sedimenting material which makes the identification of small adsorbed molecules not feasible. A swelling of the filaments, owing to absorption of solvent at the lower pH, would not be consistent with the data, which indicate an increase in the cross-sectional weight.

The physiological role of axon filaments remains unknown. It is curious that filaments dissociate under conditions which are so nearly physiological.

ACKNOWLEDGEMENT

The authors wish to express their appreciation for the encouragement and advice of Professor F. O. SCHMITT.

SUMMARY

The dissociation of axon filaments, the major fibrous protein of nerve axoplasm, has been studied by two independent methods, ultracentrifugation and viscosimetry, and light scattering. Both methods agree in demonstrating a marked decrease in molecular weight of filaments when the pH is raised from 6.0 to 7.7. During this process the length of the filaments decreases only slightly, while the diameter decreases markedly. It is not possible at present to distinguish a lateral splitting of filaments into nearly equal parts from a splitting off of much smaller material from the filaments. This dissociation of filaments is reversible and occurs in the region of physiological pH of axoplasm which was found to be 6.4. Axon filaments are shown by electrophoresis to be very highly charged compared to other macromolecular components of axoplasm.

REFERENCES

1 M. MAXFIELD, *J. Gen. Physiol.*, 37 (1953) 201–206.
2 F. O. SCHMITT AND B. B. GEREN, *J. Exptl. Med.*, 91 (1950) 499–504.
3 H. FERNANDEZ-MORAN, *Exptl. Cell Research*, 3 (1952) 5–83.
4 R. S. BEAR, F. O. SCHMITT AND J. Z. YOUNG, *Proc. Roy. Soc. (London)*, 123 (1937) 505–520.
5 J. FOSTER, *J. Neurochem.*, 1 (1956) 84–90.
6 B. H. ZIMM, *J. Chem. Phys.*, 16 (1948) 1099–1116.

Received October 22nd, 1956

MECHANISMS IN THE INTERCONVERSION OF RIBOSE-5-PHOSPHATE AND HEXOSE-6-PHOSPHATE IN HUMAN BLOOD*

I. ISOMERIZATION OF RIBOSE-5-PHOSPHATE IN HUMAN HEMOLYSATES

ZACHARIAS DISCHE AND HAROLD SHIGEURA
Department of Biochemistry, College of Physicians and Surgeons
Columbia University, New York (U.S.A.)

It has been shown that when ribose-5-phosphate (R-5-P) is added to a human hemolysate, sedoheptulose phosphate and alkaline-labile phosphate ester are formed after a short incubation at room temperature[1]. It was also found that the amount of the alkaline labile phosphate produced from R-5-P during a 30 minute incubation at room temperature at a concentration of $M/150$, was greater than that equivalent

* This investigation was supported by a grant from the Rockefeller Foundation.

References p. 99.

to the amount of sedoheptulose phosphate. It was assumed, therefore, that R-5-P is partly converted to another ester which is subsequently converted into sedoheptulose phosphate and triose phosphate. HORECKER *et al.*[2] found that R-5-P added to preparations from yeast and liver is partly isomerized to ribulose-5-phosphate (Ru-5-P). 20–25% of the latter ester was found in equilibrium with 80–75% of R-5-P at room temperature. We reported briefly in 1954[3] that when the total amounts of keto- and aldopentose were determined by the cysteine-carbazole[4] and phloroglucinol[5] reactions after a brief incubation of R-5-P in a hemolysate at 33° it was found that not about 30% as expected, but about 60% of ketopentose phosphate was in equilibrium with about 40% of the original amount of R-5-P. As the concentration of proteins in the hemolysate was found to be without influence on the equilibrium, it was assumed that the isomerization of R-5-P in the hemolysate is a complex enzymic process and that in addition to Ru-5-P, another ester of a 5-carbon keto compound is formed by enolization and subsequent inversion or by a shift of OH between vicinal carbons. While this investigation of the isomerization of R-5-P was in progress, ASHWELL AND HICKMAN[6,7] in a study of the isomerization of R-5-P in preparations from spleen, reported the isolation of D-xylulose and a 3-ketopentose in addition to ribulose from dephosphorylated isomer mixtures of R-5-P formed in these preparations. They interpreted these findings as resulting from enolization of the keto group on carbon 2 of ribulose with subsequent shift of hydroxyl to carbon 3 of the pentose. The present report deals with further results of our investigation of the isomerization of R-5-P in human hemolysates.

EXPERIMENTAL

1. *Material and general experimental procedures*

A suspension of red blood cells from pooled fluoridated blood was obtained by repeated washing with saline and removal of the white cells from the upper layer. The cells were hemolyzed by addition of 1½ volumes of water. The hemolysate was then dialyzed at 4° for 5–6 days against H_2O or against $M/40$ NaF often changed. The pH of the hemolysate was 7.1–7.2. For balance experiments, to 3 ml of the fluoridated hemolysate the designated amount of R-5-P in 1 ml of $M/40$ NaF was added and the mixture incubated in a water bath varying in temperature between 33° C and 0° C. At the end of the incubation period the hemolysate was deproteinized by TCA or $HClO_4$. Aldo- and ketopentoses were determined in the supernatant. In some experiments the pH of the experimental samples was brought up to 8.4 by addition of 1 M Tris buffer. For paper chromatography of reaction products hemolysates dialyzed against H_2O were used.

