less. The limit of satisfactory detection of either substance by the Jaffé reaction is 1 μ g. With

dilute solutions, the initial pipetting of large volumes on to chromatograms may lead to the accumulation of enough salts seriously to disturb band formation and movement. For creatinine detection under these circumstances, preliminary adsorption on to Lloyd's reagent (Behre & Benedict, 1922) is suggested.

The methods described have not been extended beyond the qualitative detection of creatine and creatinine. Preliminary experiments indicate that it is doubtful whether the direct application of the Jaffé reaction to paper chromatograms can be made sufficiently quantitative, owing to the variations obtained in the width of bands and the difficulty of spraying on standard amounts of the reagents. However, the bands of both compounds, in the particular solvents used, are discrete enough for their positions to be calculated from R_f values which have been determined from duplicate experi-

ments, the bands cut out of the strips and the creatinine extracted from the paper and estimated photocolometrically. This principle has been applied with success by Flood, Hirst & Jones (1947) in the chromatographic identification and estimation of sugars.

SUMMARY

1. A method is described for the identification of creatine and creatinine in biological material in amounts down to 1 μ g.

2. Creatine may be detected in the presence of large amounts of creatinine without resort to a quantitative estimation.

3. The effects of various factors influencing the reproducibility of R_f values are discussed.

The author wishes to thank Dr H. King for the gift of a sample of glycoeyamidine, Dr E. Work for helpful advice and Miss E. Large for technical assistance.

REFERENCES

- Behre, J. A. & Benedict, S. R. (1922). *J. biol. Chem.* **52**, 11.
Benedict, S. R. & Behre, J. A. (1936). *J. biol. Chem.* **114**, 515.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
Dent, C. E. (1946). *Lancet*, **2**, 637.
Dent, C. E. (1947). *Biochem. J.* **41**, 240.
Eggleston, P., Elsdon, S. R. & Gough, N. (1943). *Biochem. J.* **37**, 526.
Flood, A. E., Hirst, E. L. & Jones, J. K. N. (1947). *Nature, Lond.*, **160**, 86.
Hunter, A. (1928). *Creatine and Creatinine*. London: Longmans, Green and Co.
Krebs, H. A. & Eggleston, L. V. (1939). *Enzymologia*, **7**, 310.
Maw, G. A. (1947). *Nature, Lond.*, **160**, 261.
Miller, B. F. & Dubos, R. (1937). *J. biol. Chem.* **121**, 447.

The Fate of Ingested Creatinine in the Rat

By G. A. MAW, *Department of Biochemistry, University College, London*

(Received 2 February 1948)

The published observations on the excretion of ingested creatinine point to a large and unaccounted loss of this substance when it is administered to rat, dog and man, generally not more than 80% of a given dose being excreted in the urine (Hunter, 1928; Wang, 1939; Beard, 1943). Since there appear to be no concomitant changes in blood urea (Folin & Denis, 1912) or in the output of urinary urea and ammonia (Rose & Dimmitt, 1916), the fate of the unrecovered fraction of the dose has been the subject of some speculation. Claims have been made that creatinine retention takes place, or that conversion to creatine occurs (Beard & Jacob, 1939; Beard, 1943). That losses might result from bacterial decomposition in the gut has been suggested (Beard, 1943). In the earlier studies there is no evidence that intestinal absorption of an oral dose was complete and the likelihood of loss of part of a dose by

excretion in the faeces does not seem to have been considered. Dominguez & Pomerene (1945) found that in a human subject with an ileal fistula, 75% of an oral dose was recovered from the urine, in agreement with other published data on man, but, in addition, that intestinal absorption was incomplete, creatinine being recovered in the material from the fistula to the extent of 25%, sufficient to account for the apparent 'loss' of creatinine referred to by previous workers.

A study has therefore been made of creatinine absorption in the rat in order to determine whether partial recovery of ingested doses may again be explained on the basis of incomplete intestinal absorption. A series of *in vitro* experiments has also been carried out to examine the possibility of bacterial destruction of creatinine in the gut.