- Bauer, G. C. H., Carlsson, A. & Lindquist, B. (1955c). *Acta physiol. scand.* **35**, 56.
- Bauer, G. C. H., Carlsson, A. & Lindquist, B. (1956). *Metabolism* (in the Press).
- Carlsson, A. (1951). *Acta pharm. tox., Kbh.*, **7**, suppl. 1.
- Clark, E. P. & Collip, J. B. (1925). *J. biol. Chem.* **63**, 461.
- Hamilton, J. G. (1947). *Radiology*, **49**, 328.
- Kolthoff, I. M. & Sandell, E. B. (1945). *Textbook of Quantitative Inorganic Analysis*, p. 593. New York: The Macmillan Co.
- Spencer, H. (1955). *Dynamics of Calcium and Strontium Metabolism in Man*. In *Gordon Research Conf. on the Structure, Chemistry and Physiology of Bones and Teeth*. U.S.A.: N. H. Meriden.

Colorimetric Estimation of Ketopentoses and Ketohehexoses

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(Received 6 October 1955)

The introduction of the cysteine-carbazole reaction by Dische & Borenfreund (1951) has greatly simplified the estimation of ketopentoses in the presence of aldopentoses. In this reaction ketopentoses give a purplish-red colour ($\lambda_{\max}$ 540 m μ .) with little interference from aldopentoses; but a disadvantage of the method is that ketohehexoses ($\lambda_{\max}$ 560 m μ .) interfere with the estimation of ketopentoses.

Cohen (1953) and Dische (1951), using respectively the techniques of Roe (1934) and of Roe, Epstein & Goldstein (1949), have reported that ketopentoses give a green colour in the resorcinol reaction. The absorption spectrum of the green colour developed with both these methods appears to be unsuitable for the estimation of ketopentose in the presence of ketohehexose. Using the resorcinol method of Cole (see Bacon & Bell, 1948), McCorkindale & Edson (1954) also noticed that ketopentoses gave a green colour ($\lambda_{\max}$ 640 m μ .), whereas ketohehexoses gave a red colour ($\lambda_{\max}$ 480 and 415 m μ .).

Since these differences seemed to afford a basis for the estimation of ketopentoses and ketohehexoses in the same solution, the conditions of the resorcinol reaction have been investigated and a satisfactory quantitative method has been developed.

MATERIALS AND METHODS

D-Xylulose was prepared according to Levene & Tipson (1936). The monoacetone xylulose obtained (m.p. 68°) was shown to be free from other sugar derivatives by paper chromatography with ethyl acetate-acetic acid-formic acid-water (18:3:1:4, by vol.) as the mobile phase (Hough & Jones, 1953) and *m*-phenylenediamine dihydrochloride (Chargaff, Levine & Green, 1948) as spray reagent. Hydrolysis of the monoacetone derivative yielded xylulose as a colourless syrup, $[\alpha]_D^{25} + 34.8 \pm 0.6^\circ$ in water (*c*, 2.42). No aldose could be detected in the free sugar by paper chromatography with ethyl acetate-pyridine-water (Isherwood & Jermyn, 1951) as the mobile phase and aniline hydrogen phthalate (Partridge, 1949) as the spray. All solutions of

xylulose were standardized with periodate (Salo, 1953). After storage for about 2 months at 4° a solution of xylulose gave approximately 5% less colour than a portion of the same solution stored at -35°.

D-Tagatose (a gift from Mr D. R. D. Shaw) was prepared according to Reichstein & Bosshard (1934) and had m.p. 130° $[\alpha]_D^{25} - 2.9 \pm 0.3^\circ$ in water (*c*, 5.03). Paper chromatography showed the absence of aldose.

D-Fructose, L-sorbose and dihydroxyacetone were obtained from Pfanstiehl Chemical Co., U.S.A. and mannoheptulose from General Biochemicals, Inc., U.S.A. Perseulose hemihydrate was a gift from Dr N. K. Richtmyer. A solution of L-ribulose was prepared by oxidation of ribitol with an enzyme preparation made from *Acetobacter suboxydans* (Arcus & Edson, 1954).

