The Formation and Breakdown of Pentosephosphates by Liver Fractions

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In a previous paper (Dickens & Glock, 1951) it was shown that at least two liver factors are necessary for the oxidation of D-ribose-5-phosphate, one being a coenzyme Π -(triphosphopyridine nucleotide; TPN)-specific dehydrogenase and the other an aldolase. Two alternative mechanisms of oxidation were suggested. The first involves an aldolase split into triosephosphate and a C_3 fragment, synthesis of hexosemonophosphate from two C_3 molecules and subsequent oxidation through the appropriate TPN-specific system. The second alternative is the direct oxidation of D-ribose-5-phosphate to 5-phospho-D-ribonic acid followed by oxidative decarboxylation as suggested by Dickens (1936, 1938) for yeast. It has now been shown that this direct oxidative pathway is unlikely to occur, at least in liver. Synthesis of hexosemonophosphate, already demonstrated by Dische (1938, 1949) with haemolysed human erythrocytes and by Schlenk & Waldvogel (1947) and Waldvogel & Schlenk (1947, 1949) with rabbit-liver preparations, has been found to occur very rapidly and to such an extent as to exclude its formation solely from triosephosphate derived from breakdown of pentosephosphate.

MATERIALS

Coenzyme Π (TPN), containing 34.5% TPN and 0.6% coenzyme I (DPN), and the barium salts of 6-phospho-D-glucuronate (6-PG), synthetic D-glucose-6-phosphate (G-6-P), fructose-6-phosphate (F-6-P) and D-arabinose-5-phosphate were preparations used previously (Dickens & Glock, 1951).

D-Ribose-5-phosphate (R-5-P). Two preparations of the barium salt were used. One was prepared by Prof. F. Dickens from barium inosinate according to Levene & Jacobs (1911). (Found: organic P, 8.50; inorganic P, 0.21; pentose (orcinol reaction) 40.6. Calc. for $C_5H_8O_4 \cdot PO_4Ba$: P, 8.48; pentose, 41.1%.) This preparation was found to contain some ketopentose. The other preparation was synthesized by Dr C. T. Beer according to the method of Michelson & Todd (1949). (Found: organic P, 8.48; inorganic P, 0.08; pentose, 41.5%.)

5-Phospho-D-ribonate. The barium salt was prepared by Prof. F. Dickens from R-5-P by Br_2 oxidation.

Ribulose-5-phosphate. This was an equilibrium mixture of the barium salts of ribulose-5-phosphate and ribose-5-phosphate (containing respectively 65 and 35% of these two phosphates) kindly donated by Dr B. L. Horecker.

Mannoketoheptose, mannosemonophosphate, galactose-6-phosphate and trehalosemonophosphate. These were given by

Dr Marjorie Macfarlane from the collection of the late Prof. R. Robison.

D-isoCitrate. This was generously supplied by Prof. H. A. Krebs in the form of the dimethyl-D-isocitrate lactone. Hydrolysis of the lactone and conversion to the sodium salt was effected by incubating 8.08 mg. lactone in 1 ml. 0.02N-NaOH for 1 hr. at 37° and then diluting with 1 ml. water to give a 0.02M solution of the sodium salt.

Glycolaldehyde. This was prepared by Miss D. Salmony from dihydroxymaleic acid according to Fischer & Taube (1927).

Crystalline muscle aldolase, triosephosphate and 'Zwischenferment'. These were prepared by Dr P. McLean. Aldolase was prepared according to Cori, Slein & Cori (1947), 0.5 mg. enzyme liberating 24 μ g. alkali-labile P in 5 min. at 30° from 1 ml. 0.001M-hexosediphosphate. Triosephosphate (equilibrium mixture of the barium salts of glyceraldehyde-3-phosphate, and dihydroxyacetone phosphate) was prepared according to Meyerhof (1938), using crystalline muscle aldolase instead of crude muscle extract. Zwischenferment was made from bottom yeast (Anheuser-Busch) according to Kornberg (1950). This preparation, in the presence of TPN, oxidized G-6-P and F-6-P at the same rate (cf. Seegmiller & Horecker, 1951), but was completely inactive towards mannosemonophosphate, galactose-6-phosphate, trehalosemonophosphate, R-5-P, glycolaldehyde and 6-PG (after allowing for its small F-6-P content).

