# Accelerating Effect of 2,4,6-Trinitrophenol on the Glycolytic Rate of Human Red Cells

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ABSTRACT   A number of instantaneous changes occurred when picrate was added to a suspension of human red cells in steady state with respect to glycolysis and ion distribution across the membrane at pH 7.40. The rate of glycolysis increased, without change in glycolytic quotient, to a new steady-state value, the effect reaching a maximum of 1.75 times the rate of the control at 0.5 mM picrate. Inorganic phosphate ($P_i$) was released at a relatively constant rate, increasing with picrate concentration to 1.0 mmol $P_i$/liter cells × h at 5–6 mM picrate. The steady-state concentration of ATP and 1,3-diphosphoglycerate (1,3-DPG) decreased to new stable values within 15–45 min after the addition of picrate. The ATP level was affected only at picrate concentrations of 1 mM or more, and the level of ATP stabilized at 75% of the control values at 4 mM of picrate. In contrast, 1,3-DPG concentrations decreased to 40% of the control value at 0.5 mM picrate. Higher concentrations of picrate resulted in only a small additional decrease in the stationary concentration of 1,3-DPG. A net efflux of cellular potassium at constant rate took place. This net efflux was an almost linear function of picrate concentration in the range 0.1–3 mM. At the latter concentration the net efflux amounted to about 2.7 meq/liter cells × h and a further increase in picrate concentration caused only a minor increase in the potassium efflux. Possible mechanisms for the effects of picrate on human red cell glycolysis are discussed.

## INTRODUCTION

A number of substituted phenols have been shown to exhibit strong and complex effects on a number of biological membranes. One of the first such actions to be described was that of 2,4-dinitrophenol as an uncoupler of oxidative phosphorylation in mitochondria.

The substituted phenols picric acid and trinitrocresole (TNC) have been shown to inhibit carrier-mediated exchange of chloride ions across human and sheep red cell membranes at concentrations of 1 mM or lower (7, 8). A significant increase in cation permeability has also been demonstrated (8) on application of TNC at a concentration of 3–10 mM.

In *Amphiuma* red cells these substances (especially picrate) were shown to produce similar effects, although the increase in cation permeability was relatively smaller (31). In addition, ,picrate produces a strong transient hyper-

polarization of the membrane as demonstrated by direct measurements with a microelectrode. This hyperpolarization has been explained as the result of two separate mechanisms. First, there is a strong hyperpolarization accompanying the influx of picrate ions together with undissociated picric acid. Thereafter follows a steady-state period characterized by a less pronounced, stable membrane potential of about 35 mV (inside negative), caused by the increase in cation permeability introduced by the picrate which is now in equilibrium across the membrane. The degree of hyperpolarization found in the stable period is best explained by assuming a small increase in the permeability ratio between potassium and sodium that is simultaneous with a pronounced decrease in chloride conductance.

In addition to the various permeability changes, Gunn and Tosteson (8) in their study on sheep red cells found that TNC at the higher level of concentration of the phenol partially inhibited active sodium and potassium transport.

If the action of TNC on the pump was similar to that of ouabain, an ATP-sparing effect might be expected. However, preliminary experiments showed that the closely related phenol picrate (5 mM) added to a suspension of human red cells in steady state at pH 7.4 caused the level of ATP to decrease 15–20% within 30 min, whereas the glycolytic rate increased instantaneously by about 60%. This decrease in ATP concentration may lead to a very large change in the ATP:ADP ratio. It was of interest, therefore, to see whether the inhibitory effect of TNC or picrate on the cation transport system involved a specific inhibition of the ATPase system or was due to an increased consumption of ATP in different reactions and therefore decreased ATP for the pump system.

In the present paper, data on glucose consumption, lactate production, production of inorganic phosphate by degradation of 2,3-DPG, and steady-state concentration levels of ATP and 1,3-DPG are presented as functions of the amount of picrate added to suspensions of human red cells in steady state. The possible locus of attack by picrate on metabolism is discussed, especially with respect to a possible connection between the inhibitory effect on the active transport of sodium and potassium and the changes in the metabolic pattern.