2. *Analytical methods*

The total amount of isomers of R-5-P was determined by a modification of the procedure used by AXELROD AND JANG[8] for the determination of Ru-5-P formed by the purified alfalfa isomerase from R-5-P. This procedure is based on the fact that Ru-5-P is completely split in 30 minutes by 1 N HCl at 100°, while only 30% of R-5-P is hydrolyzed at the same time. By determining, therefore, the amount of inorganic phosphate split off from a mixture of Ru-5-P and R-5-P after one hour and in subsequent time intervals and comparing the values with the amounts of phosphate split from pure R-5-P, it is possible to calculate the amount of phosphate present in the form of Ru-5-P. As our isomer mixture, in addition to Ru-5-P, contained still other isomers, we first determined the time interval necessary to split completely all of these isomers. Samples of the isomer mixture and of pure R-5-P were hydrolyzed with 1 N HCl at 100° and aliquots were taken after every half hour. The amounts of inorganic phosphate split off from the isomer mixture and of the R-5-P respectively in every time interval were plotted against time and the point was determined at which the slope of the hydrolysis curve obtained from the isomer mixture became parallel to that from the R-5-P standard. At this point (2 hours) no significant amount of other esters were present in solution. Our isomer mixture, therefore, contained one or more substances less easily hydrolyzable than Ru-5-P. The amount of isomers which are formed from R-5-P is calculated from the formula

References p. 99.

$$X = \frac{A - 0.95\,F_R - 0.05}{1 - 0.95\,F_R - 0.05}$$

in which X is the fraction of the total phosphorus added to the hemolysate as R-5-P present in for of its isomers. A is the total amount of phosphorus hydrolyzed off in two hours, and F_R is the amount of phosphorus hydrolyzed in two hours from the standard solution of R-5-P which contains as much of this ester as has been added to the experimental sample. The factor 0.05 in the numerator and in the denominator represents a correction for ribose-1-phosphate present in the equilibrium mixture and calculated under the assumption that 0.05 mole of ribose-1-phosphate is in equilibrium with 0.95 mole of R-5-P. The total ketopentose in the isomer mixture was determined by the carbazole cysteine reaction[4] with Ru-5-P as standard*. That this procedure gave very nearly the true amount of ketopentose phosphate present in the isomer mixture was shown by the close agreement of the values so obtained with those obtained by the hydrolysis method. It was found, in addition, that xylulose-5-phosphate in this reaction develops the color more slowly than Ru-5-P, but after about 18 hours at room temperature the two ketopentose phosphates yield substantially the same extinction coefficients. This was established by comparing the cysteine carbazole reaction of two preparations of ketopentose phosphates obtained from our isomer mixture by chromatographic separation on paper, one of which contained, 60% xylulose- and 40% Ru-5-P, and the other 20% xylulose- and 80% Ru-5-P**.

The same reaction was used for the determination of free xylulose and ribulose. With free ketopentoses the color is developed much faster[9]. A great difference, however, is observed between the two ketopentoses as far as the rate of the color development is concerned. The maximum is reached at room temperature in about 25 minutes with ribulose and only in about 3 hours with xylulose. The latter sugar shows after 25 minutes only 70% of the maximum color, while ribulose after 3 hours shows a decline of about 7% in color. By determining, therefore, the optical density of a solution containing the two ketopentoses after 25 and 180 minutes, respectively against standards of ribulose and xylulose it is possible to determine the amount of the two ketopentoses in the experimental sample.

The second method by which the total amount of ketopentose phosphate was determined is based on the fact that ketopentose-5-phosphates are completely destroyed by an incubation of 15 minutes in 1 N NaOH at room temperature[9a]*. The alkali treatment decreases the color produced by the aldo ester only by about 10%. By determining, therefore, the optical density before and after treatment with alkali against a standard of 5-R-P containing as much R-5-P as was added to the experimental sample it is possible to determine the R-5-P present in thes ample. It must be noted, however, that the values obtained for R-5-P by this procedure are maximal values as it has not been possible to establish whether the product of the alkali treatment of ketopentose-phosphate does show a certain small absorption at 670 mμ at which wavelength the readings are carried out**.

The amount of ketopentose phosphate in the isomer mixture was finally determined by $NaIO_4$ oxidation by the micromethod of DIXON AND LIPKIN[10]. For this purpose the hemolysate was deproteinized with $HClO_4$, the main bulk of the latter was removed from the filtrate with $KHCO_3$ and after suitable dilution of the consumption of $NaIO_4$ was measured in a diluted acetate buffer of pH 5. The amount of ketopentose was calculated on the assumption that only ketopentose phosphates substituted in position 5 are present. Under these conditions 1 mole of ketopentose ester consumes 2 moles of $NaIO_4$, whereas R-5-P consumes 3 moles. The readings were carried out at 260 mμ.

In most experiments the isomerization of R-5-P was determined by following, with the phloroglucinol reaction[5], its decrease when added to the hemolysate. To this end the difference of optical densities at 552 and 510 mμ of extracts of a hemolysate incubated with 5-R-P was compared with a R-5-P standard containing as much R-5-P as the total pentose phosphate present in the isomer mixture. To obtain the true aldopentose content in the isomer mixture, it was necessary to make two corrections. One was for the amount of ribose-1-phosphate which reacts like pure ribose in the phloroglucinol reaction and has an extinction coefficient about half as great as R-5-P. This correction was made on the assumption that 5% of the total aldopentose is present as ribose-1-phosphate. The other correction, for the phloroglucinol reaction of ketopentoses, was calculated

* We are greatly indebted to Dr. B. L. HORECKER of the National Institutes of Health Bethesda, Maryland, for a sample of Ba salt of ribulose-5-phosphate.