Ketose reagent. This was a modification of Cole's (Bacon & Bell, 1948) reagent, and consisted of two solutions: A, resorcinol, 0.05% (w/v) in absolute ethanol and B, HCl (sp.gr. 1.18) containing $\text{FeNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$, 0.216 g./l. This reagent was used in all experiments unless stated otherwise.

General procedure for ketose estimation. A solution (2 ml.) containing 5–50 μg . of ketose/ml. is placed in a 12 mm. $\times$ 125 mm. test tube. To this are added 3 ml. of solution A, followed by 3 ml. of solution B. The contents of the tubes are mixed thoroughly and the tubes, covered with glass marbles, are immersed in a water bath at $80^\circ \pm 3^\circ$ for 40 min. (60 min. for sorbose). A reagent blank containing 2 ml. of water instead of ketose solution is included with each batch of tubes. Unknown solutions are compared with standards, extinctions being measured at specific wavelengths in a Beckman quartz spectrophotometer Model D.U., Corex cells with a 1 cm. light-path being used.

When ketohehexose alone is being estimated, tubes are placed in the bath simultaneously and cooled together in ice water at the end of the heating period. The extinction at 480 m μ . is stable for more than 5 hr. If ketopentose is present, tubes are placed in the bath in groups of three at 10 min. intervals, removed after 40 min. and cooled in ice water for 2 min. Extinctions at 640 m μ . are measured 5 min. after removal of the tubes from the bath by reading against the reagent blank heated with the first three tubes. This procedure avoids error due to the instability of the ketopentose colour.

RESULTS

Investigation of the resorcinol reaction under the conditions of Cole

When the fructose colour reaction was carried out under the conditions of Cole (Bacon & Bell, 1948), the red colour produced after heating the tubes for 30 min. at 75° had a two-banded absorption spectrum with one maximum at 480 m μ . and a lower maximum at 415 m μ . (cf. Roe *et al.* 1949). Small fluctuations in heating time and temperature caused considerable differences in $E_{480 \text{ m}\mu}$. When the temperature was raised to 80°, $E_{480 \text{ m}\mu}$ reached a constant value after 35 min., although $E_{415 \text{ m}\mu}$ continued to increase. Values obtained by measuring $E_{480 \text{ m}\mu}$ after heating for 40 min. at 80° were reproducible. Under the latter conditions $E_{415 \text{ m}\mu}$ was greater than $E_{480 \text{ m}\mu}$ but, as it was markedly affected by small differences in heating conditions, it was less suitable for the measurement of fructose concentration.

Investigation of the green colour (λ_{max} 640 m μ .) produced when xylulose was heated with Cole's reagents under these conditions revealed three serious disadvantages as far as the estimation of xylulose is concerned. The extinction produced by xylulose at 640 m μ . is only one-fifth of that produced by an equal concentration of fructose at 480 m μ .; solutions of xylulose deviate markedly from Beer's law in the 640 m μ . region; and the $E_{480 \text{ m}\mu}$ of xylulose is considerable (one-tenth of that of an equal concentration of fructose). It seemed desirable to find conditions more favourable for the estimation of ketopentoses.

On treatment with Cole's reagents, monoacetone xylulose gave a colour which was identical with that due to xylulose. As monoacetone xylulose is crystalline and stable, offering a convenient way of preparing xylulose-containing solutions, it was used for determination of the optimum conditions for the development of the green colour. When, however, free xylulose was tested with reagents giving optimum colour with the monoacetone derivative, the intensity of the colour given by the former compound, expressed as $E_{640 \text{ m}\mu}$, was 1.15 times as great as that given by an equivalent concentration of the latter. Since the reagents giving optimum colour with the monoacetone derivative proved to be satisfactory for the estimation of xylulose, the optimum conditions for the latter were not determined.