## MATERIALS AND METHODS

### Abbreviations

ADP, AMP, ATP: adenosine di-, mono-, and triphosphate, respectively; 2,4-DNPH: 2,4-dinitrophenylhydrazone; 1,3-DPG: 1,3-diphosphoglycerate; 2,3-DPG: 2,3-diphosphoglycerate; EGTA: ethyleneglycol-bis-($\beta$-aminoethyl ether)$N,N'$-tetraacetic acid; FDP: fructose 1,6-diphosphate; GDP: glucose 1,6-diphosphate; G-6-P: glucose 6-phosphate; LDH: lactic dehydrogenase; MOPS: morpholinopropane sulfonic acid; NAD, NADH, NADPH: oxidized and reduced forms of nicotinamide-adenine dinucleotide and nicotinamide-adenine dinucleotide phosphate, respectively; $P_i$: inorganic phosphate; PCA: perchloric acid; PEP: phosphoenolpyruvate; PFK: phosphofructokinase; 2-PG: 2-phosphoglycerate; 3-PG: 3-phosphoglycerate; PGK: phosphoglycerate kinase; PK: pyruvate kinase; TNC: trinitrocresolate; TP: triosephosphate; TRIS: Tris(hydroxymethyl)-aminomethane hydrochloride.

## Sources

Reagents were obtained from commercial sources as follows: NAD, NADH, pyruvate, LDH from Boehringer/Mannheim, West Germany; glucose oxidase peroxidase from KABI, Stockholm, Sweden; ATP, MOPS, TRIS from Sigma Chemical Co., St. Louis, Mo.; ouabain from W. G. Merck & Co., Darmstadt, West Germany; heparin from LEO, Denmark.

## Incubation

Freshly drawn blood from healthy human donors was heparinized and centrifuged for 30 min at 5°C at 1,500 $g$. The plasma and the buffy coat were removed by aspiration and the latter was discarded. About 10 ml of red blood cells were incubated in a thermostated incubation flask at 37.0 ± 0.1°C in either their own plasma or an isotonic salt solution; the cell volume fraction was adjusted to 0.40. The salt solution normally contained 2.0 mM $KH_2PO_4$, 2.0 mM $CaCl_2$, 10–15 mM $NaHCO_3$, and NaCl up to a total anion concentration of 156 meq/liter. Glucose was added to the cell suspension to give an initial concentration of about 7 mM/liter total water. By a gasometric pH stat (28), pH was maintained at 7.40 ± 0.02 with $CO_2$ as downscale titrant and $N_2$ as upscale titrant during the incubation which was accordingly conducted anaerobically.

Two different methods for producing control cells were employed. One was to incubate the suspension of cells for 3–4 h and then add picrate made isotonic with saline. The other was to perform parallel incubations with and without picrate.

## Sample Collection

For determination of metabolic intermediates 200-$\mu$l samples of cell suspension were pipetted in triplicate into a microcentrifuge tube containing 1,000 $\mu$l of ice-cold 0.6 M PCA, immediately and carefully mixed, and allowed to stand in an icebath for 5–10 min to ensure complete precipitation. After centrifugation for 45 s at 15,000 $g$ at 0–5°C, 1,000 $\mu$l of PCA supernate was pipetted into another microcentrifuge tube with 200 $\mu$l ice-cold 2.74 M KOH, 0.6 M MOPS and immediately mixed to give a final solution of pH 7.0–7.5. After 45 s of centrifugation at 15,000 $g$ the tubes were stored in an icebath for determination of total concentrations of glucose, lactate, and phosphate. For determination of ATP 1 vol of neutralized supernate was transferred into 10 vol 0.1 M MOPS, pH 7.2, and mixed. For measurements of 1,3-DPG and 3-PG concentrations a special precipitation with charcoal containing PCA was carried out (14).

For determination of extracellular concentrations of potassium and phosphate, 100 $\mu$l of extracellular phase was mixed with 500 $\mu$l of 0.6 M PCA. For determination of cellular concentrations of potassium and sodium 1 vol of cells was washed three times with 10 vol of isotonic TRIS-Cl, pH 7.4, after which 5 vol of 0.6 M PCA were added for precipitation of protein. This procedure did not cause measurable precipitation of potassium perchlorate.

## Assays of Glycolytic Intermediates

ATP was measured by the firefly luciferin luciferase method of Rasmussen and Nielsen (17) and inorganic phosphate was determined by the extraction method according to Vestergaard-Bogind (29). D-Glucose was measured by a glucose oxidase method, L-lactate by the method of Bergmeyer (1), and 3-PG and 1,3-DPG according to Momsen (14). Determination of the initially very low concentrations of pyruvate (relative to those of lactate and glucose) by oxidation of NADH catalyzed by LDH was not possible because picrate absorbs strongly at 340 and 366 nm. Another method was chosen. Samples of neutralized supernate and 1 mM 2,4-DPNH dissolved in 1 M HCl were

mixed and allowed to stand for 20 min at room temperature. These samples were then mixed with 2.5 times their volume of 0.80 M NaOH and allowed to stand for 5 min at room temperature after which the absorbance at 546 nm was determined. This method measures all intermediates with an oxogroup (except glucose), but the semiquantitative comparison with results obtained by the LDH method indicated that only very small amounts of triose phosphates were present during our incubation period.

