Effects of Acetylphenylhydrazine on Phosphate Uptake and Incorporation in Erythrocytes

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The effects of acetylphenylhydrazine on the red cell have been studied by a number of investigators since it was first suggested that this drug may act as a model for a large group of chemicals causing drug-induced hemolytic anemia. It is clear that the drug acts as a hydrogen acceptor and through this mechanism may denature proteins and accelerate or inhibit biochemical reactions. It has been shown to have several distinct actions within the red cell. In the presence of oxyhemoglobin, it is rapidly oxidized, and thereupon indirectly or directly may cause the oxidation of coenzymes, glutathione and hemoglobin, as well as oxidized groups in other protein structures.1,2 Recent studies suggest that within the red cell it generates peroxide, which leads to further oxidation of vital metabolites unless this action is neutralized by the action of catalase and glutathione peroxidase.3-5

The present studies were carried out to determine whether it affects phosphate uptake and turnover of high energy phosphate within the red cell.

Methods

Human blood was collected in heparinized syringes, centrifuged, and the plasma and buffy coat removed. The cells were resuspended in saline, washed once, then resuspended in Krebs-Ringer's solution buffered with 0.35M Tris to a pH of 7.4. The ratio of buffer to solution was 30 parts to 109. This gave a final phosphate concentration of $1.1 \times 10^{-3}$M.

Glycolytic intermediates were separated according to the method of Bartlett using a column of Dowex-1-Cl and eluting with solutions from 0.01N HCl to 0.5N NH4Cl as modified by Robinson et al. for small volumes.6 Approximately 2 ml of cells in a total volume of 5 ml were used for each aliquot studied. Fractionation was carried out by collecting the eluate in 10 ml volumes from a resin column 2.0 cm in length by 1.8 cm in diameter. Total phosphorous was measured according to the method of Bartlett,8 and adenosine phosphate compounds were measured at 260 mμ. The ultraviolet absorption maximum for adenosine. Since the pH in the first runs of the fractionation is acid, a comparison was made of the OD before and after neutralizing. The difference being small, readings are reported throughout at the pH of the given eluate without modification. To evaluate the method, an aqueous solution of acetylphenylhydrazine was allowed to stand 48 hours, at which time it had turned yellow, and was then run on the columns. This
Fig. 1.—The effect of temperatures on the inhibition of uptake of P$^{32}$O$_4^-$ by acetylphenylhydrazine. The abscissa and ordinate refer respectively to time of incubation and counts per minute adjusted to a concentration of 10 Gm. per cent hemoglobin. At 37°C, the inhibition by acetylphenylhydrazine is evident. The lines labeled 0°C refer to experiments carried out in ice water. The uptake is minimal at this temperature and no difference is seen between the acetylphenylhydrazine and control samples.

caused a significant increase in the readings of 0.01 M eluate, and, therefore, changes in AMP, which is found in this fraction, could not be evaluated. In experiments in which P$^{32}$O$_4^-$ was used, 1 ml. of the solution to be measured was dried in a planchet and counted in a windowless flow counter at preset counts. Background was 15 to 20 counts per minute. Samples were counted in triplicate at a setting of at least 400 counts. Most samples were counted for more than a minute. All were counted for more than 0.5 minutes.

RESULTS

To determine the effects of acetylphenylhydrazine on phosphate uptake, P$^{32}$O$_4^-$ (P$^{32}$-labeled inorganic orthophosphate) was added to a suspension of red cells, and the rate of uptake observed in the presence and absence of the drug. Experiments were carried out at 37°C in a Dubnoff shaker. At 0 time, acetylphenylhydrazine was added to 5 ml. of the red cell suspension in an Erlenmeyer flask to make a final concentration of 3.3 x 10$^{-5}$M. Immediately thereafter, P$^{32}$O$_4^-$ was added and measurements of uptake were made at 1, 5 and 15 minutes. To prevent further significant incorporation of the isotope at the designated time, an aliquot of the suspension was diluted to approximately 20 times its volume in ice-cold 0.1 M phosphate buffer. The red-cell suspension was then washed twice with a similar volume of this cold buffer, after which the cells were suspended in an equal volume of Krebs-Ringer solution and 1 ml. placed in planchets for counting. Within 5 minutes, an effect on uptake of P$^{32}$ by the red cells could be demonstrated.
ACETYLPHENYLHYDRAZINE EFFECTS ON PHOSPHATE

