THE EFFECT OF PHENYLHYDRAZINE ON THE ADENOSINE TRIPHOSPHATE CONTENT OF NORMAL AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE-DEFICIENT HUMAN BLOOD *

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The erythrocytes of certain individuals are peculiarly sensitive to the hemolytic effects of primaquine and a number of related compounds. A variety of biochemical abnormalities has been uncovered in such erythrocytes, principally related to deficiency of glucose-6-phosphate dehydrogenase (G6PD) (1–3) but the mechanisms responsible for the drug-induced hemolysis have not been clearly defined.

Adenosine triphosphate (ATP) is an important compound in the metabolism of the erythrocyte, and it is known that a decrease in the concentration of ATP in erythrocytes correlates well with decreased survival of these cells as they age *in vitro* (4, 5) and *in vivo* (6, 7). It therefore seems possible that a fall in the level of ATP might be associated with the decreased erythrocyte survival that occurs in drug-induced hemolytic anemia. In order to obtain evidence bearing on this point phenylhydrazine hemolysis was chosen as a model. The effect of phenylhydrazine on the ATP of whole blood from normal subjects and from individuals with G6PD deficiency was determined under a variety of *in vitro* experimental conditions. Herein are presented data from experiments which demonstrate that phenylhydrazine induces a marked fall in the ATP level of both normal and G6PD-deficient blood. The fall in ATP can be partially prevented by glucose in both types of blood, but the protective effect is less in G6PD-deficient blood.

MATERIALS AND METHODS

Subjects studied. All subjects were adult males. The G6PD-deficient subjects were all Negroes who had normal hemoglobin by electrophoretic analysis performed on starch blocks (8). In all subjects the leukocyte count was normal, the hematocrit above 40 per cent, and the reticulocyte count below 3.3 per cent. The hematologic studies were performed by standard methods (9). G6PD was assayed by following the appearance of reduced triphosphopyridine nucleotide (TPNH) spectrophotometrically under the conditions described by Motulsky (10): The reaction mixture contained 3 μmoles disodium ethylenediamine tetraacetic acid; 20 μmoles $MgCl_2$; 39.5 μmoles nicotinamide; 222 μmoles Tris-(hydroxymethyl) aminomethane; 5 μmoles glucose-6-phosphate; 0.4 mg triphosphopyridine nucleotide (TPN); and 2 ml of a 1:250 aqueous hemolysate of whole blood in a final volume of 3 ml. The reaction was carried out at room temperature in cuvets of 1 cm light path in a Beckman model DU spectrophotometer. Units of activity are defined as change in optical density at 340 mμ per minute per ml of erythrocytes.

Preparation of blood. Whole blood was collected in heparin (0.1 mg per ml of blood) under sterile conditions from fasting subjects. Whole blood was used for most experiments to keep manipulation of the erythrocytes to a minimum. The data of Bishop, Rankine, and Talbott (11) and of Whittam (12) indicate that essentially all the ATP of whole blood is in the erythrocytes, and in the present study no significant difference was found between the ATP content of whole blood and an equivalent amount of washed erythrocytes from a suspension which had a hematocrit of 35 per cent and contained 2,000 platelets and 1,850 leukocytes per mm^3.

Washed erythrocytes were prepared at 4° C as described by Whittam (12) except that balanced salt solution buffered with bicarbonate was used throughout. The erythrocytes were finally suspended in sufficient balanced salt solution to restore the volume to that of the original blood.

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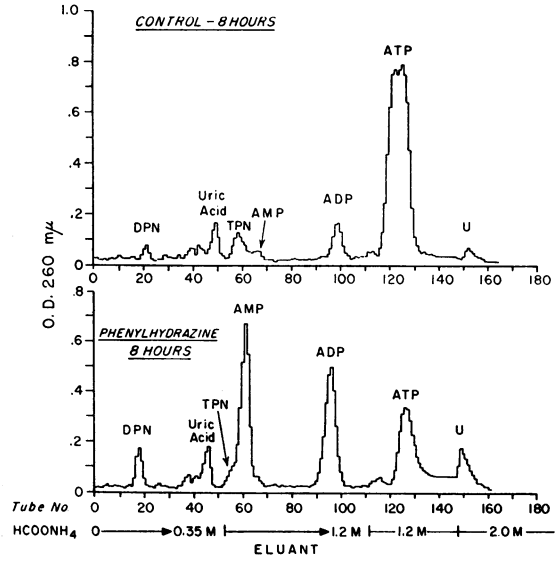