Interference in the determination of glucose and lactate caused by the presence of picrate was corrected for by using blank and sample standards. No interference was found in the determination of ATP (10 times dilution) and 1,3-DPG (precipitation with charcoal).

### Other Measurements

The degree of hemolysis was determined by measuring the concentration of hemoglobin in the medium and comparing it with the hemoglobin concentration in the total suspension. For determination of these concentrations the cyanmethemoglobin test of Boehringer/Mannheim was used. Methemoglobin was determined with the same test kit by the method of Betke et al. (2). Concentrations of picrate were determined photometrically and K and Na concentrations in the PCA supernates were determined by flame photometry by use of an Eppendorf flame photometer.

In calculations of intracellular concentrations the cells were assumed to contain 72.5% vol/vol of water or 34% wt/wt of dry matter. Based on 150 triplicate determinations, the relative errors of the mean were the following: ATP $\pm$ 2.9%; $P_i$ $\pm$1.3%; glucose $\pm$0.8%; lactate $\pm$1.3%; cell volume fraction $\pm$0.9%. The relative error of the mean of a duplicate determination of 1,3-DPG and 3-PG was about $\pm$3%.

### RESULTS

The experiments were always, as far as possible, carried out at steady state with respect to glucose consumption and lactate production at pH 7.40. Moreover, in the control experiments (pH 7.40) the concentration levels of inorganic phosphate, intra- and extracellular sodium and potassium, and cellular concentrations of ATP were constant during the period of the experiment. In blood samples to which picrate had been added it was found that a steady state as defined above was obtained immediately with respect to glucose consumption and lactate production, whereas the intracellular concentrations of ATP and 1,3-DPG reached a constant level only after a period of about 30 min. The concentration of total inorganic phosphate in the system increased with time in experiments with picrate, but after a transient period of 30 min the production of inorganic phosphate was constant with time in most experiments. The net efflux of cellular K was always constant with time in experiments with picrate.

In all experiments the effect of picrate was compared to a picrate-free incubation of the same blood sample (control). This was done in two different ways as illustrated in Fig. 1 and 2. A high degree of reproducibility was found with respect to the effect of picrate on the various metabolic parameters, irrespective of the blood donor.

Fig. 1 shows the effect on glycolysis of adding 6.85 mM picrate. There is an instantaneous stimulation of glucose consumption and lactate production, both of which increase by a factor of 1.8. Thus the glycolytic quotient stays constant, within the limits of error. This was true for all the experiments with picrate, independent of the concentration of picrate applied. The level of ATP decreases

towards a new steady-state level of 80% of the control within 30 min. Finally, a constant production of inorganic phosphate (measured as the increase in the concentration of total inorganic phosphate in the cell suspension) takes place as a result of the addition of picrate. The release of inorganic phosphate begins immediately after the addition of picrate and over 0.5 h adjusts to a constant rate which presumably is achieved at the time that the ATP level becomes constant.
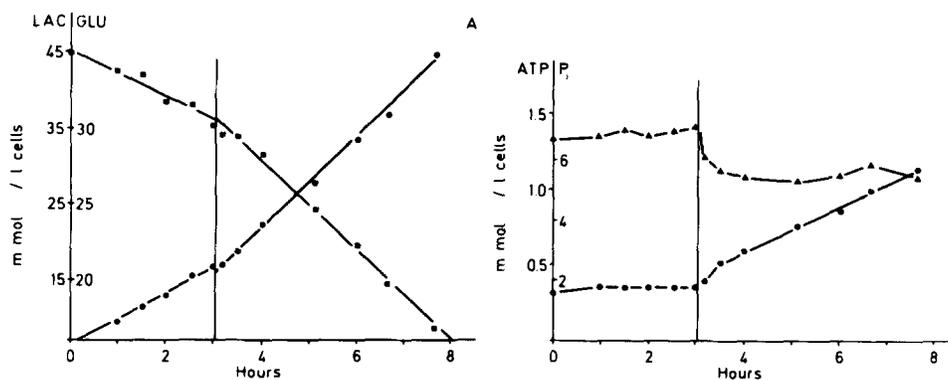