** These two preparations were obtained by paper chromatography in *iso*butanol-picric acid[17] of the isomer mixture of R-5-P. Two separate spots containing ketopentose phosphates were obtained which, after extraction, were separated from picric acid by rechromatography in 80% ethanol-glacial acetic acid and from R-5-P by Br oxidation. Xylulose and ribulose content in the two preparations were determined by the cysteine-carbazole reaction after dephosphorylation with prostate phosphatase.

References p. 99.

by comparing the value for the total ketopentose (obtained from the decrease of the phloroglucinol reaction) with the value obtained from the phosphate hydrolysis in several experiments. These determinations showed that the difference of the extinction coefficients $E_{552}-E_{510}$ of the ketopentose mixture is only about 3% of that for R-5-P. Both corrections amounted to no more than about 5% of the value for $D_{552}-D_{510}$ of the isomer mixture.

RESULTS

1. *Equilibrium between ribose-5-phosphate and its isomers and its dependence on the concentration of enzyme and substrate*

The conversion of R-5-P to its isomers in the hemolysate is a very fast process, and is completed before any significant conversion of R-5-P to sedoheptulose phosphate and triose phosphate occurs. The rate, however, of the isomerization depends not only on the concentration of the enzyme, but also upon the initial concentration of the added R-5-P. When $M/125$ of this ester is added to the hemolysate and the disappearance of aldopentose is followed by the phloroglucinol reaction, a very significant drop in the phloroglucinol reaction can be seen after only 1 minute and after 6–8 minutes a constant level corresponding to about 39% ± 1.5% of the value for the initial content of R-5-P is reached. This decrease of aldopentose which can also be demonstrated by the orcinol reaction (Expt. VIII, Table I) is accompanied by a corresponding increase in ketopentose as determined by the cysteine carbazole reaction. The maximum of the ketopentose formation is reached at the same time as the minimum of aldopentose formation, and the amount of ketopentose as compared with the standard Ru-5-P corresponds to 62.5% ± 2.5%. The position of the equilibrium did not shift significantly between 8 and 12 minutes of incubation (Expt. III, Table I). When the initial concentration of R-5-P is $M/300$ the same equilibrium is reached after 3–4 minutes, and when it is $M/60$ the necessary incubation time is at least 15 minutes. This effect of the concentration of the substrate is probably due to the complexity of the isomerization process, which involves more than one enzymic reaction and leads to several reaction products, some of which inhibit the formation of other isomers. That the main bulk of the isomers which are formed from R-5-P is represented by esters of substances which in the cysteine carbazole reaction react like ketopentoses is demonstrated by the results of determinations of the P hydrolysis curve of the isomer mixture. As can be seen from Table I, the total amount of isomers formed from R-5-P corresponds to 63.5% ± 1.5%, which is only slightly higher than the value for ketopentose phosphate obtained with the cysteine carbazole reaction.

These values for the amount of isomers are in agreement with the determination of ketopentose phosphate by $NaIO_4$ oxidation. This oxidation of an equilibrium mixture of $M/125$ pentose phosphate at 33° showed a 21.4% lower consumption of $NaIO_4$ than a R-5-P standard which contained as much R-5-P as was originally added to the hemolysate. If ketopentose phosphates substituted in position 5 consume only 2/3 as much $NaIO_4$ as an equivalent amount of R-5-P the decrease of 21.4% corresponds to the presence of 64.2% of ketopentose phosphate substituted in position 5. At the moment when the equilibrium is reached no significant amounts of sedoheptulose could be found with the cysteine H_2SO_4 reactions for this sugar. That the

* This procedure was independently developed in our laboratory and in the laboratory of Dr. HORECKER by P. STUMPF.

** Our preparation of Ru-5-P used as reference, still contained 19% R-5-P.

References p. 99.

TABLE I

EQUILIBRIUM BETWEEN RIBOSE-5-PHOSPHATE (R-5-P) AND ITS ISOMERS AT VARIOUS CONCENTRATIONS IN DIALYZED HEMOLYSATES

Expt. No.	R-5-P added μmoles/ml	Temperature °C	Time in minutes	In equilibrium			pH
				R-5-P in % of total pentose phosphate	Ketopentose phosphate in % of total pentose phosphate		
					By the cysteine carbazole reaction	By P hydrolysis curve	
I	16.0	33	7	40.5	58.0		7.1
	16.0	33	10	37.0	62.0		7.1
	16.0	33	15	37.0	64.6		7.1
II	16.0	33	20	36.5	62.0		7.1
	8.0	33	8	36.2	65.0		7.1
	8.0	33	6	37.7			
	8.0	33	8	37.6	65.0		6.4
III	8.0	33	8	36.0			7.2
	8.0	33	12	36.0			7.2
	3.2	33	3	35.2			7.2
	3.2	33	4	35.0			7.2
IV	8.0	33	8	37.5		62.0	8.4
V	8.0	33	8	36.1		65.0	8.4
VI	8.0	0	120	48.0			7.2
	3.2	0	90	50.5			7.2
VII	8.0	0	120	48.8	72.9		7.2
	8.0	33	8	36.0	61.0		7.2
VIIIa	8.0	33	8	36.6			7.1
b*	8.0	33	30	37.2			7.1
c*	8.0	33	40	36.1			7.1
a	8.0	0	120	46.5			7.1
b*	8.0	0	8 h	47.6			7.1
c*	8.0	0	10 h	47.4			7.1
IX	8.0**	33	10	39.0			8.4
	8.0**	33	10	40.0			7.2

* Hemolysate diluted fourfold.
** Determinations by orcinol reaction after alkali treatment.

equilibrium between R-5-P and its isomers is not affected by the large amount of hemoglobin in the hemolysate is shown by the fact that the position of the equilibrium is not affected by dilution of the hemolysate. The dilution of the isomerizing enzyme, of course, proportionally decreases the rate of the isomerization (Expt. VII, Table I). The position of the equilibrium is not significantly shifted by the dilution.