METHODS

Preparation of liver fractions. These were dialysed ammonium sulphate-precipitated horse-liver fractions prepared as previously described (Dickens & Glock, 1951). Fractions O, A, B and C are precipitated by 0.3–0.4, 0.4–0.5, 0.5–0.6 and 0.6–0.7 saturation with $(NH_4)_2SO_4$, respectively.

Most of the experiments described in this paper were carried out on these liver fractions prepared from the same horse-liver homogenate. For the assay of hexosemonophosphate an additional preparation of fraction A was used since this preparation was completely inactive in oxidizing R-5-P.

Dehydrogenase activity. This was determined either by manometric estimation of O_2 uptake with N-methylphenazinium methyl sulphate as carrier or by spectrophotometric measurement of the rate of reduction of TPN at 340 m μ . (see Dickens & Glock, 1951).

Breakdown of ribose-5-phosphate. This was usually followed at 37° in a system consisting of 0.25 ml. 0.05M-R-5-P, 0.25 ml. 0.25M-sodium phosphate buffer (pH 7.4), 1 ml. liver fraction and 1 ml. water. At intervals, 0.5 ml. was introduced into 4.5 ml. 5% (w/v) trichloroacetic acid or 4.0% $HClO_4$, and samples of the supernatant used for estimating pentose, glycolaldehyde, etc. Essentially the same results

were obtained when veronal acetate buffer (Michaelis, 1931) or glycylglycine buffer were used.

Hexosemonophosphate formation. After determining pentose disappearance on incubating R-5-P with liver fraction *B* (see above), hexosemonophosphate was estimated in three ways. (a) O_2 uptake. After inactivation by boiling, O_2 uptake was determined at 37° with *N*-methylphenazinium methyl sulphate as carrier, on addition of 0.2 mg. TPN and 1 ml. liver fraction *A* (inactive in oxidizing R-5-P, but very active towards G-6-P) and compared with that of an equimolar solution of G-6-P. (b) Cysteine-carbazole method. Fructosemonophosphate formation was determined by this method (Dische & Borenfreund, 1951) on a trichloroacetic acid filtrate of the R-5-P digest. (c) Reduction of TPN. In earlier experiments, reduction of TPN was followed spectrophotometrically at 340 m μ . on addition of 0.5 mg. TPN, 0.4 ml. liver fraction *A* (for substrate specificity see Fig. 9) and 0.25 ml. 0.25M-sodium phosphate buffer (pH 7.4) to 1 ml. neutralized trichloroacetic acid filtrate of the R-5-P digest in a total volume of 2.5 ml. Since liver fraction *A* is not absolutely specific for G-6-P and F-6-P, in later experiments it was replaced by purified Zwischenferment. The reaction mixture consisted of 0.5 ml. neutralized trichloroacetic acid filtrate of the R-5-P digest, 0.5 ml. 0.25M-glycylglycine buffer (pH 7.4), 0.1 ml. 0.3M-MgCl₂, 0.5 mg. TPN and 200 μ g. Zwischenferment in a total volume of 2.5 ml. Under these conditions G-6-P (or F-6-P) can be estimated quantitatively over a range of 0.025–0.15 ml. 0.025M. The reaction was started by the addition of the enzyme to both cells, the blank being devoid of TPN.

Other determinations. Pentose was determined by the orcinol method of Mejbaum (1939) with a 45 min. heating period (Albaum & Umbreit, 1947), ketopentose and ketohexose by the cysteine-carbazole method of Dische & Borenfreund (1951), glycolaldehyde by the diphenylamine method of Dische & Borenfreund (1949), glycollic acid by the chromotropic acid method of Fleury, Courtois & Perles (1951) and phosphate by the method of Fiske & Subbarow (1925).

Preparation of ketopentose-1-phosphate. Sodium triosephosphate (from 290 mg. barium salt) was incubated at 37° for 1 hr. with 50 mg. crystalline muscle aldolase, 120 mg. glycolaldehyde and 10 ml. veronal acetate buffer (pH 7.0) in a total volume of 40 ml. After protein precipitation with HClO₄, the filtrate was neutralized and one-ninth of the total volume of 10N-NaOH added. After 20 min. at room temperature (to hydrolyse any unchanged triosephosphate) it was adjusted to pH 8.0 and water-insoluble barium salts were precipitated by the addition of 4 ml. 20% (w/v) barium acetate solution. These were discarded and the barium pentosephosphate precipitated from solution by the addition of ethanol to give a final concentration of 80% (v/v). After keeping it at 0° for 2 hr., the precipitate was centrifuged, washed with ethanol and acetone and finally dried. Yield: 168 mg. barium salt. It was purified by reprecipitating with ethanol from a small volume of water. (Found (after drying *in vacuo* at 110°): organic P, 7.35; inorganic P, 0.0%). It was completely water-soluble and contained no detectable aldose (hypoidite method of Robison & King, 1931). Ketopentose was detected by the cysteine-carbazole and orcinol reactions (see Fig. 5). The reducing activity determined according to Nelson (1944) was the same as that of R-5-P, whereas the pentose estimated colorimetrically was only about 50% of the expected value. This behaviour in the orcinol reaction is probably general for ketopentoses