FIGURE 1. A, Glucose (■) and lactate (●) in the system per liter of packed cells versus time. Red cells were incubated in their own plasma at 37 ± 0.2°C, pH 7.40 ± 0.02, at a cell volume fraction of 0.40. After 3 h of incubation picrate was added to the cell suspension at a concentration of 6.85 mmol per liter total water. The production of lactate was 3.40 ± 0.11 mmol/liter cells × hour before addition of picrate (control) and 6.06 ± 0.12 mmol/liter cells × h in the presence of picrate. The corresponding consumption of glucose was 1.56 ± 0.09 and 2.93 ± 0.07 mmol/liter cells × h. The coefficient of correlation for lactate was 0.993 before and 0.997 after the addition of picrate. The corresponding values for glucose were 0.972 and 0.995, respectively. B, Concentration of ATP (▲) and amount of $P_i$ (●) in the system per liter packed cells measured in the same experiment as A. The production of $P_i$ in the presence of picrate was 0.76 ± 0.03 mmol/liter, cells × h and the coefficient of correlation 0.894.

Fig. 2 shows the results of a similar experiment in which parallel incubations were run in the presence and absence of 0.45 mM picrate. There is still a significant increase in the rates of glycolysis and the production of inorganic phosphate, but the latter rate is significantly lower than in the experiment with 6.85 mM picrate. The concentrations of ATP (not shown in the figure) was unchanged in comparison to the control. As seen in Fig. 2 B, a constant net efflux of potassium took place from the cells.

Since the glycolytic quotient was always constant (0.45–0.48), in the subsequent experiments we chose lactate production as a measure of the rate of glycolysis. In Fig. 3 the ratio between the lactate production in the picrate and control incubation is plotted against the concentration of picrate. The most prominent feature of this figure is that lactate production reaches a maximum at low picrate concentrations (0.5–0.7 mM) which cause no decrease in the steady-state concentration of ATP. This is seen by comparing Fig. 3 with the top line in Fig.

4, which shows the steady-state concentration of ATP normalized with respect to control values as a function of the picrate concentration.

The bottom curve in Fig. 4 shows the normalized steady-state concentrations of 1,3-DPG as a function of the picrate concentration. The control values were about 500 nmol/liter cells and the adjustment to the new stationary level took place within 50 min. In these experiments the concentration of 1,3-DPG was measured at about 1 h and 3 h after the addition of picrate.
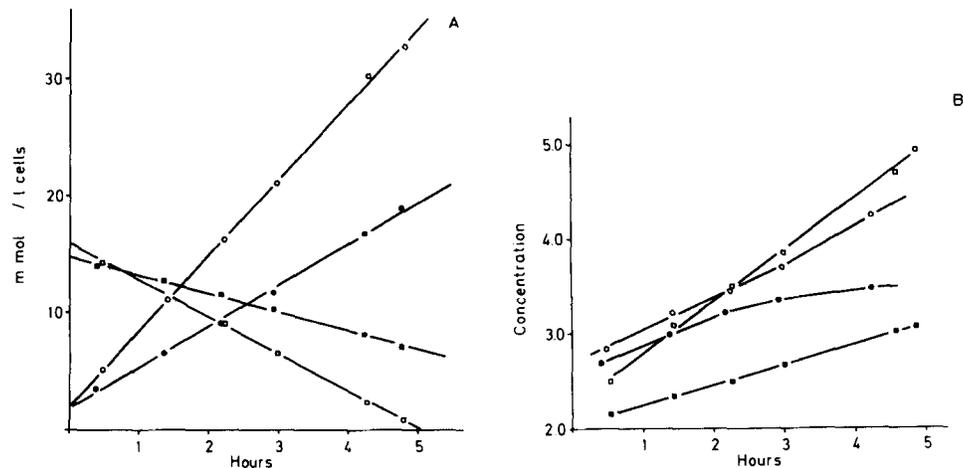


FIGURE 2. A, Amounts of glucose and lactate in the system per liter of packed cells versus time in a parallel incubation of human red cells in salt solution at 37 ± 0.2°C, pH 7.40 ± 0.02. The cell volume fraction was 0.4 in both incubations. To one of the incubations was added 0.45 mM picrate per liter total water, whereas the other incubation served as control. (●) Lactate, control. (O) Lactate, picrate. (■) Glucose, control. (□) Glucose, picrate. B, Same experiment as in A. Amount of total inorganic phosphate expressed in millimoles in the system per liter of packed cells (circles) and extracellular potassium in milliequivalents per liter extracellular phase (squares) versus time. Open signatures are from the picrate experiment. Filled in signatures are from the control experiment.

