The Phosphorus Turn-over Rate of Human Red Cell Mononucleotides

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A two dimensional paper chromatographical method was used for the study of human red cell mononucleotides *. ATP, ADP, GTP, GDP and an uridine containing mononucleotide were found constantly. The labelling of the mononucleotides was investigated after incubation with radioactive phosphate. ADP and GTP were shown to have a phosphorus turn-over rate almost as high as the ATP turn-over rate.

The presence of ATP and ADP in human red cell has been amply demonstrated (review in Whittam¹). More recently, the concentration of the two nucleotides has been adequately determined by enzymic² and paper chromatographical³⁻⁵ means and with the use of ion-exchange chromatography⁶⁻⁸. The presence of small amounts of GTP and AMP has also been verified.

The adenine nucleotide phosphorus has a high turn-over rate^{9,10}. By means of differential hydrolysis Gourley⁹ showed a high labelling of the labile phosphorus of ATP while the stable phosphorus labelling was lower. The great number of various organic phosphates present in the red cells makes, however, an adequate study of the nucleotide labelling difficult. The procedure used in the present work allowed a means for the estimation of the amount and the degree of labelling of the nucleotides after incubation of the red cells with radioactive phosphorus.

MATERIAL AND METHODS

The nucleotides were purchased from the Sigma Chemical Co, St Louis, Mo., and from Boehringer and Soehne, Mannheim, Germany, radioactive orthophosphate from the Radiochemical Centre, Amersham, England, Norite A was a produce of N.V. Norit — Vereeniging Verkoop Centrale, Amsterdam. Myokinase was purchased from Boehringer and Soehne. ATP was determined enzymatically in the presence of glyceraldehyde phosphate dehydrogenase, phosphoglycerate kinase, 3-phosphoglycerate and reduced diphosphopyridine nucleotide. The reagents were purchased from Boehringer and Soehne.

* The following abbreviations are used in the text: EDTA, ethylene diamine tetra acetic acid, pH 7.4; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; GTP, guanosine triphosphate; GDP, guanosine diphosphate.

Venous blood samples, approximately 10 ml each, were taken in tubes containing 0.5 ml of 0.2 M EDTA solution with simultaneous samples for the hematocrit and serum phosphorus determinations. Aliquots of 2 ml were immediately transferred into 50 ml Erlenmeyer bottles and about two million c.p.m. of radioactive phosphate was added. The bottles were filled with a gas mixture containing nitrogen, oxygen and carbon dioxide (75:20:5), corked and incubated for 15 min at 37°. The samples were subsequently chilled to + 2°, washed twice with physiological saline and 9 ml of 10 % trichloroacetic acid was added. The extract, after centrifugation, was neutralized to pH 8–8.5, 0.5 ml of 25 % solution of Ba-acetate and 60 ml of ethanol were added. The precipitate was allowed to settle down for 30 min, centrifuged down and washed once with ethanol. The precipitate was then suspended in 5 ml of water and enough Amberlite (IR-120, in acid form) was added to solubilize totally the precipitate.

The solution was neutralized to pH 6–7 and pressed through a Norit A column (1.5 cm length, 0.5 cm diam.). The column was washed with 10 ml of water and eluted with 5 ml of a mixture containing ethanol, ammonia and water (5:3:2). The eluate was evaporated to a small volume under a current of nitrogen and submitted to paper chromatography.

Whatman No. 3 MM chromatographical paper sheets were washed with 0.01 M EDTA solution, water and *n*-propanol by allowing the solvents to run through the paper, one day each. The sample was transferred to the paper by means of a capillary to make a spot, 0.5 cm wide and 1 cm long.

The solvents were slightly modified from those used by Hanes and Isherwood¹¹ and Miettinen¹². In the first run *tert.* butanol: formic acid: water: ammonia (8:3:4:0.03, 8 h), and in the second run *n*-propanol: ammonia: water (6:3:1, 20 h) were used.