FIG. 1. CHROMATOGRAM OF NUCLEOTIDES EXTRACTED FROM WHOLE BLOOD INCUBATED WITH AND WITHOUT PHENYLHYDRAZINE, SHOWING RISE IN AMP AND ADP AND FALL IN ATP LEVEL INDUCED BY PHENYLHYDRAZINE. The chromatograms were obtained from TCA extracts of 10 ml whole blood incubated for 8 hours at 37° C with and without phenylhydrazine, as outlined under Methods. Chromatography was carried out on 1 × 20 cm columns of Dowex 1 × 8 formate resin, 200–400 mesh. The flow rate was 0.5 to 0.8 ml per minute; 6-ml fractions were collected. The gradient elution system used achieved a concave gradient by means of a conical reservoir containing 140 ml solvent and a cylindrical mixing vessel containing 220 ml solvent. Solutions of ammonium formate at pH 4.5 were used as eluant. The first gradient was from 0 to 0.35 M ammonium formate and the second from 0.35 to 1.2 M. After this the chromatogram was completed by straight elution with 1.2 M and 2.0 M ammonium formate. Abbreviations used are: DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; and U, unknown components.

Incubation of blood. Ten ml of blood or washed erythrocyte suspension was placed under sterile conditions in silicone-coated (G. E. Drifilm) screw-capped tubes. Phenylhydrazine hydrochloride dissolved in 0.9 per cent sodium chloride solution was sterilized by filtration and was added to the appropriate vessels to a final concentration of 3.4 μmoles per ml. Sterile glucose solution in 0.9 per cent sodium chloride was added to the appropriate vessels to give a final concentration of added glucose of 20 μmoles per ml. All reaction mixtures were made to a final volume of 11.0 ml with 0.9 per cent sodium chloride solution when necesary. Incubation was carried out at 37° C in an atmosphere of air with intermittent inversion of the tubes to maintain suspension of the cells.

At the end of each period of incubation the pH, per cent hemolysis (13), and Heinz body formation (14) were determined on a 1.0 ml aliquot of the reaction mixture, and a cold trichloroacetic acid (TCA) extract was made from the remainder by the method of Bishop and co-workers (11). Glucose was determined at intervals throughout the incubation period by a glucose oxidase method (Glucostat, Worthington Biochemical Corp., Freehold, N. J.). Pyruvic acid was measured by the method of Bueding and Wortis (15) and lactic acid by the method of Barker and Summerson (16).

Column chromatography. The acid-soluble nucleotides were chromatographed on 1 × 20 cm columns of Dowex 1 × 8 formate resin (200–400 mesh) (17). After the extract had passed through the resin, the column was washed with water until the effluent no longer absorbed ultraviolet light. Elution of the nucleotides was then carried out with solutions of ammonium formate at pH 4.5, with a flow rate of 0.5 to 0.8 ml per minute. Separation of all the major nucleotides was achieved with a concave gradient system (18) employing a conical reservoir and a cylindrical mixing vessel (19). The details of the technique are given in Figure 1.

In order to simplify the estimation of ATP, a chromatographic system was developed which separated the nucleotides into a pre-ATP fraction, ATP, and a post-ATP fraction. The chromatogram was developed with 1.1 M ammonium formate at pH 4.5 until ATP was recovered, and then with 2.0 M ammonium formate at pH 4.5 to recover the post-ATP fraction. A chromatogram of this type is illustrated in Figure 2.

Fractions of 6 ml were collected for both types of chromatogram with an automatic fraction collector. The optical density at 260 and 275 mμ was determined for each fraction. Nucleotides were identified by their ultraviolet absorption spectra and by their position on the chromatogram as compared with standard compounds (purchased from Sigma Chemical Co., St. Louis, Mo.).

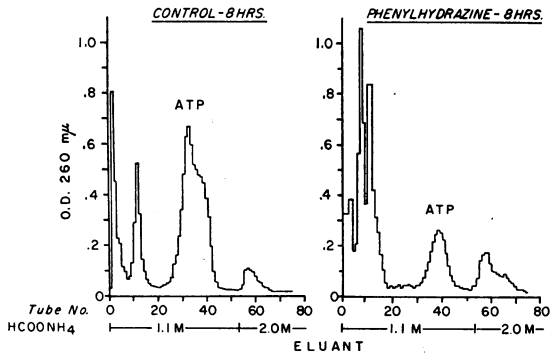

FIG. 2. SIMPLIFIED CHROMATOGRAPHIC PROCEDURE FOR MEASURING ATP. Blood was incubated as described in Figure 1. The chromatographic procedure was that used in Figure 1 except that elution was performed with a nongradient system utilizing 1.1 M and 2.0 M ammonium formate at pH 4.5, as indicated on the figure.