Fig. 5 shows the interdependence between the steady-state rate of inorganic phosphate production and the concentration of picrate. At low concentrations of picrate (0.1–0.7 mmol/liter total water) the release of inorganic phosphate sometimes began with one constant rate and then, after about 2 h, adjusted to a new constant rate during the remaining part of the experiment. In such cases the average of the two rates was used.

In an experiment conducted at pH 7.75, the addition of 6.05 mM picrate induced an increase in the rate of glycolysis by a factor of 1.77, resulting in a lactate production of 8.5 ± 0.1 mmol/liter cells × h. This acceleration is within the range found at pH 7.40. At pH 7.75 the stationary concentration of ATP was about 60% of the normal concentration at pH 7.4. The addition of picrate resulted, however, in an increase in ATP concentration to a new stationary level about 30% higher than that of the control at pH 7.75.

Potassium net efflux was measured at various concentrations of picrate (Fig. 6). It should be noted that the effluxes of K given in Fig. 6 are the differences between the net effluxes in picrate and control media. The rise in the extracellular K concentration also seen in control experiments is mainly due to a small but significant outflow of KCl from the calomel electrode. Since the outflow of KCl from the different calomel electrodes used in these experiments was essentially the same, correction for this phenomenon was incorporated into
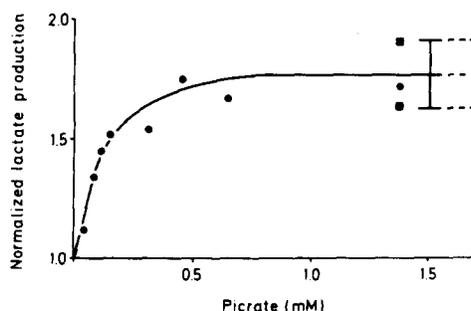


FIGURE 3. Lactate production as a function of picrate concentration. *Ordinate*, Ratio between lactate productions in the picrate and control incubations. *Abscissa*, Concentration of picrate. The bar indicates the range for eight experiments at picrate concentrations from 1.5 to 6.85 mM. (●) Picrate added. (■) Picrate and ouabain added, the latter also added to the control.
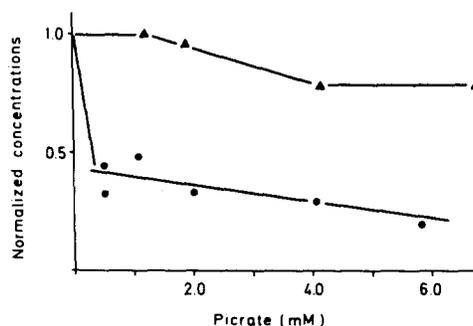


FIGURE 4. Cellular steady-state concentrations of ATP and 1,3-DPG as functions of the concentration of picrate. *Abscissa*, Concentration of picrate. *Ordinate*, Concentrations of ATP (▲) and 1,3-DPG (●) normalized with respect to control values.

the method for determining the net efflux just described. The efflux of cellular potassium was always constant during experimental periods lasting up to 4 h (the coefficient of correlation was >0.98 and the variance of the individual data points with regard to the regression line was on average 2.5%). In some experiments cellular concentration of sodium was determined two or three times and found to be significantly increased with time in cells incubated in picrate medium. The magnitude of the net influx of sodium into the cells could not be determined as accurately as that of the cellular potassium net

efflux. However, there was no change in the cell volume fraction during the experiments, indicating that the potassium efflux was osmotically counterbalanced by the sodium influx.

Evidently, a net efflux of potassium took place even at the lowest concentrations of picrate, that is within the low range of concentrations where no change
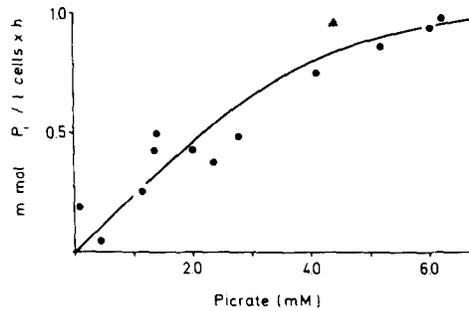


