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ADDUCT FORMATION BETWEEN HEMOGLOBIN AND 5-DEOXY-D-XYLULOSE-1-PHOSPHATE

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SUMMARY

5-deoxy-D-xylulose-1-phosphate, which is formed in human erythrocytes from added acetaldehyde and endogenous dihydroxyacetone phosphate, reacts with hemoglobin Ao to form an adduct that is stable to repeated precipitation by trichloracetic acid and to prolonged dialysis against dilute phosphate buffer. Formation of this hemoglobin derivative in individuals who consume alcohol would serve to integrate the blood acetaldehyde concentration and thereby reflect a dose-time record of alcohol consumption.

In the preceding communication formation of 5-deoxy-D-xylulose-1-phosphate * from added acetaldehyde and endogenous DHAP was reported to take place in human erythrocytes (1). The possibility that DXP reacts with hemoglobin to form a stable adduct, as does its analogue, fructose-1-phosphate, prompted the present study (2).

MATERIALS AND METHODS

($1-^3H$) acetaldehyde: This was prepared by oxidation of ($2-^3H$) alanine (3) with ninhydrin, as described by Arnstein and Crawhill for the synthesis of tritiated formaldehyde from tritiated glycine (4) but with the exception that, before distilling over acetaldehyde, 50 ml of water were added to the reaction mixture. From 5.0 mmoles of ($2-^3H$) alanine, having a specific activity of 4.1×10^5 dpm/μmole were obtained 60 ml of an aqueous solution of 26.0 mM ($1-^3H$) - acetaldehyde having a specific activity of 4.00×10^5 dpm/μmole, a radioactive yield of 30%. Acetaldehyde was assayed with yeast alcohol dehydrogenase (5).

($4-^3H$) DXP: 10 ml of ($1-^3H$) acetaldehyde, 50 mg of LiDHAP (Sigma), 2 ml of 0.5M TEA - 0.001M EDTA, pH 7.6, and 20 mg of aldolase (Sigma 10 units/mg) dissolved in 2.5 ml of 0.1M TEA-0.0002M EDTA, pH 7.6, were mixed in a glass-stoppered centrifuge tube that was then kept at 37° for 3 hours. After cooling the reaction mixture in ice, aldolase was precipitated with cold perchloric acid, the solution neutralized with KOH

Abbreviations:

Dihydroxyacetone phosphate: DHAP; fructose-1, 6-diphosphate: FDP; 5-deoxy-D-xylulose-1-phosphate, DXP.

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to remove perchlorate, and then lyophilized. The residue was extracted with small portions of cold water, which were combined. From this solution radioactive DXP was isolated by chromatography on Dowex-1-formate. Using this procedure (6), which employs a 0-6N gradient of formic acid to elute anions less acidic than simple phosphate esters, DXP was eluted by passing 6N formic acid through the column after gradient elution had ended. The DXP fraction was pooled and lyophilized. The residue was dissolved in 10 ml of water and the solution brought to pH 7.01 by adding solid bis-Tris (Sigma). The concentration of DXP, determined by enzymatic analysis (1), was 11.6 mM and the specific activity, 4.21×10^5 dpm/μmole, a radioactive yield of 47%.

Hemoglobin Ao: This was prepared from a hemolysate of freshly drawn heparinized venous blood of a health donor by chromatography on Biorex 70 as described by McDonald, et al (7). Hemoglobin concentration was measured by the method of Drabkin (8).

Reaction between hemoglobin Ao and (4-^{3}H) DXP: 2.0 ml of (4-^{3}H) DXP (23.2 μmoles) were added to 5.0 ml of 2.35 mM hemoglobin Ao (11.8 μmoles) dissolved in 0.14M NaCl-0.001M bis-Tris, pH 7.00. The concentration of DXP in this solution was thus 3.3 mM and of hemoglobin Ao, 1.69 mM. The solution was sterilized by passage through a 0.45μ membrane filter and collected in a sterile container which was then placed in a 37° water bath. Each day for 3 consecutive days aliquots were withdrawn, taking sterile precautions, for measurement of the radioactivity of any DXP-hemoglobin adduct that may have formed. Hemoglobin concentration was also determined.

Precipitation of hemoglobin: To 0.1 ml of sample were added 0.1 - 0.2 ml of 30% hydrogen peroxide. The frothy mixture was stirred with a glass rod until the hemoglobin was completely bleached. The decolorized hemoglobin was then dissolved in 2.0 ml of water, adding 0.05 - 0.1 ml of 1M NaOH when needed to form a clear solution, and then precipitated by addition of 2.0 ml of 10% trichloracetic acid. The flocculent precipitate was sedimented by centrifugation, the resulting pellet dissolved as before, and the protein again precipitated with trichloracetic acid. By carrying out this procedure for a third time, virtually all non-protein radioactivity was removed. To assay the decolorized hemoglobin for radioactivity, a 1 ml aliquot was withdrawn from 2.05 - 2.10 ml of hemoglobin solution and pipetted into 10 ml of a compatible scintillation mixture (Hydromix). Because of short-lived scintillations unrelated to the radioactivity of the sample, counting was carried out overnight. To correct for quenching the samples were recounted after adding an internal standard. Agreement between duplicate analyses was within 2%.