Table 1.—Effect of Increasing Concentration of Acetylp phenylhydrazine on Erythrocyte Phosphate Uptake. In All Samples, 10,000 Counts Were Counted in Triplicate

<table>
<thead>
<tr>
<th>APH Molarity</th>
<th>P^32 Uptake in cpm/ml.</th>
<th>1 Minute</th>
<th>5 Minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>2330</td>
<td>14,900</td>
</tr>
<tr>
<td>6.6 x 10^-3</td>
<td></td>
<td>2200</td>
<td>13,700</td>
</tr>
<tr>
<td>1.33 x 10^-2</td>
<td></td>
<td>2050</td>
<td>12,300</td>
</tr>
<tr>
<td>2 x 10^-2</td>
<td></td>
<td>1910</td>
<td>11,400</td>
</tr>
<tr>
<td>2.66 x 10^-2</td>
<td></td>
<td>1680</td>
<td>10,600</td>
</tr>
<tr>
<td>3.3 x 10^-2</td>
<td></td>
<td>1610</td>
<td>8,950</td>
</tr>
</tbody>
</table>

In figure 1, it can be seen that there is inhibition of uptake in the presence of the drug.

Addition of amounts of acetylp phenylhydrazine in a final concentration varying from 3.3 x 10^-2 M to 6.6 x 10^-3 M demonstrated a correlation between the degree of inhibition and concentration of the drug. At the lowest concentration, inhibition was only slight under the conditions of the experiment (table 1).

The effect of temperature was also observed by carrying out parallel experiments at 37 C., at room temperature, and in ice water. In the cold, rate of uptake was almost nil, and inhibition by the drug was not evident. At room temperature, uptake was evident in both samples with equivocal difference between control and experimental samples. At 37 C., when the rate of uptake was much greater, the inhibition by acetylp phenylhydrazine was apparent. A comparison of results at 0 C., and 37 C. can be seen in figure 1.

Evaluation of uptake demands some knowledge of egress under similar circumstances. Therefore, an experiment was carried out to observe the effects of acetylp phenylhydrazine on phosphate loss. Normal red cells were incubated for 1 hour with P^32O_4 to allow incorporation of the isotope, after which the cells were washed twice with 0.1 M phosphate buffer and then resuspended in Krebs-Ringer’s. The drug was added to one aliquot, and the rate of appearance of radioactivity in the supernatant was then compared to that in a control aliquot. These studies demonstrated a diminution in rate of egress of phosphate. In figure 2, the results of such an experiment are plotted. Thus, the inhibition of uptake cannot be explained as an artifact due to increased egress.

Since the drug may act as a sulphydryl inhibitor for glycolytic phosphorylation, comparative studies were done with monooiodoacetic acid, a known inhibitor of triose phosphate dehydrogenase. This was found to have no effect on the phosphate uptake at 1 x 10^-2 M, a concentration 10 times greater than that known to inhibit glycolysis and just below that found to produce spontaneous hemolysis. Moreover, 1 x 10^-5 M sodium fluoride, an inhibitor of glycolysis at the enolase reaction, did not inhibit P^32 uptake.

Parachlorohydroxybenzoate, a known SH inhibitor, which does not cross the red cell membrane, was incubated with the red cell suspensions and its effect studied. It was found that this drug also had no effect on P^32O_4 uptake.
in concentrations as high as $5 \times 10^{-3}$ M, beyond which point hemolysis occurs.

To determine further whether the decreased phosphate uptake was related to decreased rate of glycolytic phosphorylation, observations were made on the phosphorylated intermediates in the presence and absence of acetylphenylhydrazine. The drug was added to the red cell suspension in a final concentration of $3.3 \times 10^{-2}$ M, with 1 mg of glucose added per ml and the suspension incubated in an open Erlenmeyer flask in a Dubnoff shaker. Changes in glycolytic intermediates were then observed over a 2-hour period.