FIGURE 5. Production of inorganic phosphate as a function of picrate concentration. *Abscissa*, Concentration of picrate in millimoles/liter total water. *Ordinate*, Production of inorganic phosphate. (●) Experiments at an intracellular $Ca^{++}$ concentration of about 1.8 mM. (▲) Experiment in $Ca^{++}$-free salt solution.
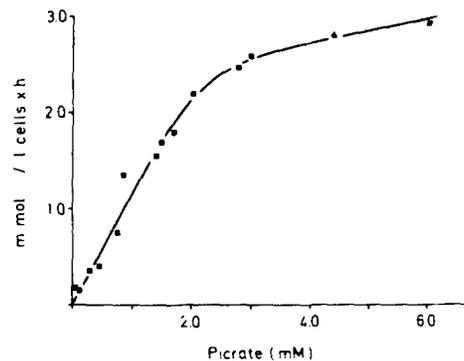


FIGURE 6. The net efflux of cellular potassium as a function of picrate concentration. *Abscissa*, Concentration of picrate. *Ordinate*, Net efflux of cellular potassium. The experimental points have been corrected for the efflux in the corresponding control. (■) Experiments in normal salt solution. (▲) Experiment in $Ca^{++}$-free salt solution.

in stationary ATP level was as yet detectable. Moreover, the main effects of picrate as represented in Fig. 1 could be reproduced without significant changes by using erythrocytes suspended in an equilibrium Ringer (100 mM K, 54 mM Na) or cells treated with ouabain (5 × 10⁻⁴ M). In order to see whether the K net efflux was induced by an enhanced Ca influx caused by picrate (5, 18), an experiment (4.4 mM picrate) was conducted in Ca-free, 2 mM EGTA Ringer. The increase in glycolytic rate and the K net efflux were found to be within the usual range.

To determine whether, in spite of the anaerobic conditions during incubation, picrate had been reduced and methemoglobin formed, the recovery of picrate was measured. After 2 h of incubation at a concentration of 5.6 mM the recovery of picrate was measured. After 2 h of incubation at a concentration of 5.6 mM the recovery of picrate was 98 ± 2%, if one assumed all picrate to be distributed in the water phase only. It is likely that some picric acid was dissolved in the lipid phase of the membrane and that some was bound to hemoglobin. When one assumed an even distribution of picrate between the water phase and the phases represented by the dry weight, the recovery was 100 ± 2%.

In two experiments the concentration of methemoglobin was measured both before and about 1 h after addition of either 1.8 or 5.5 mM picrate. The percent of methemoglobin in the control was 1.9 ± 0.2, and the average at the two picrate concentrations was 2.0 ± 0.2%. Thus, the addition of picrate induced no change in the steady-state concentration of methemoglobin.

In a few experiments pyruvate production was measured. Since the absorbance of picture disturbed the enzymatic determination of pyruvate and the colorimetric determination likewise had a high error, highly accurate determination of the kinetics of pyruvate production was not possible. Nevertheless, it was found that the total pyruvate production had a general magnitude of half the amount of inorganic phosphate released, that is equimolar with the 2,3-DPG metabolized.

In four experiments with picrate added in concentrations from 0.7 to 2.5 mM, the steady-state concentrations of 3-PG in the cells were found to be equal to the values in the control.

The inhibition of the anion exchange mechanism manifested itself clearly with respect to the equilibration of released inorganic phosphate across the membrane. In experiments with large productions of inorganic phosphate ratios of intra- to extracellular concentrations of phosphate as high as 3.6 were observed.

In spite of the inhibition of the anion exchange mechanism the gasometric pH stat in all picrate experiments compensated sufficiently for the production of lactic acid. Direct measurements of pH on a sample of freeze-thawed, packed erythrocytes from an incubation with picrate gave the value 7.2, which corresponds to a membrane potential of about $-15$ mV. This value is close to the $-13$ mV found by Funder and Wieth (6) with respect to protons and agrees well with the idea that picrate (picric acid) constitutes an efficient proton carrier mechanism (31).

Addition of picrate which had been adjusted to pH 7.4 caused a high rate of down-scale titration ($CO_2$) for about 30 s which was followed by a period of upscale titration ($N_2$) for up to 15 min. However, at 37°C and a pH value maintained by the pH stat at 7.40, the equilibration of picrate across the human erythrocyte membrane took place within 2-3 min. The equilibration was so fast that no kinetic analysis was attempted. The period of upscale titration after the addition of picrate was thus much longer than that of the equilibration of picrate across the membrane.

DISCUSSION

The changes in metabolic patterns induced by picrate are not easily explained on the basis of the present data. However some possible mechanisms can apparently be rejected.