TABLE I

Changes in the pH, per cent hemolysis, and Heinz body formation of whole blood incubated with phenylhydrazine

Time of incubation, hours	0		4		6		8	
	Control	Phenyl-hydrazine	Control	Phenyl-hydrazine	Control	Phenyl-hydrazine	Control	Phenyl-hydrazine
pH	7.6	7.6	7.52	7.58	7.45	7.4	7.35	7.35
Hemolysis, %	0.8	1.2	1.3	1.0	0.8	1.8	1.0	1.9
RBC with 5 or more Heinz bodies, %	0	0	0	90	0	94	0	96

In some experiments the adenine nucleotides were analyzed for ribose (20) and phosphate (21) content, and DPN and TPN were further identified by the change in extinction coefficient at 340 mμ which occurs on reaction with cyanide (22). Recovery of ultraviolet-absorbing material was greater than 90 per cent in all experiments with both types of chromatography. Also, recovery of known amounts of an ATP standard was greater than 94 per cent with both chromatographic systems.

RESULTS

Effect of phenylhydrazine on the ATP of whole blood in vitro. Data from an experiment on the effect of incubation of whole blood with phenylhydrazine on the pH, per cent hemolysis, and Heinz body formation are shown in Table I. In no experiment was the final pH less than 7.3; hemolysis never exceeded 2 per cent during an 8 hour incubation; and in most experiments after 4 hours' incubation with phenylhydrazine, more than 90 per cent of the erythrocytes contained more than 5 Heinz bodies.

Illustrated in Figure 1 are typical chromatograms obtained from the TCA extracts of normal whole blood incubated for 8 hours with and without phenylhydrazine. The chromatogram of blood incubated for 8 hours without phenylhydrazine does not differ significantly from that found with blood at zero time, and the quantities of nucleotides recovered at both 0 and 8 hours agree well with the data of Bishop and co-workers (11). Two definite changes in the nucleotide pattern of blood incubated with phenylhydrazine can be detected: first, there is a marked decrease in ATP and a corresponding increase in adenosine diphosphate (ADP) and adenosine monophosphate (AMP); and second, there is an increase in the material eluted from the column after ATP. This material has not been identified but appeared, from a study of the ultraviolet absorption spectrum, to be a mixture of a guanine compound with an adenine-like compound. In these experiments the final concentration of phenylhydrazine was 3.4 μmoles per ml; higher concentrations, up to 6.8 μmoles, caused a greater fall in ATP; lower concentrations, down to 1.7 μmoles per ml, were less effective in this regard.

TABLE II

Reciprocal rise in AMP and ADP after the fall in ATP occurring after 8 hours' incubation of whole blood with phenylhydrazine *

Subject	AMP	ADP	ATP	AMP & ADP & ATP	Adenine nucleotide recovered, %†
D.M. (normal) Control	7.5	35.2	372.5	415.2	93.1
Phenylhyd.	127.9	125.6	133.2	386.7	
R.D. (G6PD-def.) Control	5.9	54.5	339.5	399.9	85.6
Phenylhyd.	94.6	126.2	121.7	342.5	

* Concentrations are given as μmoles/L whole blood. See text for abbreviations.
† In each experiment 100% recovery was assumed for the control vessels.

Quantitative data on the adenine nucleotides from this experiment and a similar one on blood from a G6PD-deficient subject are presented in Table II. Most of the ATP which disappeared was found as ADP and AMP in both experiments, indicating that the fall in ATP was due primarily to loss of terminal phosphate groups.

Figure 2 shows chromatograms from experiments to determine the time course of the decrease in ATP which occurs when blood is incubated with phenylhydrazine. The simplified chromatographic system was used. In extracts of blood incubated with phenylhydrazine, the total amount of ultraviolet-absorbing material remained constant, but there was an increase in material eluted from the column before and after ATP and a decrease in ATP. Quantitative data on the ATP level obtained from such experiments on three normal and one G6PD-deficient subject are presented in Figure 3. It can be seen that the ATP of blood incubated with phenylhydrazine decreases, beginning at about 6 hours, and by 8 hours has fallen to about 35 per cent of the original amount present. In blood incubated without phenylhydrazine the ATP remained nearly constant. The results were the same in blood from normal subjects and a subject with G6PD deficiency.