The spots were localized under ultraviolet light, outlined with pencil, cut off and eluted with 0.1 M tris buffer, pH 7.1, by means of a capillary. The volume was adjusted to 0.5 ml. The absorption spectra of the spots were determined in Beckman DU spectrophotometer in 0.5 ml cuvettes having a light path of 1 cm. The radioactivity of the samples was subsequently determined in an Ekco Model M-6 Geiger-Müller tube. Generally, the counts were taken over a period of 5 min.

Suitable blank areas on the chromatograms were treated similarly.

The total amount of ATP in blood samples was determined by the glyceraldehyde phosphate dehydrogenase reaction. The total amount of the other nucleotides was computed using the ratio of total ATP to ATP in the chromatogram as a correction factor.

Fig. 1. Diagrammatic representation and radioautogram of red cell mononucleotides after two-dimensional separation by paper chromatography.

RESULTS AND DISCUSSION

Eight ultraviolet light absorbing spots were visible in the chromatograms.

A) was the largest spot with a spectrum identical to that of adenosine. It was identified as ATP by means of the glyceraldehyde phosphate dehydrogenase reaction.

B) also showed the absorption spectrum of adenosine. It's R_F value was identical with that of ADP. It also showed the glyceraldehyde phosphate dehydrogenase reaction after addition of myokinase.

C) A weak spot with a spectrum of adenosine was occasionally found at this area. The R_F values of AMP coincided with that of this spot. No radioactivity was found in the spot.

D) and E) were not well separated from each other. The two spots had spectra identical with that of guanosine. D) has been further identified as GTP by means of it's R_F value. E) has been tentatively identified as GDP.

F) The weak spot has not been identified.

G) has a spectrum identical with that of uridine. The compound has not been further identified, however. The spot showed no radioactivity.

H) was a weak spot and it was found inconsistently. It had a spectrum of adenosine, and is presumed to be ADP, a breakdown product of ATP, formed during the run with the acid solvent.

A weak and indistinct spot was seldom found in the area between A, D and E. The eluate of the area showed consistently a spectrum of adenosine. The area also had radioactivity, which, however, mostly was due to the phosphate liberated from ATP during the run.

The amount of the four nucleotides that were found constantly in the blood samples are shown in Table 1. The ratio of ATP/ADP, roughly 8:2, is similar to the value found by Greenwalt ⁵, Bishop ⁶, Mills ⁷ and Hannon ² but is somewhat lower than that found by Vogel ⁴. The amount of GTP is consistent with the results of Mills ⁷.

Table 1. The amount of labelling of mononucleotides after incubation with radioactive phosphorus in eight samples of human red cells.

The amounts expressed as μg of mononucleotide/ml of red cells. Amount of radioactive phosphorus was two million counts per min. for 1 ml of red cells. The labelling corrected for 3 mg phosphorus in 100 ml plasma.

ATP			ADP			GTP			GDP	
μg	c.p.m.	Counts per μg	μg	c.p.m.	Counts per μg	μg	c.p.m.	Counts per μg	μg	c.p.m.
229	35 350	154	32	4 370	136	6.8	860	108	1.9	80
425	34 450	81	85	6 170	72	14	304	20	3.7	—
347	44 030	126	35	4 580	130	21	810	39	5.1	—
587	65 214	111	77	6 362	82	22	1 150	50	6.5	103
533	42 100	78	61	3 105	50	32	1 406	43	5.7	43
634	60 500	95	62	2 580	41	38	1 010	23	8.5	—
560	88 950	158	94	12 460	132	26	1 834	70	7.4	71
400	74 226	185	69	9 769	141	9.3	1 227	130	3	70

The highest uptake of phosphorus was found in ATP. It suggests, in accordance with Gourley⁹ that ATP is one of the key substances in the uptake of phosphorus from plasma into the cell. ADP, however, showed a labelling almost as high as that of ATP. It suggests that, in addition to the adenylate kinase system, another pathway exists in the red cell for the labelling of the ADP-phosphorus. The lack of any detectable AMP in most of the samples also suggests that the adenylate kinase pathway is inhibited in the red cell.

A remarkably high phosphorus uptake was found also in GTP.

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