2. Effect of temperature on the equilibrium between ribose-5-phosphate and its isomers

AXELROD AND JANG[8] found that the equilibrium between Ru-5-P and R-5-P obtained with the purified alfalfa isomerase is strongly dependent upon the temperature. Thus, at 0° C they find a ratio Ru-5-P to R-5-P of 0.14, while at 37° it shifts to 0.32.

References p. 99.

They explain this strong dependence on temperature by the fact that Ru-5-P cannot form a furanoid ring, and must be present in another form, probably as a straight chain sugar. The conversion of R-5-P to Ru-5-P can be assumed to be an endothermic process, and the equilibrium would, therefore, be shifted in the observed sense. As this consideration applies to other 5-carbon keto compounds substituted in position 5 it seemed of particular interest to examine the effect of temperature on the equilibrium between R-5-P and its isomers in the hemolysate. As can be seen from experiments VI and VII of Table I, the dependence of this equilibrium on temperature is much smaller than that reported by AXELROD AND JANG. At $M/125$ the equilibrium at 0° was reached after 90 minutes and did not change in the following 90 minute period. The ratio R-5-P to its isomers, however, rose only from 39 to 50.5%. This indicates that at least one of the isomers is formed by a reaction which by its thermodynamic conditions significantly differs from the simple isomerization to Ru-5-P.

3. *Tentative identification of the ribose-5-phosphate isomers*

(a) *Separation of two groups of esters.* For the tentativei dentification of the isomers of R-5-P in the reaction mixture 20 ml of $M/15$ solution of R-5-P was added to 60 ml of a hemolysate which had been dialyzed against distilled water; the mixture was incubated for 15 minutes at 33° and deproteinized by heating for 3 minutes in a boiling waterbath. The supernatant was then concentrated *in vacuo* to a very small volume. The mixture of isomers was then separated into two main groups by chromatography on paper, using as solvent a mixture of 80% ethanol and acetic acid according to COHEN AND SCOTT[11]. Four distinct spots were obtained with the orcinol spray (Fig. 1). The uppermost, very narrow blue spot corresponded to sedoheptulose-7-phosphate which was formed in this experiment in very small quantities. The second spot beneath the first (S II) was grey in appearance; it represented the main bulk of the esters and proved to be a mixture of ketopentosephosphates and R-5-P. A third, much smaller, lower spot showed an intensive pink color with a bright orange fluorescence in ultra-violet light. It became intensely yellow after a few days and corresponded to still unidentified phosphate esters, which will be referred to as pentose phosphate analogues (PAP) (Fig. 1). The fourth lowest spot on the chromatogram was grey-purple and contained small amounts of free ribose, xylulose and ribulose.

(b) *Xylulose and ribulose in the ketopentose phosphate fraction of the hemolysate.* For the identification of the ketopentose phosphate fraction a concentrate corresponding to about 20 ml of the original hemolysate incubated for 15 minutes with R-5-P was chromatographed in 80% ethanol acetic acid. The S II spot containing the main bulk of the ketopentose phosphates and R-5-P was extracted from the paper with a small volume of water; this solution was dephosphorylated with prostate phosphatase* in acetate buffer of pH 5 and then deproteinized with $HClO_4$. The supernatant was neutralized with potassium bicarbonate, which precipitated most of the perchloric acid as the K salt. The filtrate was then deionized with Amberlite IR-4B (OH) and Amberlite IR-120 (H) exchange resins, and concentrated again *in vacuo* and in a desiccator over P_2O_5 until it contained at least 2 mg of ketopentose per ml. Paper chromatography was then carried out with 3 different solvents, namely (I) 80% phenol containing 8-hydroxyquinoline[12], (II) butanol, pyridine and water (10:3:3)[13] and (III) butanol, ethanol and water (10:1:2)[14]. The orcinol TCA

* We are greatly indebted to Dr. E. CHARGAFF for the preparation of prostate phosphatase.

References p. 99.

reagent was used for spraying. Standards of xylulose and ribulose, prepared from the *p*-bromophenylhydrazone and *o*-nitrophenylhydrazone* respectively according to

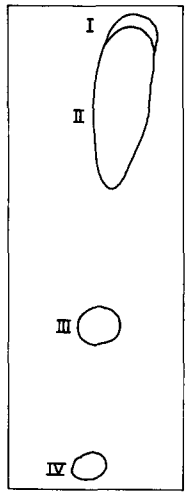

Fig. 1. Chromatogram of the mixture of phosphate esters in the hemolysate incubated with R-5-P 16 μmoles/ml for 15′ at 33°. I. Blue sedoheptulosemonophosphate. II. Gray R-5-P and ketopentose-5-phosphates. III. Ester reducing I_2 at pH 5 at 22° (PAP). IV. Free ketopentose. Solvent 80% ethanol + glacial acetic acid. Orcinol TCA spray.

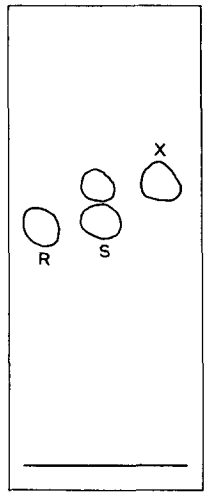

Fig. 2. Chromatogram of the dephosphorylated spot II of Fig. 1 with phenol-8-hydroquinolin as solvent. R = ribulose standard. X = xylulose standard. S = Concentrate of dephosphorylated spot II. Orcinol TCA spray.

the procedure of SCHMIDT AND TREIBER[15] were run simultaneously with the concentrate. With all three solvents two different spots were obtained, one of which when sprayed with TCA orcinol reagent showed a brown, the other a purple color. As can be seen from Table II, the R_F values of the two spots were, with each solvent, identical with those of xylulose and ribulose respectively. The purple spot corresponding to ribulose showed the characteristic orange fluorescence in ultra-violet light. The spots corresponding to the two keto sugars obtained from the concentrate of the hemolysate were separately extracted from the paper with water. When tested with the cysteine carbazole reaction the spots showed the same rates of color development as the corresponding controls.