Observations during the first 15 minutes revealed no measurable change in the amount of intermediates as measured by total phosphate and adenine in either experimental or control flasks. The appearance of $^{32}$P in the ATP fraction could be documented in the first measurements at 5 minutes with an increase noted on further observation throughout the 2-hour incubation period.

At the end of this time, a significant difference was seen between the ATP fraction in the acetylphenylhydrazine-treated cells and in the control cells. One such experiment is shown in figure 3 in which OD at 260 m$\mu$ is plotted against eluate fractions. Whereas, in the control fraction, the eluate containing ATP (0.5N NH$_4$Cl) showed little change, the treated, cells showed a significant decline in this value. Concomitant readings in $^{32}$P activity demonstrated radioactivity in this fraction to be less in the treated cells. Specific activity was measured to evaluate the rate of incorporation. The isotope has been found to be present predominantly in the beta and gamma phosphates in short-term incubation experiments. Table 2 shows the results of such an experiment and demonstrates that there is a depression of the specific ac-
Fig. 3.—Effect of acetylphenylhydrazine on adenosine compounds and on $^{32}$P incorporation into erythrocyte glycolytic intermediates. In the designated eluates, the predominant intermediates are adenosine monophosphate and inorganic phosphate in the 0.01 HCl fraction, adenosine diphosphate in the 0.02 HCl fraction, fructose diphosphate in the 0.1N NH$_4$Cl fraction, 2, 3 diphosphoglycerate in the 0.2N fraction, and adenosine triphosphate in the 0.5N fraction. The open circles represent the values of the control after 2 hours incubation, and the closed circles refer to the values of the samples incubated with acetylphenylhydrazine. The ordinate in the upper chart refers to counts per minute per ml. of eluate and that on the lower chart to optical density at 260 m$\mu$, the maximum for adenosine. There is a decrease in the absolute amount of ATP and in the radioactivity in the drug-treated cells and a corresponding increase in these parameters in the ADP fraction.

Activity in the treated samples, though not a striking one. The amount of $^{32}$P in the fraction containing ADP was greater in the cells incubated with acetylphenylhydrazine, but it should be emphasized that the eluate containing ADP contains other phosphorylated intermediates, predominantly monophosphoglycerate, which makes it difficult to interpret the $^{32}$P values. However, the adenine readings are a reliable measure of ADP in this eluate because it contains no other interfering substances, and the total ADP as measured by adenine was higher than in the control preparation. This resulted in a depression in the ATP: ADP ratio in drug-treated cells as can be seen from the experiment in table 3. The degree of depression of this ratio varied in different experiments.

There was little difference in 2, 3 DPG as measured by total phosphate,
Table 2.—Effect of Incubation of Red Cells with Acetylphenylhydrazine for 2 Hours on the Specific Activity of ATP. CPM Refers to the Sum of Counts per Minute per ml. and O.D. Refers to the Sum of Optical Density Units at 260 m\(\mu\) for Each 10 ml Aliquot of the 0.5N NH\textsubscript{4}Cl Eluate

<table>
<thead>
<tr>
<th></th>
<th>cpm</th>
<th>OD</th>
<th>cpm/1.00 OD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2276</td>
<td>1.355</td>
<td>1680</td>
</tr>
<tr>
<td>APH</td>
<td>665</td>
<td>.790</td>
<td>841</td>
</tr>
</tbody>
</table>

Table 3.—Effect of Incubation of Red Cells with Acetylphenylhydrazine for 2 Hours on ATP:ADP Ratio. ADP and ATP Are Expressed as the Sum of Optical Density Readings of the 10 ml Aliquots of the Specific Eluate. Observations Were Carried Out after 2 Hours Incubation in Drug-treated (APH) and Control Samples

<table>
<thead>
<tr>
<th></th>
<th>ADP</th>
<th>ATP</th>
<th>ATP/ADP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>.505</td>
<td>1.355</td>
<td>2.68/1</td>
</tr>
<tr>
<td>APH</td>
<td>1.370</td>
<td>.790</td>
<td>0.57/1</td>
</tr>
</tbody>
</table>

and the radioactivity of the drug-treated sample at 2 hours was usually slightly less than the control with a slight decrease in specific activity of this intermediate. The decreased specific activity of 2, 3 DPG and of ATP is consistent with a depression of glycolytic phosphorylation by the drug, but the degree of depression of ATP values suggests increased utilization of ATP as well.