Since it is highly probable that chloride-bicarbonate exchange across the human red cell membrane is strongly inhibited (7), one might expect a disturbance of the transmembrane pH equilibration. For example, a decrease in intracellular pH would presumably lead to dephosphorylation of 2,3-DPG (32) but at the same time to a decrease rather than an increase in glycolytic rate. Despite the inhibitory effect of picrate on the anion exchange, hydroxyl ion (and pH) equilibrium across the membrane was maintained in the present experiments even during maximum proton production from glycolysis. This is possibly due in part to the ability of picrate itself to operate as a proton carrier. A transient change in the intracellular pH seems to occur as the picric acid permeates the membrane. But in contrast to *Amphiuma* red cells, where the equilibration of picrate across the membrane was not completed until 90 min at 17°C (31), the equilibration period in suspensions of human red cells at 37°C took about 2 min. This further indicates that in contrast to the membrane of the *Amphiuma* red cell the human red cell membrane is not substantially hyperpolarized for a longer period. A hyperpolarization of substantial magnitude and duration might result in an extended decrease in intracellular pH by passive adjustment of the hydroxyl ion distribution across the membrane to the existing membrane potential. Direct measurements of intracellular pH showed a value corresponding to a membrane potential of about −15 mV.

The quantitative recovery of picrate after 2 h of incubation showed that picrate had not been reduced in significant amounts. This, together with the finding of a constant, almost normal percentage of methemoglobin during 2 h of anaerobic incubation was taken as an indication that no acceleration of the pentose phosphate shunt had taken place. The finding that the production of pyruvate was equal in magnitude to that which could be calculated from the degradation of 2,3-DPG indicates rather an inhibition of the pentose phosphate shunt. In other work we have shown that human red cells anaerobically incubated at pH 7.0 normally show a degradation of 2,3-DPG of 0.8 mmol/liter cells × h, with a comparable extraproduction of lactate. No accumulation of pyruvate was found under these conditions, where the shunt appears to furnish the necessary electrons for reduction of the pyruvate originating from 2,3-DPG (32).[1]

The rate of glycolysis in human red cells is mainly regulated at two enzymatic reactions far from equilibrium, namely phosphofructokinase (PFK) and pyruvic acid kinase (PK) (11, 19). PFK is strongly inhibited by ATP (12, 27) and the physiological activity of the enzyme is almost entirely determined by the positive effectors, GDP, FDP, G-6-P, AMP, ADP, and $P_i$, all of which release the ATP inhibition (cf. reference 9). PK is also inhibited by ATP and FDP acts as a positive effector (9, 25).

[1] Momsen, G., and B. Vestergaard-Bogind. Manuscript in preparation.

It is important that the glycolytic quotient did not change detectably when the glycolytic rate was increased by addition of picrate. Indeed, activation of the PFK in red cells may well lead to an increase in the total glycolytic rate with no change in the glycolytic quotient. This is possible because the PK reaction normally operates at a PEP concentration far below the saturation value for PK. Therefore, the concentration of PEP during moderately increased flow at the PFK step increases up to a balance point at which the rate of the PK reaction is equal to the rate of the PFK reaction (cf. 14). Since the changes in glycolytic rates on addition of picrate were almost instantaneous, the only possible positive effectors at the PFK step, apart from picrate itself, are ADP, AMP, and $P_i$. However, the rise in intracellular inorganic phosphate was too slow and therefore must be secondary. Also, if inorganic phosphate released from 2,3-DPG acted as a rate-determining positive effector, one would expect the glycolytic rate to increase steadily with time. Rose et al. (20) have shown that inorganic phosphate can act as an activator of hexokinase by modulating the effectiveness of G-6-P as an inhibitor of this enzyme. However, the above-mentioned arguments against $P_i$ as the primary activator of PFK in the presence of picrate should hold true also in the case of the hexokinase reaction.

An increased level of ADP and AMP as the cause of the acceleration by glycolysis would be in agreement with the facts that both compounds exert strong activation on PFK and that the stationary level of ATP was found to decrease at picrate concentrations of 2 mM and upwards. Furthermore, the increased permeabilities to Na and K would probably cause an increase in consumption of ATP by the Na-K pump and in steady-state ADP-AMP concentrations.

The following observations weaken the presumption of ADP-AMP as primary positive effectors. The maximum increase in glycolytic rate was obtained at a picrate concentration of about 0.5 mM, whereas a detectable decline in stationary ATP level was found only at picrate concentrations greater than 1 mM. In the experiment conducted at pH 7.75 the increase in the glycolytic rate induced by picrate (6.05 mM) was 1.77 times the control, i.e. within the normal range. In this experiment the stationary concentration of ATP in the control, in agreement with earlier observations (32), was about 60% of the control value at pH 7.4. But in this case the addition of picrate induced an increase in the stationary ATP concentration and, presumably, a decrease in the ADP concentration (see Results).