TABLE II

SEPARATION ON PAPER OF XYLULOSE AND RIBULOSE OBTAINED FROM DEPHOSPHORYLATED ISOMER MIXTURES FROM BLOOD HEMOLYSATES

	Solvents		
	80% Phenol	*BuOH-pyridine-H_2O (10:3:3)*	*BuOH–EtOH–H_2O (10:1:2)*
1. Brown spot	0.60	0.42	0.35
2. Purple spot	0.65	0.37	0.30
3. Ribulose standard	0.65	0.37	0.30
4. Xylulose standard	0.60	0.42	0.35

(c) *The presence of xylulose and ribulose as 5-phosphate esters.* The presence in large amounts of another ketopentose phosphate ester in addition to Ru-5-P in the

* We are greatly indebted to Dr. O. TOUSTER, Vanderbilt University, for the preparation of the *p*-bromophenylhydrazone of D-xylulose, and to Dr. S. S. COHEN, University of Pennsylvania, for the preparation of the *o*-nitrophenylhydrazone of ribulose.

References p. 99.

isomerization mixture suggested the possibility that a shift of phosphate may take place during the isomerization process. This possibility was tested by determining the ratio D_{540} to D_{710} in the carbazole reaction[16] of a TCA extract of a hemolysate containing the mixture of isomers and comparing it with the corresponding ratios from R-5-P and Ru-5-P. This determination was carried out on an extract from the experiment I of Table I. The ratio for R-5-P in the carbazole reaction in this determination was found to be 0.52 and for pure Ru-5-P 1.47. The same ratio for the mixture of the isomers was found to be 1.33 for the total ketopentose phosphate after subtracting the optical density due to R-5-P in the isomer mixture. When the optical density due to Ru-5-P, the concentration of which was calculated from the values obtained by AXELROD AND JANG[8] on their alfalfa isomerase, was subtracted from the total, the ratio for the remaining ketopentose phosphate was 1.28, *i.e.* about 15% lower than the value for Ru-5-P. In this form of the carbazole reaction the ratio for free pentoses does not differ significantly from that for esters substituted in position 1 and 3 and is about 15 to 20 times higher than for the corresponding 5-phosphate esters. Ketopentose esters substituted in position 4 could barely be expected to behave differently from esters substituted in position 3 as the phosphate in beta position to the carbonyl group could be very easily split off by the acid. It is reasonable, therefore, to assume that any ester of ketopentose not substituted in position 5 would yield about the same ratio D_{540} to D_{710} as the corresponding free ketopentose. The value of 1.33, therefore, for the ketopentose phosphate fraction of the isomer mixture indicates that the main bulk of the ketopentose in this mixture must be present as 5-phosphate ester. If only 20% of it were present as another ester the ratio D_{540}/D_{710} of the ketopentose-5-phosphate present in addition to Ru-5-P would have to be lower than that of R-5-P itself, and this appears highly improbable in view of the fact that Ru-5-P shows a ratio three times as high as R-5-P.

The presence in the isomer mixture of the ketopentoses as 5-phosphate esters is finally borne out by the results of the oxidation of the isomer mixture with periodate. The mixture of isomers formed from R-5-P showed that the keto isomers used up 2 moles $NaIO_4$ per 1 mole sugar ester. This excludes the possibility that significant amounts of the ketopentose esters substituted in position 4 are present in addition to 5-phosphate esters. The possibility that an equimolar mixture of 1 and 4-phosphates is present which on the average also would up 2 moles $NaIO_4$ per mole sugar appears excluded by the result of the determination of the D_{540}/D_{710} ratio in the carbazole reaction. The presence of significant amounts of ketopentose-3-phosphate also appears incompatible with this ratio for the isomer mixture as substitution of ribose in position 3 was shown not to influence the D_{540}/D_{710} ratio.

(d) *The phosphate esters of pentose analogues (PAP).* The isomers present in the third spot on the chromatogram in Fig. 1, which gave a bright red color with the TCA orcinol spray, gave after extraction from the paper an intense brownish green color with Bial's orcinol reaction, according to DISCHE AND SCHWARTZ[9b]. This color showed one maximum at 670 mμ like that produced by pentoses and another at 500 mμ. The different extracts of the PAP did not, however, have an identical composition. This is demonstrated by the reactivity in the phloroglucinol reaction. While the extract of Experiment I in Table III showed a very low phloroglucinol reaction, the intensity of this reaction was about twice as high in Experiment II and 4½ times as high in Experiment III. In all 3 experiments the extracts reduced

References p. 99.

TABLE III

FORMATION OF PENTOSE-5-PHOSPHATE FROM PAP DURING ITS INCUBATION WITH THE HEMOLYSATE AT 33°

0.8 ml hemolysate + 0.2 ml of PAP solution + 0.2 ml of H_2O or adenosine
all values in μmoles/ml.