This was studied, therefore, by inhibiting incorporation of phosphate into glycolytic intermediates with monoiodoacetic acid and observing the effect of the acetylphenylhydrazine on rate of disappearance of ATP\textsuperscript{32}. A concentration of the inhibitor of 1 \(\times 10^{-3}\) M caused a marked inhibition of incorporation of P\textsuperscript{32}O\textsubscript{4} into ATP over a 2-hour period (fig. 4). The control samples showed significant incorporation into ATP during this period, and at 2-hours monoiodoacetic acid was added to prevent further synthesis. At this point acetylphenylhydrazine was added to one of the samples, and a comparison of the rate of disappearance of ATP\textsuperscript{32} was noted. Figure 4 demonstrates that at 15 minutes there is a striking difference in radioactivity in the treated and control samples. Addition of the drug caused an increased rate of disappearance of ATP with a corresponding increase in radioactivity in the ADP fraction. Thus, increased utilization of ATP accounts for a significant degree of the loss of the high energy phosphate compound on incubation with acetylphenylhydrazine.

In summary, the drug both inhibits incorporation of phosphate to form ATP, and also increases the rate of disappearance of this intermediate.

**DISCUSSION**

These investigations demonstrate an effect of acetylphenylhydrazine both on phosphate uptake and phosphate turnover in the erythrocytes. The former does not appear to be directly dependent upon the latter, but seems to be a separate phenomenon of the red cell membrane. The action of phosphate uptake is demonstrable within 1 minute, whereas depression in ATP is seen
Fig. 4.—Effect of acetylphenylhydrazine on breakdown of ATP. Cells were incubated with P\(^{32}\)O\(_4\) for 2 hours, and then further incorporation into ATP was prevented by adding monooiodoacetate. At this point acetylphenylhydrazine was added to one aliquot and the rate of disappearance of radioactivity from the ATP fraction compared to the control. It is evident that the drug caused an increased rate of disappearance of P\(^{32}\) from this fraction with an increase in radioactivity in the ADP fraction. The broken line from 0 to 2 hours, labeled ATP and IAA, demonstrates the inhibition of incorporation of P\(^{32}\) into ATP in the presence of iodoacetate.

only after longer incubation. While the decrease in specific activity of ATP which was observed could best be interpreted as inhibition of phosphate incorporation, incubation with monooiodoacetate acid, which has a profound effect on phosphate incorporation into ATP, showed no analogous effect on phosphate uptake.

Previous reports have demonstrated biochemical trauma on red cell stroma by acetylphenylhydrazine. Weed has documented a potassium leak in the presence of concentrations between 3 \(\times\) 10\(^{-4}\) and 2.4 \(\times\) 10\(^{-2}\).\(^{10}\) Jandl and his colleagues have shown that SH inhibitors which are known not to enter the red cell cause in vitro changes and lead to splenic sequestration and in vivo destruction of these cells.\(^{11}\) Whether the present study demonstrates another
abnormality or another manifestation of a common underlying defect produced by these drugs is not clear. Known SH inhibitors had no analogous effect on uptake, suggesting that the action, if of an oxidant nature, has other specificities.