Chromatographic separation of hemoglobins: Separation of hemoglobins in the reaction mixture was carried out by the method of Trivelli, Ranney, and Lai (9) as modified by Gabay, et al (10).

RESULTS

This experiment showed that a radioactive product, stable to precipitation by trichloracetic acid, was formed by reaction of labeled DXP with hemoglobin Ao. A plot of the specific activity of hemoglobin as a function of time of reaction gave the straight line shown in Figure 1. The values in parentheses express, in percent, the

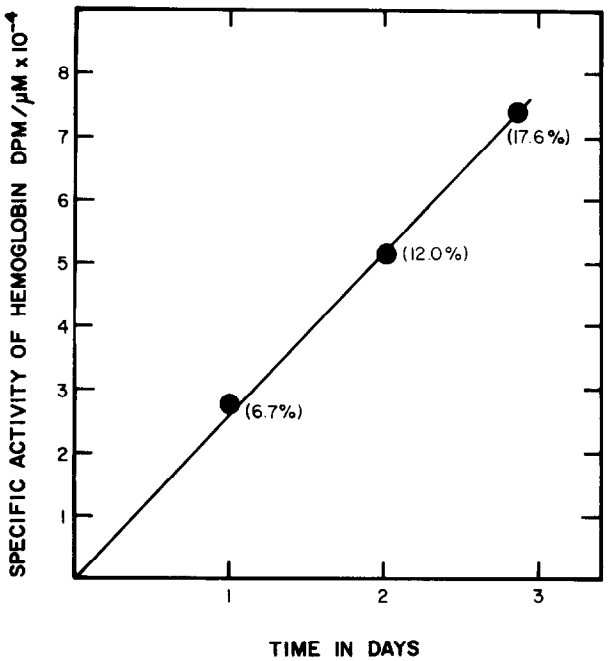

Figure 1 Formation of a radioactive adduct between hemoglobin Ao and (4-^{3}H) DXP. See text for experimental details.

proportion of total hemoglobin that had formed an adduct with DXP. They were calculated by dividing the specific activity of hemoglobin at each time point by the specific activity of labeled DXP and multiplying the result by 100. In making this calculation it was assumed that DXP combined with hemoglobin in a 1:1 molar ratio.

On the beginning of the second day of the experiment an aliquot of the reaction mixture was withdrawn and dialyzed for 24 hours at 4° against 500 volumes of 0.04M Na phosphate, 0.01M KCN, pH 6.4. Separation of hemoglobins from this solution was then carried out as indicated above. Coincident peaks of radioactivity and optical density appeared in the chromatogram soon after elution with dilute phosphate-cyanide was begun. In this respect DXP-hemoglobin was indistinguishable from phosphate-containing hemoglobins normally found in hemolysates of human erythrocytes (9, 10).

From values of the absorbance at 421 nm of the DXP-hemoglobin and hemoglobin Ao fractions, it was determined that DXP-hemoglobin comprised 6.4% of the total, a

value in good agreement with that calculated from the specific activity of hemoglobin isolated from the reaction mixture at the end of the first day of incubation. However, although this result suggested that the radioactive fraction consisted only of DXP-hemoglobin, the specific activity of the fraction was found to be 3.0×10^5 dpm/μmole, corresponding to 71% rather than 100% purity.

DISCUSSION

(4-^{3}H) DXP was found to react with hemoglobin Ao to form a radioactive adduct stable to repeated precipitation by trichloracetic acid and to 24 hour dialysis against dilute phosphate-cyanide buffer. Formation of a DXP-hemoglobin adduct to the extent of 17.6% in 3 days at 37° indicates that the affinity of DXP for hemoglobin is greater than has so far been observed in experiments with other phosphate esters. An interesting example for comparison is fructose-1-phosphate, whose steric configuration from C1 to C4 is the same as that of the corresponding carbon atoms of DXP. Under conditions not remarkably different from those presently employed, fructose-1-phosphate combined with hemoglobin Ao to the extent of 9.8% (2). It is tempting to speculate that the greater affinity of DXP for hemoglobin relates to the fact that DXP has an open-chain configuration whereas fructose-1-phosphate exists predominantly as the furanoside. Thus, in contrast with DXP, a ring must open before fructose-1-phosphate can react to form a Schiff base, creating the bond which, it is widely assumed, is responsible for the glycosylation of hemoglobin by a variety of sugar phosphates.

Having observed that DXP is formed in human red blood cells exposed to acetaldehyde and that it is bound by hemoglobin, the question arises whether synthesis and binding of DXP occur in alcoholic individuals. Formation in vivo of a DXP-hemoglobin adduct would serve to integrate the concentration of acetaldehyde in the blood and thus reflect a dose-time record of alcohol consumption. In this connection it is of interest that, when the blood of a non-diabetic individual who had been drinking

heavily for a month was analyzed for hemoglobins by the Trivelli-Gabay procedure, 20% of the total hemoglobin was eluted in the phosphate-containing hemoglobin fraction.

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