Expt. No.	*Substance added*	*Time of incubation*	*Aldopentose phosphate as R-5-P by phloroglucinol reaction*		*Total PP formed calculated from phloroglucinol reaction*	*Total PP as by orcinol reaction*		*Total PP formed calculated from orcinol reaction*
		min	*Before incubation*	*After incubation*		*Before incubation*	*After incubation*	
662	(a) PAP *M*/150 phosphate	3	0.018	0.140	0.310	0.052	0.326	0.274
	(b) PAP + 1. *M*/150 phosphate + 1.2 μmoles/ml adenosine			0.590				
	2. *M*/150 phosphate + 1.2 μmoles/ml adenosine			0.530				
698	PAP	2	0.083	0.100	0.042	0.475	0.590	0.115
714	PAP	2	0.173	0.113		0.520	0.780	0.260

ferricyanide in 1/10 *N* $NaHCO_3$ at room temperature. Iodine was reduced at room temperature in an acetate buffer of pH 5, and the oxidation was complete after about 1 hour. As both ketopentose esters were found on the chromatogram in spot S II, which had a much lower R_F, the strongly reducing esters in spot S III could not be identical with either of the ketopentoses-5-phosphates. Free ribulose and xylulose were shown (Fig. 1) to have a higher R_F than spot S III. That this fraction was not due to a decomposition of the ketopentose phosphate during chromatography was demonstrated by repeating this procedure on the concentrated extract from spot S II containing the two ketopentose phosphates. When this concentrated mixture was rechromatographed with 80% ethanol acetic acid no spot corresponding to PAP appeared. That in hemolysates these esters were in equilibrium with R-5-P and ketopentose phosphate was demonstrated in the following way.

4. *Conversion of PAP to R-5-P and ketopentose phosphate*

The spot, S III, of the chromatogram was extracted and concentrated to a small volume required by the sensitivity of the analytical methods and this concentrate was added to a hemolysate and incubated for 2–4 minutes at 33°. After deproteinization with $HClO_4$ the phloroglucinol and orcinol reactions were simultaneously carried out on the supernatant and on the original concentrate diluted with $HClO_4$ to the same final volume as in the experimental sample. The results of three such experiments, listed in Table III, show that this short incubation led to a considerable increase in the orcinol reaction. In two of the experiments, particularly Experiment 1, there was also a considerable increase in the phloroglucinol reaction. It will be noted that in this experiment the initial phloroglucinol reaction of the concentrate was very low, whereas in other experiments in which the increase in the phloroglucinol reaction was not so marked or could not be observed at all, the initial intensity of the reaction

References p. 99.

of the phloroglucinol reaction was much higher. If we calculate from the increase in phloroglucinol reaction in Experiment I, the amount of R-5-P and from the increase in the orcinol reaction the total pentose phosphate formed in the hemolysate, we find that the ratio of the two values corresponds to that in the equilibrium mixtures of isomers in the hemolysate.

These results indicate the presence in the isomer mixture of an ester, probably a 5-carbon compound, which is not identical with pentose-5-phosphate, but which is converted into the equilibrium mixture of R-5-P and its isomers. The fact that the phloroglucinol reaction did not always increase in these conversion experiments can be interpreted as the result of a partial conversion to ribose phosphate during the prolonged concentration and extraction procedures. The conversion of R-5-P to ketopentose phosphate, which takes place simultaneously with the conversion of the analogue ester, would result in an increase in the orcinol reaction without a corresponding increase in the phloroglucinol reaction. This interpretation appears to be supported by an experiment carried out with an extract used in Experiment I in Table III in which, in addition to PAP, adenosine and inorganic phosphate were added to the hemolysate. Under these conditions adenosine continuously produced R-1-P and R-5-P. As can be seen from Table III in this case the decrease in the phloroglucinol reaction, after subtraction of the optical density due to adenosine and the R-5-P formed from it, was much less than in the experimental sample which contained no adenosine.

5. *The ratio between ribulose-5-phosphate and xylulose-5-phosphate in the equilibrium mixture*

The amounts of xylulose and ribulose which could be isolated on paper from the hemolysate do not necessarily indicate the true ratio of these two ketopentoses in the equilibrium mixture under our experimental conditions, as for the purpose of paper chromatography the deproteinization had to be carried out by heating at 100° and the ratio of the two ketopentoses should be altered by temperature. The ratio at 33° between Ru-5-P and xylulose-5-phosphate (Xu-5-P) or the ester in the equilibrium mixture, which produces xylulose after dephosphorylation and behaves like Xu-5-P in the cysteine carbazole and cysteine H_2SO_4 reactions, can be determined in the following way. AXELROD AND JANG[8] have shown for the purified alfalfa R-5-P isomerase that at 37° C 32% of Ru-5-P is in thermodynamic equilibrium with 68% of R-5-P. The ratio between the first and the seond ester, therefore, under these conditions is 0.47. We found that the change from 33° to 37° in our experiments decreases the aldopentose fraction by only 1% of the total pentose. At 37°, therefore, in our equilibrium mixture we have 35% of R-5-P instead of 36% at 33°, and the amount of Ru-5-P in the equilibrium mixture would be, on the basis of the data of AXELROD AND JANG, 16.5% of the total pentose phosphate. This leaves 48.5% for all the other isomers except R-5-P and Ru-5-P. If we assume that 3-ketopentose and PAP represent about 3%*, the amount of Xu-5-P or the ester producing it during dephosphorylation would be 45.5%, corresponding to a ratio of Xu-5-P to Ru-5-P

* The determination of 3-ketopentose was carried out by the characteristic color reaction of this substance discovered by Dr. GILBERT ASHWELL. We are greatly indebted to him for giving us the procedure used in this still unpublished reaction. The amount of PAP was calculated from the amount of pentose-5-phosphates formed after its incubation in the hemolysate.