Optimum conditions for xylulose estimation

Concentration of Fe³⁺. Table 1 shows the effect of increasing ferric-ion concentration on the intensity of colour obtained with a solution of monoacetone xylulose. The ferric ammonium sulphate used in the

modified Cole's reagent gives approximately the same ferric-ion concentration (25 mg./l.) as 75 mg. of FeCl₃/l.

Concentration of resorcinol. Maximum colour was obtained when the resorcinol concentration was between 0.05 and 0.025 % (Table 2). The extinctions were consistently lower when the same experiment was carried out with hydrochloric acid containing 7.5 mg. of ferric chloride/l. (the concentration in Cole's reagent). The intensity of the colour developed with fructose was not markedly decreased until the resorcinol concentration was lowered to 0.025 %.

Behaviour of sugars with the modified ketose reagent

Xylulose. The absorption spectrum of the characteristic green xylulose reaction product is shown in Fig. 1. The time course of colour formation is illustrated in Fig. 2. Although the $E_{640 \text{ m}\mu}$ was only 95 % maximal after heating for 40 min., results with this heating period were consistently reproducible. Beer's law is obeyed at 480 m μ . but not at 640 m μ . (Fig. 3). The deviation from Beer's law is, however, much less marked than with Cole's reagents, and if unknown solutions are compared with standards of similar strength, little error will be introduced by assuming Beer's law to be obeyed over a small concentration range. If greater accuracy is desired a series of standards may be

Table 1. *Effect of ferric-ion concentration on intensity of xylulose colour reaction*

Tubes containing 2 ml. of monoacetone xylulose solution (30 $\mu\text{g./ml.}$), 3 ml. of resorcinol 0.15 % (w/v) in absolute ethanol and 3 ml. of conc. HCl containing FeCl₃, as indicated, were heated for 40 min. at 80°. Each tube was read against a reagent blank of the same reagent.

Ferric chloride (mg./l.)	$10^3 E_{640 \text{ m}\mu}$	$10^3 E_{480 \text{ m}\mu}$
7.5	109	57
37.5	147	71
75	158	95
150	160	105

Table 2. *Effect of resorcinol concentration on intensity of xylulose colour reaction*

Tubes containing 2 ml. of monoacetone xylulose solution (15 $\mu\text{g./ml.}$), 3 ml. of ethanol containing resorcinol as indicated and 3 ml. of conc. HCl containing 75 mg. of FeCl₃/l. were heated for 40 min. at 80°. Each tube was read against a reagent blank of the same reagent.

Resorcinol (%, w/v)	$10^3 E_{640 \text{ m}\mu}$	$10^3 E_{480 \text{ m}\mu}$
0.01	112	50
0.025	127	56
0.05	125	55
0.10	102	55
0.25	40	38
0.50	13	17

heated with the unknown and used to construct a standard curve. Extinction values obtained with the same batch of reagents showed remarkable consistency, but differed by as much as 20 % from extinction values obtained with reagents prepared at a different time. These inconsistencies were traced to variations in the quality of the absolute ethanol used.

Ribulose. A green colour was produced having an absorption maximum at 640 $m\mu$. and an absorption curve similar to that of xylulose. McCorkindale (1952) states that ribulose gives approximately the same intensity of colour as xylulose.

Fig. 1. Absorption spectra of the reaction products formed after heating with the modified ketose reagent for 40 min. at 80°. ●, Fructose (25 $\mu\text{g./ml.}$); ○, xylulose (24 $\mu\text{g./ml.}$); ×, perseulose hemihydrate (26.6 $\mu\text{g./ml.}$).