There is still uncertainty as to the mechanism of phosphate uptake by the erythrocyte. These experiments suggest that it is not by passive diffusion, but depends on temperature-dependent enzyme processes. A shuttle via stromal glycolytic intermediates has been postulated as the mechanism of phosphate entry. Specific glycolytic steps have been demonstrated in stromal preparation, but it is difficult to interpret such studies because of the impossibility of working with a physiologic preparation. The present studies demonstrate the first detectable radioactivity to be in the ATP fraction of the glycolytic intermediates, but the technic of study does not allow separation of stromal and intracellular ATP, if, indeed, they differ. Moreover, the technic does not separate extracellular from intracellular inorganic phosphate, and, therefore, one cannot rule out this as being the first form in which phosphate is found upon entrance into the cell. However, the work of others has demonstrated the latter to be unlikely.

The present studies add evidence to the multiple effects of acetylphenylhydrazine on erythrocyte metabolism. Mohler and Williams have previously reported a decline in ATP on incubation of red cells with acetylphenylhydrazine. The change in ATP was much less marked in these reports and occurred only after longer incubation, perhaps due to the fact that the present experiments were carried out with constant aeration of blood samples, while the previous studies were carried out in closed test tubes without agitation. It has been previously demonstrated that aeration has a profound effect on the GSH levels on incubation with APH and one might expect more striking changes in all other effects of the drug dependent on the presence of oxygen and possibly the generation of peroxide. The decrease in specific activity suggests inhibition of incorporation of phosphate into ATP, as one might expect, since the GSH-dependent enzyme, triose phosphate dehydrogenase, plays a role preceding the formation of this high energy compound. The evidence is equally impressive, however, that there is also an increased rate of breakdown of ATP. This is consistent with the theory that the drug is a glycolytic stimulant. Marks, Prankerd, and Kellermeyer have shown that it stimulates the pentose shunt in a manner similar to methylene blue. Mohier has described increased glycolysis in the presence of the drug, which demands an increase in phosphate donation by ATP. Waller has suggested that it may also oxidize DPNH thereby stimulate the Embden-Meyerhof cycle, since it has at least been shown that the pyruvate-lactate ratio is low in the APH-treated red cell, a situation suggesting that the presence of the drug maintains DPN in the oxidized form.

It should also be noted that ATP plays a role in membrane metabolism in the energy-requiring system which continually maintains the normal intracellular electrolyte environment with the constant extrusion of sodium, and possibly with other transfer mechanisms including phosphate itself. It has been suggested that the stromal enzyme causing the extrusion of sodium
is ATPase, an enzyme known to be present in stroma and normally activated by hemolysis. One might postulate that membrane damage by acetylphenylhydrazine could result in some activation of this enzyme. More recently Hokins and Hokins have suggested that phosphodiglyceride and ATP interact in membrane sodium and potassium transfer. Thus, the increased rate of disappearance of ATP may be related to internal glycolysis, to membrane metabolism, or to both.

SUMMARIO IN INTERLINGUA

Le effectos de acetylphenylhydrazina super le erythrocytos esseva studiate per vari investigatores de pos que il esseva suggestionate que iste pharmaco pote esser prendite como modello pro un grande gruppo de drogas que causa anemia hemolytic. Il es clar que le pharmaco age como acceptor de hydrogeno e que, per iste mechanismo, illo pote disnaturar proteinas e accelerar o inhibir reactiones chimic. Il ha essite monstrate que illo exerce plure distincte effectos intra le erythrocyto. In le presentia de oxyhemoglobina illo es oxydate rapidemente, e postea—directe- o indirectemente—illo pote causar le oxydation de co-enzymes, glutathiona, e hemoglobina e etiam le formation de oxydate gruppis in altere structuras proteinic. Recente studios suggestiona que intra le erythrocyto illo genera peroxydo, lo que duce al oxydation additional de metabolitos vital si iste action non es neutralisate per le action de catalase e de peroxydase de glutathiona.

Le presente studios esseva interprendite pro determinar si acetylphenylhydrazina affice le acceptation e le metabolismo de phosphato de alte energia intra le erythrocyto. Le experimentos, que es describite in detalio, demonstra un inhibition del acceptation de phosphato in le presentia del pharmaco mentionate e etiam un inhibition del incorporacion de phosphato in triphosphato de adenosina. In plus, le disparition de triphosphato de adenosina es accelerate per acetylphenylhydrazina.

REFERENCES

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