since Horecker, Smyrniotis & Seegmiller (1951) found that the extinction coefficient of ribulose at 670 m μ . was only 50% of that given by aldopentoses. The constitution is discussed later.

RESULTS

Oxidation and breakdown of ribose-5-phosphate by liver fractions

The ability of the liver fractions, used in most of the experiments in the present study, to oxidize G-6-P, 6-PG, R-5-P and isocitrate is shown in Table 1, the TPN specificity of the oxidation of R-5-P having already been established (Dickens & Glock, 1951). These results corroborate the previous findings that at least two factors are necessary for the oxidation of R-5-P, although in this case fractions *O*, *A* and *B* singly produce some oxidation of this substrate. By further fractional precipitation with ammonium sulphate it is possible to obtain fractions which singly are completely inactive and which have to be combined to produce any oxidation (Dickens & Glock, 1951). The fractions most active in oxidizing R-5-P are clearly those with the highest G-6-P dehydrogenase activity.

Table 1. *The oxidation of D-glucose-6-phosphate, 6-phosphogluconate, D-ribose-5-phosphate and D-isocitrate by horse-liver fractions*

(Total volume 3.0 ml. including 1 ml. (or 0.5 ml.*) liver fraction, 0.2 ml. 0.05M substrate and 0.2 mg. TPN. The values have been corrected for the O_2 uptake in the absence of TPN which in all cases was very small.)

Liver fraction	O_2 uptake (μ l. O_2 in 45 min. at 37°)			
	G-6-P	6-PG	R-5-P	isoCitrate
<i>O</i>	42	1	11	—
<i>A</i>	122	22	40	132
<i>B</i>	10	64	10	75
<i>C</i>	0	89	0	1
<i>A + B</i>	—	—	146	—
<i>A + C</i>	—	—	133	—
<i>A* + B* + C*</i>	—	—	90	—
<i>O + C</i>	—	—	97	—
<i>B + C</i>	—	—	19	—

The breakdown of R-5-P, as determined by pentose disappearance, by the same liver fractions is shown in Fig. 1. Fraction *B* is the most active and fraction *C* the least active in this respect. The most vigorous oxidation of R-5-P is, as one would expect, produced by fraction *A* which is highest in G-6-P dehydrogenase in combination with fraction *B*, the most active in splitting R-5-P. Although the oxidation of R-5-P is completely TPN specific, the breakdown is independent of the presence of coenzyme. The breakdown is, moreover, the same under aerobic and anaerobic conditions and never proceeds beyond 66–72% disappearance of pentose. This indicates the existence of some equilibrium

which is unaffected either by increasing the concentration or the duration of action of the enzyme. Crystalline muscle aldolase is completely inactive in splitting R-5-P.

Fig. 1. Breakdown of ribose-5-phosphate by liver fractions and crystalline muscle aldolase at 37°. Digestion mixture (see Methods section) included 0.25 ml. 0.05 M-R-5-P, 1 ml. horse-liver fractions O, A, B or C or 10 mg. crystalline muscle aldolase, in total volume of 2.5 ml. Pentose disappearance measured by orcinol method. —, horse-liver fractions; - - -, crystalline muscle aldolase.

Direct oxidation of ribose-5-phosphate to 5-phosphoribonic acid

Since direct oxidation of R-5-P to 5-phosphoribonic acid followed by oxidative decarboxylation has been suggested by Dickens (1936, 1938) to occur in yeast, the ability of liver fractions to oxidize 5-phosphoribonic acid has been investigated. It has been found, however, that combined (A + B + C) liver fractions do not oxidize 5-phosphoribonic acid at all, even in the presence of TPN, although such liver fractions always oxidize R-5-P. It is thus unlikely that this direct oxidative pathway exists in liver.