Fig. 2. Change in extinction with heating time of ketose solutions heated with the modified ketose reagent at 80°. Tubes were set up in pairs, one tube containing ketose solution and reagents and the other being a reagent blank. Pairs of tubes were removed from the bath after the times indicated and cooled in ice water; the extinction at 480 $m\mu$. of the ketose reaction product was read against the reagent blank. ●, Fructose (25 $\mu\text{g./ml.}$); ●, tagatose (25 $\mu\text{g./ml.}$); ○, sorbose (25 $\mu\text{g./ml.}$); △, xylulose (24 $\mu\text{g./ml.}$); ×, $E_{640 m\mu}$ for xylulose.

Fructose. The spectral characteristics of the fructose reaction product (Fig. 1) are essentially the same as were obtained with Cole's reagents under the same conditions of heating. As with Cole's reagents $E_{480 m\mu}$ was more satisfactory than $E_{415 m\mu}$ as a measure of fructose concentration. The time course of colour development is shown in Fig. 2. Beer's law is obeyed within a concentration range of 0–60 $\mu\text{g./ml.}$ (Fig. 4). Inulin gives a colour the intensity of which is 93 % of that given by an equivalent concentration of fructose, but its behaviour is in other respects identical.

Sorbose and tagatose. The absorption spectra of the red colours developed are similar to that of fructose, the values of $E_{480 m\mu}$ for sorbose and tagatose being respectively 73 and 83 % of that given by an equal concentration of fructose under the same conditions of heating. With a 40 min. heating period the sorbose colour obeys Beer's law within a concentration range of 0–30 $\mu\text{g./ml.}$, but this range can be increased to 0–100 $\mu\text{g./ml.}$ (Fig. 4) by heating for 60 min. (Fig. 2). The tagatose colour obeys Beer's law within a concentration range of 0–60 $\mu\text{g./ml.}$ (Fig. 4).

Perseulose and mannoheptulose. It was observed in this Laboratory (McCorkindale & Edson, 1954) that when ketoheptoses are heated with Cole's reagents a purple-red colour is developed. With Cole's and the modified reagent the purple-red colour was transient and insensitive as a test for

Fig. 3. Change in extinction with concentration of xylulose. Solutions were treated with the modified ketose reagent according to the standard procedure. ●, $E_{640 m\mu}$; ○, $E_{480 m\mu}$.

ketoheptose. After heating for 10 min. the colour became brown-red and then brown. The absorption spectrum of the perseulose reaction product after heating for 40 min. (Fig. 1) has maxima at 440 and 615 $m\mu$. The reaction product of mannoheptulose has absorption maxima at 430 and 615 $m\mu$; but the ratio of the absorption at 430 $m\mu$. to the absorption at 615 $m\mu$. is much higher than in the case of perseulose. Beer's law is obeyed by both compounds within a concentration range of 0–100 $\mu g./ml$. (Fig. 4).

The presence of ketoheptose interferes considerably with the estimation of both ketohexose and ketopentose. The values of $E_{480\ m\mu}$. and $E_{640\ m\mu}$., determined with a mixture of approximately equal concentrations of xylulose and perseulose, were equal to the sum of the extinctions at the respective wavelengths observed with the two sugars separately. The values of $E_{480\ m\mu}$. for fructose and perseulose were similarly additive. If the concentration

of ketoheptose can be determined independently, appropriate corrections may be applied.

Dihydroxyacetone. This gives a pink colour (λ_{max} . 520 $m\mu$.), which interferes slightly with the estimation of fructose (8% error due to a twofold excess). Values for xylulose tend to be low if dihydroxyacetone is present.

Other sugars. Glucose, galactose and lactose produce approximately 1% of the colour of an equal concentration of fructose. They do not interfere measurably with the estimation of ketopentoses.

Aldopentoses give a green colour. The intensities of absorption at 640 $m\mu$. for arabinose, xylose and ribose respectively are 0.8, 2.3 and 3.4% of that due to an equal concentration of xylulose; at 480 $m\mu$. the intensities are 0.3, 0.9 and 1.4% respectively of that due to an equal concentration of fructose.

Sorbitol, mannitol and gluconic acid give no appreciable colour.