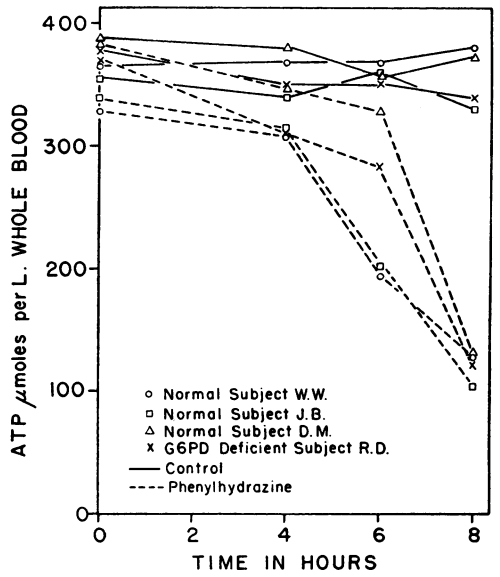

FIG. 3. RATE OF FALL OF ATP IN NORMAL AND G6PD-DEFICIENT BLOOD INCUBATED WITH AND WITHOUT PHENYLHYDRAZINE. ATP was determined on extracts prepared from 10 ml of blood incubated as outlined under Methods.

The effect of glucose on the ATP of phenylhydrazine-treated blood. The delay of several hours before the decrease in ATP of phenylhydrazine-treated blood suggested that the fall might be related to the depletion of glucose. Accordingly, experiments were carried out to assess the role of glucose in the changes in ATP in blood incubated with phenylhydrazine. Table III presents data from such experiments on three normal and three G6PD-deficient subjects. Glucose utilization averaged 0.66 μmole per ml of blood per hour for samples incubated without phenylhydrazine and 0.85 μmole per ml of blood per hour for samples incubated with phenylhydrazine. Blood from two normal and one G6PD-deficient subject was incubated at 37°

TABLE III

The effect of added glucose on the ATP content of normal and G6PD-deficient whole blood incubated with phenylhydrazine for 8 hours *

Subject	G6PD activity‡	No glucose added					Glucose added†				
		Control		Phenylhydrazine		% Decrease in ATP§	Control		Phenylhydrazine		% Decrease in ATP§
		ATP	Glucose	ATP	Glucose		ATP	Glucose	ATP	Glucose	
Normal											
W.W.	6.9	382.6	0.6	135.3	<0.1‖	64.6	389.4	19.3	343.4	16.6	11.8
B.F.	7.0	331.5	0.4	115.4	<0.1	65.2	341.1	22.9	297.6	21.7	12.8
E.B.	7.0	247.1	<0.1	79.2	<0.1	67.9	326.8	20.8	289.7	18.0	11.4
G6PD-def.											
R.D.	1.1	315.1	0.9	120.0	<0.1	61.9	348.6	22.0	224.8	20.5	35.5
L.J.	1.4	265.7	0.7	99.0	<0.1	62.7	287.4	23.4	172.7	21.2	39.9
J.C.	0.5	332.6	1.0	179.0	0.2	46.2	309.0	20.8	188.4	20.0	39.0

* ATP concentration expressed as μmoles/L blood, glucose concentration as μmoles/ml blood.
† The blood was supplemented with 20 μmoles per ml glucose.
‡ Glucose-6-phosphate dehydrogenase activity expressed as change in optical density/min/ml RBC.
§ Expressed as % fall in ATP content of phenylhydrazine-treated blood compared with control blood.
‖ Measurement of glucose not accurate below 0.1 μmole per ml.

C with or without phenylhydrazine (3.4 μmoles per ml) and with added glucose (20 μmoles per ml), and the amount of lactic and pyruvic acids formed was compared with the amount of glucose which disappeared. Although 88 to 103 per cent of the glucose utilized over an 8-hour period by both types of blood incubated with or without phenylhydrazine could be accounted for by lactic and pyruvic acids, phenylhydrazine altered the relative amounts of these two compounds formed. Thus, in both types of blood incubated without phenylhydrazine, pyruvic acid accounted for only 1 to 2 per cent of the glucose utilized, while in blood incubated with phenylhydrazine, 10 to 18 per cent of the glucose utilized appeared as pyruvic acid. These data indicate that glucose is metabolized principally by normal pathways in blood incubated with phenylhydrazine, although there appears to be interference with the reduction of pyruvic acid to lactic acid. The accumulation of pyruvic acid has been previously reported for phenylhydrazine-treated avian (25) and human (23) erythrocytes.