Formation of ketopentosephosphate by liver fractions

Ketopentose formation has been detected both from the oxidative decarboxylation of 6-PG and during the initial stages of R-5-P breakdown by liver fractions. In this connexion, the recently published cysteine-carbazole method of Dische & Borenfreund (1951) has proved particularly useful. Ketopentoses give a pink colour with maximum absorption at 545 m μ . in this reaction and also show absorption at 540 m μ . in the orcinol reaction in addition to the much greater absorption at 670 m μ . characteristic of pentoses in general. When R-5-P was incubated with liver fraction B at 37°, no keto-

pentose was detected even during the first few minutes. If, however, incubation was carried out at 28° in order to slow down the breakdown of R-5-P, which has a very high temperature coefficient (Dickens & Glock, 1951), ketopentose was detected.

Fig. 2. Ketopentosephosphate formation from ribose-5-phosphate at 28° detected by cysteine-carbazole reaction. Digestion mixture (see Methods section) included 0.25 ml. 0.05 M-R-5-P and 1 ml. horse-liver fraction B in total volume of 2.5 ml. Incubation period: - - -, 0 min.; ▲—▲, 5 min.; —, 10 min.; ●—●, 30 min.; ○—○, 120 min. (545 m μ . peak = ketopentose. 560 m μ . peak = ketohexose).

Fig. 2 gives absorption curves obtained with the cysteine-carbazole reaction after 5, 10, 30 and 120 min. incubation periods, the percentage breakdown of R-5-P at these times being, respectively, 21, 39, 60 and 71. The presence of the 545 m μ . ketopentose maximum is quite evident after 5 and 10 min., but after 30 min. the maximum has shifted to 560 m μ ., characteristic of ketohexose. This ketopentose does not appear to be alkali-labile since the 545 m μ . maximum is still shown after treatment with N-sodium hydroxide for 10 min. at room temperature, although under these conditions ribulose-5-phosphate no longer gives a ketopentose reaction. Identification of the ketopentosephosphate, however, awaits its isolation.

Ketopentose is also formed from the oxidative decarboxylation of 6-PG. After measuring the rate of oxidation of 6-PG with liver fraction C at 37° for 60 min., pentose was determined, after trichloroacetic acid precipitation. In this instance 'apparent' pentose formation accounted for 28% of the oxidized substrate, but since ketopentose was present, which gives a much lower extinction coefficient at 670 m μ . in the orcinol reaction than aldopentose, the 'true' pentose formation must be greater than this. Fig. 3 shows the absorption curves of the digestion mixtures before and after incubation, the 670 m μ .

peak in the unincubated mixture being due to TPN. Fig. 3 also includes absorption curves of R-5-P and an equilibrium mixture of ribulose-5-phosphate and R-5-P. Both ribulose-5-phosphate and the incubated digest give an absorption maximum at 540 $m\mu$, characteristic of ketopentoses. The rate of colour development of the incubated digest in the orcinol reaction is given in Table 2, and is very similar to that of R-5-P, which Horecker *et al.* (1951) state is identical with that of ribulose-5-phosphate.

Fig. 3. Ketopentosephosphate formation from 6-phosphogluconate at 37° detected by orcinol method. Digestion mixture included 0.2 ml. 0.05M-6-phosphogluconate and 1 ml. horse-liver fraction C in total volume of 2.5 ml. ---, unincubated digestion mixture; —, after 60 min. incubation; ●—●, 100 μ g. R-5-P (barium); ×—×, 50 μ g. equilibrium mixture of ribulose-5-phosphate and R-5-P (barium salts). (540 $m\mu$. peak = ketopentose.)

Aldolase condensations with glyceraldehyde

Since R-5-P breakdown products are very readily converted into hexosemonophosphate without any accumulation of ketopentosephosphate, it was decided to attempt to synthesize ketopentosephosphate from liver fraction B, triosephosphate and glyceraldehyde. Ketopentosephosphate forma-

tion was very rapid and almost quantitative, the absorption curves in the orcinol and cysteine-carbazole reactions being given in Fig. 4. No hexosemonophosphate was formed. This ketopentosephosphate synthesized with liver fraction B is