Stability of colour

The extinction at 480 $m\mu$. of the fructose reaction product remained unaltered after standing for 5 hr. at room temperature in dim light. The value of $E_{640\ m\mu}$. for the xylulose reaction product, on the other hand, decreased after 0.5 hr. to 97%, after 1 hr. to 95% and after 5 hr. to 86% of its initial value.

Accuracy of the method

An analysis of the results obtained when twenty tubes containing equal amounts of ketose were treated at the same time is shown in Table 3.

Estimation of fructose and xylulose in a binary mixture

The protocol of an experiment showing recoveries of fructose and xylulose in presence of each other is given in Table 4. The value of $E_{640\ m\mu}$. was taken to be due only to xylulose. By comparison with a standard xylulose solution of similar strength the concentration of xylulose in the unknown and its contribution to $E_{480\ m\mu}$. could be calculated. Subtraction gave the $E_{480\ m\mu}$. due to fructose.

A more rapid method for finding both the xylulose concentration and its contribution to $E_{480\ m\mu}$., as well as the concentration of fructose, is to use standard extinction-concentration curves (Figs. 3, 4); particular care must be taken to ensure uniformity of the reagents and conditions of reaction. In view of the inconsistencies observed in the intensity of the colour given by xylulose with different batches of reagents, new standard curves should be constructed for each batch of reagents.

Accurate estimation of xylulose without correction of $E_{640\ m\mu}$. is possible only if there is less than

Fig. 4. Change in extinction with concentration of ketose. Solutions were treated with the modified ketose reagent according to the standard procedure. The heating time was 40 min., except for sorbose (60 min.). ●, $E_{480\ m\mu}$. fructose; ○, $E_{480\ m\mu}$. tagatose; □, $E_{480\ m\mu}$. sorbose; ×, $E_{440\ m\mu}$. perseulose hemihydrate; △, $E_{430\ m\mu}$. mannoheptulose. Broken lines represent straight lines required by Beer's law.

Table 3. *Error of estimations with the modified ketose reagent*

Solutions of fructose (25 $\mu\text{g./ml.}$) and of xylulose (16 $\mu\text{g./ml.}$) were treated with ketose reagent according to the standard procedure. For each ketose 20 tubes were heated at the same time.

	Fructose $E_{480 \text{ m}\mu}$	Xylulose	
		$E_{640 \text{ m}\mu}$	$E_{480 \text{ m}\mu}$
Mean extinction	0.444	0.245	0.099
Range of extinctions	0.434–0.454	0.237–0.250	0.091–0.106
S.E.	± 0.0045	± 0.0031	± 0.0043
Percentage error	± 1.0	± 1.3	± 4.3

Table 4. *Estimation of fructose and xylulose in a binary mixture*

Tubes containing 2 ml. of ketose solution were treated with the ketose reagents according to the standard procedure. Ketose concentrations were calculated as described in the text with standards (Std.) whose extinctions are included in the table for comparison.

Xylulose ($\mu\text{g./ml.}$)	Fructose ($\mu\text{g./ml.}$)	$10^3 E_{640 \text{ m}\mu}$	$10^3 E_{480 \text{ m}\mu}$	Xylulose found ($\mu\text{g./ml.}$)	Xylulose recovered (%)	Fructose found ($\mu\text{g./ml.}$)	Fructose recovered (%)
8	0	126	48	Std.	(100)	—	—
16	0	241	100	Std.	(100)	—	—
32	0	455	195	Std.	(100)	—	—
0	5	3	46	—	—	Std.	(100)
8	5	128	93	8.1	101.5	4.8	96
16	5	244	143	16.2	101	4.7	94
32	5	455	245	32.0	100	5.4	108
0	25	16	441	—	—	Std.	(100)
8	25	136	498	8.6	108	25.3	101
16	25	246	535	16.3	102	24.7	99
32	25	451	621	31.7	99	24.2	97
0	50	48	875	—	—	Std.	(100)
8	50	155	922	9.8	123	49.5	99
16	50	266	975	17.6	110	49.6	99
32	50	468	1057	32.8	103	49.0	98

twice as much fructose present. If the ratio of fructose to xylulose is greater than 2:1, empirical corrections must be determined.