References p. 99.

of 2.95. The use of the data of AXELROD AND JANG for this calculation appears justified by the fact that the ratio Ru-5-P to R-5-P in alfalfa isomerase is about the same as that found by HORECKER and his associates for isomerase preparation from yeast and liver and by the fact that in our experiments a 4-fold dilution of the hemolysate did not significantly affect the equilibrium, which excludes the possibility that a shift in the equilibrium might be brought about in the hemolysate by an interaction between ketopentose phosphate and hemolysate proteins*.

DISCUSSION

The isomerization of R-5-P in the hemolysate appears as a complex system of reactions catalyzed by at least two different types of enzymes, of which one appears to be identical with or closely related to the R-5-P isomerase prepared from alfalfa by AXELROD AND JANG, and from yeast and liver by HORECKER and associates. With these preparations R-5-P was reversibly converted to Ru-5-P and thermodynamic equilibrium was established in which at room temperature the ratio between Ru-5-P and R-5-P was about 0.20 to 0.25 and at 37° 0.32. In our hemolysates, on the other hand, the ratio between R-5-P and the isomers appears completely reversed as only about 36% of the total pentose esters appears in the form of the aldo ester at 33°. This equilibrium is also reversible and the equilibrium mixture consists, in addition to R-5-P and Ru-5-P, and small amounts of at least two other phosphate esters of 5-carbon compounds, of a 5-carbon phosphate ester which either is identical with Xu-5-P or behaves like the latter in the cysteine carbazole reaction and yields xylulose after dephosphorylation with phosphatases. As the equilibrium between Ru-5-P and R-5-P itself in the purified preparation of isomerase from alfalfa and liver appears to be a thermodynamic one, the formation of Xu-5-P and the other isomers must be catalyzed by one or more enzymes different from the isomerase which catalyzes the interconversion of R-5-P and Ru-5-P.

It seems most probable that the formation of Xu-5-P or another ester yielding xylulose after dephosphorylation is due to the intermediate formation of a 2, 3-enediol which in turn can be converted to either Xu-5-P or Ru-5-P or into a 3-ketopentose phosphate as suggested by ASHWELL AND HICKMAN[7].

The I_2 reducing phosphate ester PAP which on incubation with the hemolysate is converted to a mixture of R-5-P and its isomers could be identical with this 2, 3-enediol. It cannot be identical either with ketopentose-5-phosphate which has a different R_F with the ethanol acetic acid solvent or with another ketopentose phosphate ester as these would not reduce I_2 at pH 5.

RACKER[18] and his associates have shown that the isomerization of R-5-P is a necessary step in its breakdown to sedoheptulose phosphate and triose phosphate. While this paper was being prepared, a report appeared by HORECKER *et al.*[19] according to which a conversion of Ru-5-P to Xu-5-P by an epimerase is necessary for the breakdown of R-5-P and Ru-5-P to sedoheptulose-7-phosphate and triose phosphate by liver transketolase. The authors, therefore, considered Xu-5-P as a donor of the 2-carbon residue for R-5-P. This consideration is based on the assumption that

* The determination of the ratio of Xu-5-P to Ru-5-P based on the rate of the color development in the cysteine-carbazole reaction yielded in the same experiment a value of 2.65, which does not differ significantly from the value obtained on the basis of the equilibrium constants.

References p. 99.

Xu-5-P is the only isomer formed from R-5-P and Ru-5-P, and that the transfer of the 2-carbon residue to R-5-P is preceded by the formation of an active glycolic aldehyde. The authors do not take into consideration the possibility, that a phosphate ester of a 2,3-enediol rather than Xu-5-P might be the substrate of the transketolase.

Our data concerning the ratio between Ru-5-P, R-5-P, and the other isomers in the equilibrium mixture in the hemolysate appear difficult to reconcile with the assumption that Xu-5-P itself represents the main bulk of the isomers formed in addition to Ru-5-P. According to AXELROD AND JANG the low ratio of Ru-5-P to R-5-P and its strong dependence on temperature are due to the existence of the ketopentose-5-phosphate in form of a straight chain component, the formation of which is thermodynamically not favored. The same consideration obviously applies also to the formation of Xu-5-P if we are not to assume that the latter ester differs considerably from Ru-5-P in its heat of solution. This latter possibility appears incompatible with findings of STUMPF AND HORECKER[20] on the equilibrium between Xu-5-P and Ru-5-P in presence of an epimerase from *Lactobacillus pentosus*. These authors found that these ketopentose-5-phosphate esters are in equilibrium with each other at 25° in a ratio of about unity. In the equilibrium mixture in the hemolysate, however, the total amount of the isomers formed in addition to Ru-5-P and which react like Xu-5-P is about three times as high as the amount of Ru-5-P calculated from the ratio between Ru-5-P and R-5-P in equilibrium in presence of the isomerase from alfalfa, yeast and liver. The ratio between Xu-5-P and Ru-5-P at 33° cannot be assumed to shift with the increase of the temperature from 25° C tot 33° C sufficiently to explain this discrepancy. HORECKER *et al.*, furthermore, found the same high ratio between the esters which yield xylulose after dephosphorylation and Ru-5-P in a system containing the epimerase from *Lactobacillus pentosus* and a R-5-P isomerase from spinach. To reconcile these findings with the ratio between Xu-5-P and Ru-5-P in presence of the epimerase from *Lactobacillus* alone it seems necessary to assume that in the hemolysate at least one part of the phosphate esters which behave like Xu-5-P and yield xylulose after dephosphorylation are 5-carbon keto-compounds which after dephosphorylation are immediately transformed into xylulose and in the presence of concentrated H_2SO_4 react like Xu-5-P in the color reactions with cysteine and carbazole and carbazole alone.