Fig. 4. Ketopentose-1-phosphate formation from triosephosphate, glyceraldehyde and horse-liver fraction B at 37°. Digestion mixture included 1.5 ml. 0.025M-triosephosphate and glyceraldehyde and 0.5 ml. liver fraction B in total volume of 2.5 ml. Cysteine-carbazole reaction: —, unincubated digestion mixture; ●—●, after 30 min. incubation; ○—○, after 120 min. incubation. ○—○—○, after 120 min. incubation by orcinol method.

probably the same as that synthesized under the same conditions with crystalline muscle aldolase (see Fig. 5 for absorption curves and Methods section for method of preparation and analysis). Both are alkali-stable under the conditions which

Table 2. Rate of colour development in the pentose orcinol reaction

	Time of boiling (min.)							
	5	10	15	20	30	45	60	90
	Colour development (% of maximum)							
Ribose-5-phosphate	37	64	78	96	100	—	—	—
Arabinose-5-phosphate	—	24	—	47	62	81	92	100
Ketopentose-1-phosphate*	33	67	82	94	100	—	—	—
6-Phosphogluconate digestion mixture†	34	68	—	89	94	100	—	—

* Synthesized from triosephosphate and glyceraldehyde with crystalline muscle aldolase.

† After determining O_2 uptake at 37° for 1 hr. in a system including 1 ml. liver fraction C, 0.2 ml. 0.05M-6-PG and 0.2 mg. TPN, pentose determined in neutralized trichloroacetic acid filtrate.

destroy ribulose-5-phosphate. The rapid rate of acid hydrolysis (Fig. 6) indicates that it is a ketopentose-1-phosphate and is presumably the same as that synthesized by Racker (1948), which Dische &

Fig. 5. Ketopentose-1-phosphate synthesized from triose-phosphate and glyceraldehyde with crystalline muscle aldolase. For preparation see Methods section. 100 μ g. barium salt: $\circ$ - $\circ$, cysteine-carbazole reaction; $\circ$ - $\circ$, orcinol reaction.

Fig. 6. Rates of hydrolysis of pentosephosphates in N -HCl at 100° . $\circ$ - $\circ$, ketopentose-1-phosphate synthesized from glyceraldehyde and triosephosphate with crystalline muscle aldolase ($k = 86 \times 10^{-3}$). $\bullet$ - $\bullet$, ribose-5-phosphate (synthetic) ($k = 2.5 \times 10^{-3}$).

Borenfreund (1951) refer to as xyloketose-1-phosphate. Racker (1948), however, gave no analysis or characterization of the sugar, but Forrest, Hough & Jones (1951) have synthesized it with pure aldolase and identified the sugar chromatographically as xyloketose.

This ketopentose-1-phosphate is oxidized slowly by liver fractions. Fig. 7 shows the oxidation of

R-5-P and ketopentose-1-phosphate by combined liver fractions *A* and *C*. Whereas the oxidation of R-5-P is negligible in the absence of added TPN, the slow oxidation of ketopentose-1-phosphate in the absence of TPN accounts for about half of the total oxygen uptake in the presence of TPN. The initial breakdown of ketopentose-1-phosphate presumably leads to formation of glycolaldehyde and triose-phosphate, which could be synthesized to F-6-P by the action of aldolase. The oxidation of the glycolaldehyde is independent of TPN (see later) whilst the oxidation of the latter is TPN-specific.

Fig. 7. Oxidation of ketopentose-1-phosphate and ribose-5-phosphate by liver fractions. O_2 uptake determined at 37° . Total volume 3.0 ml. including 1 ml. each of liver fractions *A* and *C*, 0.1 ml. 0.05M-substrate with and without 0.4 mg. TPN. —, with TPN; ---, without TPN; $\bullet$, R-5-P; $\circ$, ketopentose-1-phosphate.

Since it seemed theoretically possible that pentosephosphate might condense with glycolaldehyde to form a heptosephosphate, liver fraction *B* was incubated with R-5-P and glycolaldehyde, but with essentially the same results as with triose-phosphate and glycolaldehyde. Again, an alkali-stable ketopentosephosphate was formed and no hexosemonophosphate or ketoheptosephosphate. As Fig. 8 shows, mannoketoheptose gives an absorption maximum in the cysteine-carbazole reaction at $565 m\mu$. and in the orcinol reaction at $570 m\mu$. in the presence of ferric chloride and a much smaller one at $595 m\mu$. in the absence of ferric chloride. If, however, the orcinol reaction product is extracted into amyl alcohol, the maximal absorption both in the presence and absence of iron is at $660 m\mu$., which is in approximate agreement with the result of Robison, Macfarlane & Tazelaar (1938) who used a similar procedure. These negative results, however, do not exclude the possibility of

the formation of heptosephosphate or ketohexosephosphate from the breakdown products of R-5-P. Although fructose and fructose-6-phosphate give an absorption maximum at 560 $m\mu$. in the cysteine-carbazole reaction, the maximum absorption with the R-5-P digestion mixtures is often at 565–570 $m\mu$. (see the curve after 120 min. in Fig. 2), which would correspond more closely with a mixture of ketohexose and ketoheptose.