Recoveries of ketoses added to blood

Fresh oxalated human blood to which NaF had been added was used. The Somogyi (1945) method of deproteinization was satisfactory for the estimation of fructose but recoveries of xylulose were only 70–80%. Satisfactory recoveries of both sugars were obtained when metaphosphoric acid was used as deproteinizing reagent (Table 5). Recoveries would tend to be low if the original concentration of ketopentose in undiluted blood was less than 100 $\mu\text{g./ml.}$ When metaphosphoric acid is used the 'apparent fructose concentration' of normal blood, based on the measurement of $E_{480 \text{ m}\mu}$, is about twice that found with the Somogyi reagents, and may probably be ascribed, at least in part, to non-ketose materials in the filtrate. In determining the recoveries of xylulose the small value of $E_{640 \text{ m}\mu}$ (0.006) observed with normal blood was ignored.

Table 5. *Recoveries of ketoses from blood with metaphosphoric acid as deproteinizing agent*

Blood (1 ml.) was laked in 8 ml. of water containing the ketose and 1 ml. of freshly prepared 7% (w/v) metaphosphoric acid was added with shaking. The precipitated proteins were removed by filtration. Ketoses were estimated by the standard procedure (see Methods) with a distilled-water reagent blank. The ketose added is expressed in terms of undiluted blood.

Ketose added ($\mu\text{g./ml.}$)	Ketose found ($\mu\text{g./ml.}$)	Ketose recovered (%)
Fructose		
0	32	—
50	85	106
100	129	97
250	282	100
500	529	99.5
1000	1047	101.5
Xylulose		
40	36	90
80	73	91.5
240	236	98.5
600	596	99
1200	1207	100.5

This extinction, being non-additive with respect to the ketopentose extinction, is apparently due to substances other than ketopentose.

Estimation of ketoses in urine

The method has not been applied successfully to the estimation of fructose in urine owing to the high concentration of interfering substances normally present. The value of $E_{480\text{ m}\mu}$ for normal urine treated with the ketose reagents is of the order of 0.3. The acid-washed activated charcoal method (Roe, 1934) was only partially effective in removing interfering substances, and recoveries of fructose from urine treated in this manner were low. As normal urine gave with the ketose reagents an $E_{640\text{ m}\mu}$ value of the order of 0.03, recoveries of xylulose from untreated urine were not attempted. Recoveries of xylulose from urine after treatment by the acid-washed charcoal method were low.

DISCUSSION

As a means of estimating fructose the procedure described above compares favourably with methods in current use. Under the new conditions of heating Cole's reagent and the modified reagent have the same sensitivity, which is about 1.2 times that of Cole's original procedure (Bacon & Bell, 1948). The sensitivity of the colour reaction is greater than that of the resorcinol reaction of Roe *et al.* (1949), approximately equal to that of the diphenylamine method of Rolf, Surtshin & White (1949) and is exceeded only by that of the cysteine-carbazole reaction (Dische & Borenfreund, 1951). The interference due to glucose in presence of an equal concentration of fructose causes an error of 1%. Errors due to glucose interference given by other authors are approximately 0.7% (Dische & Borenfreund, 1951), 0.6% (Roe, 1934) and 0.05% (Roe *et al.* 1949). The last value has been questioned by Rolf *et al.* (1949), who found with the same technique an error of about 2%. These workers describe a diphenylamine reaction in which glucose gives about 1.5% of the colour of an equal concentration of fructose. Pogell (1954) describes a method of estimating fructose by the skatole reaction, in which glucose interference is less than 0.05%, but this has the disadvantage of being much less sensitive than the other methods mentioned above.