As shown in Table III, in blood incubated for 8 hours with phenylhydrazine but without added glucose, there was less than 0.1 μmole of glucose per ml remaining in all vessels except one (Subject J.C.). The ATP level was markedly reduced in all vessels compared with the controls, although in the blood from Subject J.C., where there was glucose still remaining after the incubation period, the fall was not so great as in the others. In the control vessels incubated for 8 hours without phenylhydrazine or added glucose, glucose remained in all vessels except one (Subject E.B.). The ATP level in the blood from Subject E.B. was lower than in the other control vessels but was still much higher than in the comparable vessel with phenylhydrazine added.

In the experiments on blood with glucose added, a large quantity of glucose remained after 8 hours' incubation with or without phenylhydrazine. In blood from normal subjects there was an average fall in ATP of 12 per cent, while in blood from G6PD-deficient subjects there was an average fall in ATP of 38 per cent. Thus, glucose partially protected G6PD-deficient erythrocytes and almost completely protected normal erythrocytes from the fall in ATP induced by phenylhydrazine after 8 hours' incubation.

TABLE IV

Normal whole blood incubated for 12 hours with phenylhydrazine and glucose added

Subject	Control		Phenylhydrazine		% Decrease in ATP*
	ATP	Glucose	ATP	Glucose	
	μmoles/L	μmoles/ml	μmoles/L	μmoles/ml	
D.M.	394.1	19.0	302.3	16.8	23.3
C.C.	323.5	18.0	202.9	17.5	37.3
B.F.	314.8	21.2	222.4	19.0	29.4

* Expressed as % fall in ATP content of phenylhydrazine-treated blood compared with control blood.

Prolonged incubation of normal blood with phenylhydrazine. Because there was a slight fall in ATP in normal blood incubated with phenylhydrazine and added glucose, additional experiments were performed to determine whether the protective effect of glucose on the ATP of normal blood would extend over a longer period of incubation. The results are shown in Table IV and demonstrate that, even with an adequate glucose concentration, incubation with phenylhydrazine for 12 hours causes a definite fall in the ATP content of normal blood.

Washed erythrocytes incubated with phenylhydrazine. Figure 4 illustrates the results of an experiment performed to determine the effect of phenylhydrazine on the ATP of erythrocytes which had been washed to remove most of the glucose. The washed erythrocyte suspension con-

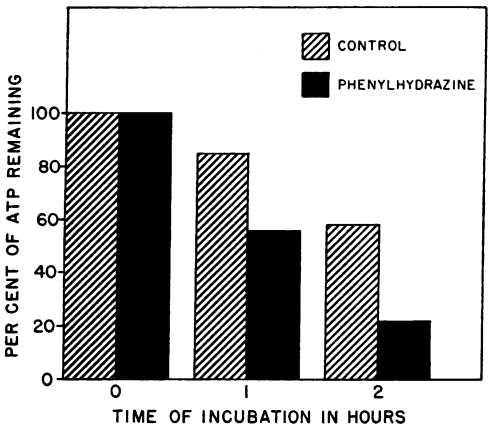

FIG. 4. THE EFFECT OF PHENYLHYDRAZINE ON THE ATP OF WASHED ERYTHROCYTES. The data are plotted as the per cent of zero time ATP remaining after each period of incubation. The hematocrit of the erythrocyte suspension was 36 per cent and the initial glucose concentration was 0.2 μmole per ml of suspension. The erythrocyte suspension was incubated as outlined under Methods.

tained 0.2 μmole of glucose per ml at the beginning of the incubation period. The fall in ATP level was more rapid in the samples incubated with phenylhydrazine than in the controls. The rate of disappearance of ATP from the control suspension was similar to that observed by Whittam (12).

DISCUSSION

The mechanism by which phenylhydrazine diminishes the ATP level of whole blood is at present unknown, although ultimately there must be either a decrease in ATP regeneration or an increase in ATP breakdown, or both. Phenylhydrazine is known to increase the rate of glucose utilization by blood (24) and, in those experiments in which blood was incubated with phenylhydrazine without added glucose, the fall in ATP might be accounted for in part by decreased regeneration of ATP as a result of exhaustion of glucose. In the experiments in which glucose was added to the incubation mixture in sufficient quantity to provide substrate throughout the experiment, a fall in ATP could still be demonstrated after 12 hours in normal blood and 8 hours in G6PD-deficient blood, which suggests that the effect of phenylhydrazine on the ATP level of blood cannot be ascribed entirely to increased glucose utilization. This point is further emphasized by the demonstration that a more rapid fall in ATP level occurred in washed erythrocytes incubated with phenylhydrazine than was found in the controls. Since essentially no glucose was available for ATP regeneration in these experiments, the results suggest that phenylhydrazine increases ATP breakdown instead of interfering with ATP regeneration. Further investigation will be necessary, however, to establish this point.