Fig. 8. Cysteine-carbazole and orcinol reactions with mannoketoheptose and ribose. Orcinol reaction with 40 μ g. ribose (●) and 100 μ g. mannoketoheptose (▲); —, in presence of $FeCl_3$ in a final concentration of 0.048%; ---, in absence of $FeCl_3$; Δ, cysteine-carbazole reaction with 100 μ g. mannoketoheptose (ribose does not react).

Breakdown products of ribose-5-phosphate

It is generally considered that R-5-P is split enzymically into triosephosphate and a C_3 fragment, as yet unidentified, but assumed to be glyceraldehyde.

Experiments devised to detect the formation of glyceraldehyde from R-5-P liver fractions have in all cases been negative, whether carried out under aerobic or anaerobic conditions, although the Dische (1949) diphenylamine reaction is extremely sensitive. This is not due to rapid oxidation to glycollic acid. Moreover, glyceraldehyde does not disappear when incubated with fraction B. As already described, when glyceraldehyde is incubated with liver fraction B and R-5-P or triosephosphate, a ketopentose-1-phosphate accumulates very rapidly. This does not accumulate when R-5-P is split by fraction B and, moreover, the addition of glyceraldehyde completely inhibits the resynthesis of hexosemonophosphate from the R-5-P break-

down products. This would indicate that free glyceraldehyde is not formed during R-5-P breakdown, although the rapid synthesis of ketopentosephosphate is further evidence that the C_3 fragment is triosephosphate.

Additional evidence for triosephosphate formation is provided by the fact that DPN is reduced in addition to TPN in the presence of R-5-P and liver

Fig. 9. Substrate specificity of liver fraction A used for hexosemonophosphate assay. Reduction of TPN measured at 340 $m\mu$. at 20° in system containing 0.1 ml. 0.05M substrate, 0.5 mg. TPN and 0.4 ml. liver fraction A in total volume of 2.5 ml. x—x, D-isocitrate; ○—○, G-6-P or F-6-P; ▲—▲, 6-phosphogluconate; ---, glyceraldehyde; ●—●, R-5-P.

fractions. Sable (1951) has shown that DPN reduction can be inhibited by poisoning the triosephosphate dehydrogenase with iodoacetate, whereas the TPN specific oxidation is unaffected (Sable, 1951; Dickens & Glock, 1951).

Glyceraldehyde is oxidized slowly by combined (A + B + C) liver fractions, but this oxidation (which in three experiments averaged 70 μ l. oxygen in 90 min. from 0.2 ml. 0.05M substrate) is unaffected by the addition of DPN or TPN. Breusch (1943) has also reported oxidation of glyceraldehyde by liver suspensions.

Fig. 9 shows the rate of reduction of TPN on the addition of D-isocitrate, G-6-P, 6-PG and a R-5-P digestion mixture to a liver fraction A, but no reduction is produced on the addition of either glyceraldehyde or R-5-P. This, and the previous evidence, proves conclusively that the TPN-specific oxidation of the breakdown products of R-5-P is not due, even in part, to the oxidation of free glyceraldehyde.

Table 3. *Hexosemonophosphate formation from ribose-5-phosphate by horse-liver fraction B*

(For composition of R-5-P digestion mixture and for details of hexosemonophosphate determination see Methods section: (1) by O_2 uptake with liver fraction A; (2) cysteine-carbazole method; (3) reduction of TPN at 340 m μ . with liver fraction A; (4) reduction of TPN at 340 m μ . with purified Zwischenferment.)