The present method allows accurate estimation of fructose in the presence of ketopentose. Dische (1951) reports interference due to ketopentose in the resorcinol (Roe *et al.* 1949) and diphenylamine (Dische, 1929) reactions to be 33 and 9% respectively in the presence of the same concentration of fructose. In the present work it was found that very little colour was produced by xylulose when the usual 0.05% ethanolic resorcinol solution was

replaced by one containing 1.0%, and the colour produced by fructose remained almost unchanged. This observation suggests a method of estimating fructose in the presence of ketopentose which eliminates the necessity of correcting for the latter.

As a means of estimating ketopentose the cysteine-carbazole reaction (Dische & Borenfreund, 1951) has, in the absence of other ketoses, some advantages over the method described in this paper. The colour development, which does not necessitate heating, is rapid, reaching a stable maximum intensity with ribulose after 15 min. The reaction is about 1.6 times as sensitive as the present method, and Beer's law is obeyed for ribulose within a concentration range of 1–40 $\mu\text{g./ml.}$ (Cohen, 1953). The interference due to arabinose is about twice that observed with the present method. The cysteine-carbazole reaction cannot, however, be applied to the estimation of ketopentose in the presence of ketoheptose and estimations in the presence of triose are complicated. By the present method ketopentose can be estimated in the presence of a twofold excess of either fructose or dihydroxyacetone. Interference due to ketoheptose is of a similar order of magnitude to that in the cysteine-carbazole reaction (Glock, 1952), but may be corrected for if the concentration of ketoheptose is known. The failure of ketopentose to obey Beer's law in the present method did not cause any great practical difficulties. The extent of deviation from Beer's law was consistent and reproducible for any given batch of reagents. Increase in the ferric-ion concentration beyond the recommended amount improved the Beer's-law range for xylulose, but the interference due to fructose at 640 $\text{m}\mu$. was simultaneously increased.

SUMMARY

1. A modification of the resorcinol reaction is described which is suitable for the estimation of ketopentoses and ketoheptoses in binary mixtures provided that there is less than a twofold excess of one ketose over the other.
2. The characteristics of the colours given with the new reagent by fructose, sorbose, tagatose, xylulose, perseulose and mannoheptulose are described.
3. The interference due to aldoses is small. Ketoheptoses interfere appreciably with the estimation of ketoheptoses and ketopentoses.
4. Satisfactory recoveries of ketoses added to blood have been obtained by this method.

The author is grateful to Professor N. L. Edson for his constant advice and encouragement throughout this work.

REFERENCES

- Arcus, A. C. & Edson, N. L. (1954). *Proc. Univ. Otago med. Sch.* **32**, 6.
- Bacon, J. S. D. & Bell, D. J. (1948). *Biochem. J.* **42**, 397.
- Chargaff, E., Levine, C. & Green, C. (1948). *J. biol. Chem.* **175**, 67.
- Cohen, S. S. (1953). *J. biol. Chem.* **201**, 71.
- Dische, Z. (1929). *Mikrochemie*, **7**, 33.
- Dische, Z. (1951). *Phosphorus Metabolism*, vol. 1, p. 171. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.
- Dische, Z. & Borenfreund, E. (1951). *J. biol. Chem.* **192**, 583.
- Glock, G. E. (1952). *Biochem. J.* **52**, 575.
- Hough, L. & Jones, J. K. L. (1953). *J. chem. Soc.* p. 343.
- Isherwood, F. A. & Jermyn, M. A. (1951). *Biochem. J.* **48**, 515.
- Levene, P. A. & Tipson, R. S. (1936). *J. biol. Chem.* **115**, 731.
- McCorkindale, M. J. (1952). M.Sc. Thesis: University of New Zealand.
- McCorkindale, M. J. & Edson, N. L. (1954). *Biochem. J.* **57**, 518.
- Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.
- Pogell, B. M. (1954). *J. biol. Chem.* **211**, 143.
- Reichstein, T. & Bosshard, W. (1934). *Helv. chim. acta*, **17**, 753.
- Roe, J. H. (1934). *J. biol. Chem.* **107**, 15.
- Roe, J. H., Epstein, J. H. & Goldstein, N. P. (1949). *J. biol. Chem.* **178**, 839.
- Rolf, D., Surtshin, A. & White, H. L. (1949). *Proc. Soc. exp. Biol., N.Y.*, **72**, 351.
- Salo, T. P. (1953). *Arch. Biochem. Biophys.* **42**, 106.
- Somogyi, M. (1945). *J. biol. Chem.* **160**, 69.