One possibility particularly which could explain the discrepancies in the nature of the equilibrium as obtained with bacterial epimerase and with the isomerizing enzymes of the blood respectively, is the presence of ketopentose-5-phosphates, not only in their keto form, but also as hydrates stabilized by the phosphate groups in position 5. These hydrates obviously after dephosphorylation would yield the corresponding ketopentoses and would react in color reactions like ketopentose phosphate themselves. We would, therefore, have to assume that the complete isomerizing enzyme system in the living cells, in addition to the Ru-5-P producing isomerase and the epimerase contains still one or several enzymes which catalyze the formation of the hydrates. This situation would also explain why in paper chromatography of the isomer mixture with *iso*butanol-picric acid as solvent[17] we find both ketopentoses although in varying proportions in two sometimes completely separated spots. Such stable hydrates of ketopentose phosphates probably would significantly differ in their R_F's from the keto forms. The breakdown of R-5-P to sedoheptulose phosphate and triose phosphate consists in a transfer of the two first

References p. 99.

carbons of 1 molecule of pentose phosphate to another molecule of pentose phosphate. Whatever the mechanism of this reaction may be, it will at a certain stage involve a condensation of an aldehydic of ketonic group with another carbon atom. A reaction of this kind could be preceded by the hydration of aldehydic or ketonic groups. A high concentration, therefore, of the hydrated aldo- and ketopentose-5-phosphates in such a case could serve to increase the rate of such transketolizing and transaldolizing reactions.

SUMMARY

1. Ribose-5-phosphate added to the hemolysate undergoes a rapid isomerization to a mixture of isomers.

2. The mixture of isomers consists predominently of ribulose- and xylulose-5-phosphate, and probably of closely related compounds which after dephosphorylation yield ribulose and xylulose.

3. The ratio between the two ketopentose phosphates was found to be much higher than that calculated from the equilibrium constants of the ribose-5-phosphate isomerase from other tissues and epimerase from *lactobacillus pentosus*. These results suggest that xylulose-5-phosphate is either present in two different forms in the equilibrium mixture, namely as the ester of the keto sugar and that of its hydrate or that another 5-carbon ester closely related to xylulose-5-phosphate is present in addition to this latter ester.

4. In addition to the two ketopentose esters, the presence was demonstrated by paper chromatography of small amounts of another ester which reduces iodine at pH 5 at room temperature, and when added to the hemolysates is rapidly converted to an equilibrium mixture of pentose phosphate esters which, on the basis of its extinction coefficients in Bial's orcinol and phloroglucinol reactions. is assumed to be a mixture of ribose-5-phosphate and its isomers.

REFERENCES

[1] Z. DISCHE AND E. POLLACZEK, *2nd Intern. Congr. Biochem., Paris, 1952, Abstr. of Communs.* p. 289.
[2] B. L. HORECKER, P. Z. SMYRNIOTIS AND J. E. SEEGMILLER, *J. Biol. Chem.*, 193 (1951) 383.
[3] Z. DISCHE AND H. SHIGEURA, *Presented at the 126th Meeting of the Am. Chem. Soc. at New York*, Sept., 1954.
[4] Z. DISCHE AND E. BORENFREUND, *J. Biol. Chem.*, 192 (1951) 583.
[5] Z. DISCHE AND E. BORENFREUND, (in the press).
[6] G. ASHWELL AND J. HICKMAN, *J. Am. Chem. Soc.*, 76 (1954) 5889.
[7] G. ASHWELL AND J. HICKMAN, *J. Am. Chem. Soc.*, 77 (1955) 1062.
[8] B. AXELROD AND R. JANG, *J. Biol. Chem.*, 209 (1954) 847.
[9] S. MITSUHASHI AND J. O. LAMPEN, *J. Biol. Chem.*, 204 (1953) 1011.
[9a] Unpublished results.
[9b] Z. DISCHE, in E. CHARGAFF AND J. N. DAVIDSON, *The Nucleic Acids*, Vol. I, Academic Press, Inc., New York, 1955, p. 301.
[10] J. S. DIXON AND D. LIPKIN, *Anal. Chem.*, 26 (1954) 1092.
[11] S. S. COHEN AND D. M. SCOTT, *Science*, 111 (1950) 543.
[12] R. J. BLOCK, R. LE STRANGE AND G. SWEIG, *Paper Chromatography*, Academic Press, Inc., New York, 1952, p. 53.
[13] E. CHARGAFF, C. LEVINE AND C. GREEN, *J. Biol. Chem.*, 175 (1948) 67.
[14] K. T. WILLIAMS AND A. BEVENUE, *Science*, 113 (1951) 582.
[15] O. T. SCHMIDT AND R. TREIBER, *Ber.*, 66 (1933) 1765.
[16] Z. DISCHE AND E. LANDSBERG, (in the press).
[17] C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107.
[18] E. RACKER, G. DE LA HABA AND J. G. LEDER, *J. Am. Chem. Soc.*, 75 (1953) 1010.
[19] B. L. HORECKER, J. HURWITZ AND P. Z. SMYRNIOTIS, *J. Am. Chem. Soc.*, 78 (1956) 68.
[20] P. K. STUMPF AND B. L. HORECKER, *J. Biol. Chem.*, 218 (1956) 753.
[21] B. L. HORECKER AND P. Z. SMYRNIOTIS, *J. Am. Chem. Soc.*, 75 (1953) 1009.
[22] B. L. HORECKER, M. GIBBS, H. KLENOW AND P. Z. SMYRNIOTIS, *J. Biol. Chem.*, 207 (1954) 393

Received September 26th, 1956