Exp. no.	Time of incubation at 37° (min.)	Pentose disappearance (%)	Percentage conversion of R-5-P broken down into	
			Hexosemono-phosphate	Fructosemono-phosphate (2)
1	60	70	80 (1) 74 (3)	—
2	60	38*	83 (1) 78 (3)	—
3	5†	22	—	64
	15	40	—	45
	120	71	—	30
4	5	40	—	68
	10	60	—	44
	30	66	—	29
5	5	35	—	68
	15	58	—	45
	60	72	—	29
6	30	69	73 (4)	28
	60	69	78 (4)	30
	120	68	75 (4)	28

* A less active enzyme.

† Incubated at 28°.

Synthesis of hexosemonophosphate

Synthesis of hexosemonophosphate from the products of R-5-P breakdown by liver fraction B has been determined by measuring the oxygen uptake on addition of TPN and liver fraction A (rich in G-6-P dehydrogenase but completely inactive in oxidizing R-5-P, see Fig. 9), or by measuring the reduction of TPN at 340 m μ . in the presence of liver fraction A or purified Zwischenferment. Both these enzyme preparations oxidized F-6-P and G-6-P at identical rates so that the above methods determine total hexosemonophosphate. Fructosemonophosphate was determined in addition, in some cases, by the cysteine-carbazole reaction. Table 3 gives the results of these experiments. The average conversion of the R-5-P broken down into hexosemonophosphate is at least 75%, which is much greater than the 50% yield obtainable by simple aldolase condensation of triosephosphate derived from R-5-P. The results obtained with the cysteine-carbazole method indicate that fructosemonophosphate is formed first and is gradually converted into G-6-P, an equilibrium mixture of these two esters being formed after about 30 min. incubation, as is seen by the proportion of fructosemonophosphate to total hexosemonophosphate (Table 3).

DISCUSSION

From the results of the present and previous work (Dickens & Glock, 1951) it would appear that at least three enzymes are necessary for the oxidation

of ribose-5-phosphate (R-5-P) by liver fractions. Since R-5-P is not split by crystalline muscle aldolase, and ketopentosephosphate formation precedes both formation and breakdown of R-5-P by liver fractions, both an aldolase and a pentose-phosphate isomerase are required for the breakdown of R-5-P. Hexosemonophosphate dehydrogenase is necessary, in addition, for the oxidation of R-5-P. Horecker & Smyrniotis (1950) and Horecker *et al.* (1951) have indicated the occurrence of a pentose-phosphate isomerase, concerned with the inter-conversion of ribulose-5-phosphate and R-5-P, in phosphogluconic acid dehydrogenase preparations from yeast. Their statement that the ribulose is in the form of the 5-phosphate is not based on evidence which would exclude the 1-phosphate, and since liver contains a phosphoribomutase (Abrams & Klenow, 1951*a, b*) which converts ribose-1-phosphate into R-5-P, it is conceivable that it might also contain a pentosephosphate isomerase for converting ribose-1-phosphate into ribulose-1-phosphate. In addition, another enzymic equilibrium must exist since R-5-P is never broken down by liver fractions beyond 66–72% disappearance of pentose.

Approximately 75% of the whole carbon skeleton of R-5-P broken down by liver fraction B has been accounted for by conversion into hexosemonophosphate. Fructosemonophosphate is formed first, and is gradually converted into G-6-P. Synthesis of fructosemonophosphate cannot proceed via fructosediphosphate since, as previously shown (Dickens & Glock, 1951), liver fractions which readily oxidize

both F-6-P and G-6-P do not oxidize fructose-diphosphate, these fractions all being deficient in fructosediphosphatase. Fructosediphosphate, if formed at all, can only be present as a minor component. Dische (1949) also showed that hexose-monophosphate was formed from R-5-P by haemolysed erythrocytes independently of fructose-diphosphate formation. The high yield of hexose-monophosphate indicates that it cannot only be formed by aldolase condensation of two triose molecules derived from two molecules of R-5-P and at present one can only speculate as to how it might occur. Dische (1951) postulates not only the formation of tetrose and tetrosephosphate but also of C₇ sugar phosphates. In spite of the fact that no free glycolaldehyde has been detected during the breakdown of R-5-P by liver fractions, it is conceivable that it might condense immediately it is formed with a molecule of glycolaldehyde phosphate to give a tetrosephosphate, and this possibility is being investigated. It does not seem possible that two glycolaldehyde molecules would condense since glycolaldehyde does not disappear when incubated with liver fraction B, the most active in splitting R-5-P. Hough & Jones (1951*a-c*) have emphasized the possible importance of glycolaldehyde as a sugar precursor, but all their experiments were carried out under mildly alkaline conditions in the absence of enzymes. The only enzymic condensation of glycolaldehyde so far reported is with triosephosphate (Racker, 1948; Forrest *et al.* 1951; and the present paper) and irrespective of the source of aldolase the product is xyloketose-1-phosphate, which has not yet been shown to occur naturally. Marmur & Schlenk (1951) found that both glycolaldehyde and glycolaldehydephosphate condensed with triosephosphate in the presence of an enzyme preparation from *Micrococcus pyogenes*, but the pentosephosphate was not characterized.