When phenylhydrazine is added to blood it reacts with oxyhemoglobin to form, among other products, hydrogen peroxide or equivalent free radicals (25–27). Hydrogen peroxide oxidizes hemoglobin (25–27) and other proteins (28, 29) including, perhaps, enzymes concerned with ATP metabolism. If hydrogen peroxide is the toxic agent responsible for the decline in ATP content of blood exposed to phenylhydrazine, then the difference in protective effect of glucose on the ATP of normal blood compared with G6PD-deficient blood may be tentatively explained on the following grounds. Hydrogen peroxide formed in the erythrocyte must be reduced by catalase or peroxidase, and we would expect the activity of both these enzymes to be suboptimal in G6PD-deficient blood incubated with phenylhydrazine. There are reports that catalase activity is decreased in the G6PD-deficient erythrocyte (30) and that phenylhydrazine is a catalase inhibitor (31). The "glutathione peroxidase" (32) of erythrocytes requires reduced glutathione (GSH) as a cofactor, and when hydrogen peroxide is reduced by this enzyme, oxidized glutathione (GSSG) is formed. GSSG must be continually reduced to GSH for peroxidase to remain active, and TPNH is the chief pyridine nucleotide necessary for this reduction (33). The G6PD-deficient cell is limited in its capacity to regenerate TPNH because metabolism of glucose via the pentose phosphate pathway, which is a source of TPNH, is interfered with by the enzyme deficiency. Hence, we would expect glucose to be less effective in protecting against the effects of hydrogen peroxide in the G6PD-deficient cell than in the normal cell, because the limiting factor would be the deficient enzyme.

The biochemical events leading to hemolysis in normal or G6PD-deficient erythrocytes exposed to phenylhydrazine or related compounds are still undefined. It seems possible that a decreasing level of ATP in the erythrocyte could lead eventually to hemolysis in view of the studies that have correlated a low level of ATP with impaired erythrocyte survival in stored blood (4, 5) and with aging of erythrocytes *in vivo* (6, 7). Other studies have shown that older erythrocytes (which have a low ATP content) (6, 7) are more susceptible to immune hemolysis (34), osmotic hemolysis (35, 36), and drug-induced hemolysis (37). The fall in ATP observed in these studies may be directly related to the hemolytic mechanism, but the relatively long time required for the gross changes in ATP concentration to occur may weigh against this concept. It is also possible that the altered ATP metabolism occurs coincidentally with other chemical changes which lead directly to hemolysis.

In this study attention has been directed primarily to the changes induced by phenylhydrazine in the concentrations of the adenine nucleotides of the erythrocyte. However, in blood incubated with phenylhydrazine there was also an increase

in the quantity of ultraviolet-absorbing material appearing after ATP in the chromatograms. The chemical nature and biologic significance of this material are at present unknown, although it appears to be at least quantitatively important, since it represented up to 28 per cent of the total ultraviolet-absorbing material in these extracts.

SUMMARY

1. Incubation of normal and glucose-6-phosphate dehydrogenase (G6PD)-deficient blood with phenylhydrazine induced a decrease in adenosine triphosphate (ATP) concentration and a corresponding increase in adenosine diphosphate and adenosine monophosphate. In addition, there was an increase in the amount of ultraviolet-absorbing material of unknown chemical composition which was eluted from Dowex-1 formate resin columns after adenosine triphosphate.

2. Phenylhydrazine enhanced glucose utilization and pyruvate accumulation in both normal and G6PD-deficient blood.

3. The fall in ATP concentration induced by incubating normal blood with phenylhydrazine for 8 hours was largely prevented by adding glucose to the incubation mixture but, in normal blood incubated for 12 hours with phenylhydrazine, a definite fall in ATP concentration was demonstrated even with glucose added.

4. Glucose was less effective in protecting G6PD-deficient blood from the ATP-lowering effect of phenylhydrazine than it was with normal blood.

5. Phenylhydrazine induced a more rapid fall in the ATP concentration of erythrocytes washed free of glucose than occurred in the controls.

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