Purification of the *iso*Citric Enzyme (Triphosphopyridine Nucleotide-linked *iso*Citric Dehydrogenase–Oxalosuccinic Carboxylase)

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(Received 9 December 1955)

Oxidative decarboxylation of D-*isocitric* acid in animal tissues occurs chiefly through the mediation of triphosphopyridine nucleotide (TPN)-linked *isocitric* dehydrogenase, which catalyses the oxidation of D-*isocitric* acid, and oxalosuccinic carboxylase, which catalyses the decarboxylation of oxalosuccinic acid (Martius, 1937; Adler, Euler, Günther & Plass, 1939; Ochoa & Weisz-Tabori, 1945; Lynen & Scherer, 1948).

Interest in the purification of *isocitric* dehydrogenase and oxalosuccinic carboxylase has largely centred about the question of whether they are two distinct enzymes, or whether one enzyme catalyses both reactions. The two enzymes have now been obtained in a highly purified state, as reported briefly in a preliminary note (Moyle & Dixon, 1955), and shown to be a single protein, the *isocitric* enzyme. The details of the method of purification are described in this paper.

EXPERIMENTAL

Materials

isoCitrate. Sodium DL-*isocitrate* was obtained by hydrolysis of DL-*isocitric* acid lactone in N-NaOH at 60° for 20 min. The solution was cooled, and brought to pH 7 with N-HCl.

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Triphosphopyridine nucleotide. TPN used was the commercial preparation of the Sigma Chemical Co. It was 80% pure as determined spectrophotometrically.

Oxalosuccinate. Potassium triethyloxalosuccinic ester was prepared by the method of Wislizenus & Waldmüller (1911). It was converted into the free ester by the method of Neuberg & Ringer (1915), and then into barium oxalosuccinate by the method of Ochoa (1948). The purity of the preparation, tested by the aniline citrate method (Edson, 1935), was 66%. Sodium oxalosuccinate was obtained by decomposition of the stable barium salt with N-H₂SO₄ at 0°, removal of BaSO₄ by centrifuging, followed by neutralization to pH 6 with N-NaOH. The solution was used immediately.

Calcium phosphate gel. This was prepared essentially as described by Keilin & Hartree (1951).

Methods

isoCitric-dehydrogenase activity. Reduction of TPN was measured in the Beckman model DU spectrophotometer by following the increase in optical density at 340 mμ. The reaction was carried out in cuvettes of 1 cm. light path, held at 24° in a temperature-controlled unit designed by Dixon (1954). The reaction mixture [similar to that of Grafflin & Ochoa (1950)] contained 0.7 ml. of 0.05M aminotrihydroxymethylmethane chloride (tris buffer) pH 7.3, 0.2 ml. of 0.02M-MnCl₂, 0.1 ml. of 0.0013M TPN, 0.1 ml. of enzyme, 1.9 ml. of water. Determinations of optical density were made at 30 sec. intervals after the addition of 0.1 ml. of 0.00525M sodium DL-*isocitrate*. The amount of enzyme was adjusted so that the initial rate of reduction of TPN was linear with time and proportional to enzyme concentration. A dehydrogenase unit was defined as the amount of enzyme causing an increase in optical density of 0.01 in 1 min. under these conditions.