The possibility suggested by Fig. 2 that heptosephosphate might be synthesized in addition to hexosemonophosphate is also of interest, particularly since Robison *et al.* (1938) isolated a ketoheptosephosphate from the products of yeast-juice fermentation and more recently Benson, Bassham & Calvin (1951) have found that sedoheptulosemonophosphate is formed during photosynthesis and is indeed a normal constituent of all plants.

SUMMARY

1. The breakdown of ribose-5-phosphate (R-5-P) by dialysed ammonium sulphate-precipitated horse-

liver fractions is independent of coenzyme II (TPN), the same under aerobic and anaerobic conditions and never proceeds beyond 66–72% pentose disappearance.

2. Oxidation of R-5-P is completely TPN-specific and is greatest with a combination of liver fraction B, most active in splitting R-5-P, and fraction A, which has the highest glucose-6-phosphate dehydrogenase activity.

3. Crystalline muscle aldolase does not split R-5-P.

4. Ketopentosephosphate formation has been detected both from the oxidative decarboxylation of 6-phosphogluconate and during the initial stages of breakdown of R-5-P by liver fractions.

5. Attempts to synthesize ketopentosephosphate from triosephosphate and glycolaldehyde with liver fractions have resulted in the rapid accumulation of a ketopentose-1-phosphate probably the same as that synthesized by crystalline muscle aldolase under the same conditions. This does not accumulate during the breakdown of R-5-P by liver fractions.

6. The main product of R-5-P breakdown by liver fractions is hexosemonophosphate which accounts for approximately 75% of the pentose disappearing. This conversion is much greater than could be accounted for by aldolase condensation of triosephosphate derived from R-5-P, and indicates that the rest of the pentose molecule must be incorporated.

7. Fructosemonophosphate is formed first and is then gradually converted into glucose-6-phosphate. Synthesis of fructosemonophosphate does not proceed via fructosediphosphate.

8. Free glycolaldehyde is not formed during the breakdown of R-5-P by liver fractions and the TPN-specific oxidation of the breakdown products cannot be due, even in part, to oxidation of glycolaldehyde.

9. Direct oxidation of R-5-P to 5-phosphoribonic acid followed by oxidative decarboxylation is unlikely to occur in liver since 5-phosphoribonic acid is not oxidized by liver fractions even in the presence of TPN.

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Comparative Studies of 'Bile Salts'

5. BILE SALTS OF CROCODYLIDAE

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The Crocodylidae are reptiles which are believed to have persisted without much alteration for a very long period. Accordingly it was expected, on the hypotheses put forward in these studies, that their bile salts would show primitive characteristics. A brief report of work on *Alligator mississippiensis* has already appeared (Haslewood, 1952*a*) and details of this, together with some findings in two other species, namely the spectacled cayman (*Caiman crocodilus*) and the Nile crocodile (*Crocodylus niloticus*) are now presented.

RESULTS

The work described shows that the bile acids of each of the three species examined are present in similar complex mixtures. Crystalline material was easily

obtained, but this, too, was very impure. By chromatography on alumina it proved in every case simple to isolate the ethyl ester of an acid $C_{27}H_{46}O_5$, named 'trihydroxycoprostanic acid'; this was further characterized as the parent acid and its methyl ester. The dehydro derivatives obtained after oxidation with chromic acid contained three carbonyl groups in the molecule. These groups could be removed by Kishner-Wolff reduction, which gave the 'stem acid', coprostanic acid, $C_{27}H_{46}O_2$; and this has been prepared by partial synthesis from cholanic acid (Bridgwater & Haslewood, 1952).

Through the kindness of Prof. T. Shimizu of the University of Okayama Medical School, Japan, it has been possible to show that trihydroxycoprostanic acid is identical with the ' α -trihydroxybisanorsterocholanic acid', isolated by Kurauchi & Kazuno