For two reasons, the substantial decrease observed in steady-state concentrations of 1,3-DPG invalidates the possibility discussed above of obtaining an increased rate of glycolysis while maintaining the glycolytic quotient only by way of decreased PFK inhibition. First, since 1,3-DPG, 3-PG, 2-PG, and PEP are intermediates in that glycolytic sequence where all the reactions are proceeding close to the equilibrium (11, 13), the sharp decline in the stationary concentration of 1,3-DPG indicates that the concentration of PEP was probably not increased. The finding that the concentrations of 3-PG in preliminary experiments were identical in picrate and control incubations substantiates this assumption. Thus no acceleration of PK by way of increased substrate concen-

tration should be possible. Second, according to the crossover principle (4), the PFK step is not the primary reaction accelerated (by ADP, AMP, or picrate), since in that case elevated concentrations of the intermediates (e.g., 1,3-DPG) in the subsequent sequences of glycolysis would be expected.

An increased consumption of ATP by a normally operating Na-K pump cannot be the source of extra ADP immediately after addition of picrate, since no appreciable change in intracellular Na takes place within the first 30 min or so, and the rate change in glycolysis occurs instantaneously. In addition, one would expect a gradually increasing effect from the pump concurrently with the increasing demand placed on it, but a stationary glycolytic rate was always observed. Finally, the lack of effect of extracellular substitution of K for Na or treatment of the cells with ouabain ($5 \times 10^{-4}$ M) (Fig. 3) substantiates the view that ADP from the pump is not the trigger of the increased glycolytic rate.

The arguments listed above indicate that it is most unlikely that the action of ADP or AMP as positive effector is the primary cause of the acceleration of glycolysis induced by picrate. On the other hand, the possibility that the action of ADP and AMP as positive effectors plays an additional role cannot be definitely ruled out. Unfortunately, because of its strong absorbance at 340–360 $\mu$m, picrate interferes with the determination of ADP by an NADH-linked assay so that it was not possible to detect a 10% change in ADP level. An alternative ADP assay using the firefly method is too insensitive. Our conclusion at present, therefore, is that the rate of the PK as well as the PFK reaction is increased by the addition of picrate. In vitro biochemical assay of the direct effect of picrate on these enzymes is currently under investigation in our laboratory.

The decreased stationary concentration of 1,3-DPG and its dependence on the concentration of picrate explain in a qualitative and also in a semiquantitative way the release of inorganic phosphate from 2,3-PDG which results from the addition of picrate. The net degradation of 2,3-DPG equals the difference between a zero-order diphosphoglycero-phosphatase reaction in which the products are inorganic phosphate and 3-PG, and a 2,3-DPG formation from 1,3-DPG catalyzed by diphosphoglyceromutase that proceeds at a rate which, on the basis of the work of Rose (21, 22), can be taken as roughly proportional to the concentration ratio (1,3-DPG:2,3-DPG). The diphosphoglycero-phosphatase activity has been found to increase strongly with the concentration of inorganic phosphate (23).

In the present experiments the concentration of 2,3-DPG decreased by 0.45 mmol/liter cells × h or less. That is a decrease of 10% × h or less. On the other hand, the intracellular concentration of inorganic phosphate, because of the inhibition of the anion exchange mechanism, increased as much as 1.0 mM × h. These combined changes in cellular concentrations of 2,3-DPG and inorganic phosphate may well explain why we most often found a production of inorganic phosphate constant with time in picrate media with concentrations of 1 mM and upwards.

The marked variation in production of phosphate found at picrate concentrations from 0.1 up to about 1 mM (see Fig. 5) may be explained by highly variable steady-state concentrations of 1,3-DPG (see Fig. 4).

Gunn and Tosteson (8) found that TNC added to sheep red cells in concentrations of up to 2 mM induced little or no increase in Na or K permeability. Preliminary results from $^{42}$K experiments in our laboratory with human red cells indicate that picrate at a concentration of 1–2 mM increases the K permeability by a factor of about 2. Within the same range of picrate concentrations we found a net efflux of cellular potassium of 1–2 mmol/liter cells × h. If we assume that the normal passive efflux of cellular potassium, corrected for potassium-potassium exchange, is around 1.5 mmol/liter cells per h, then a doubling of the permeability should lead to an extra (positive) efflux of 1–2 mmol/liter cells × h. This is, however, the magnitude of the net efflux determined (Fig. 6). Thus the pump apparently does not respond to the increased efflux of cellular potassium induced by picrate.

As mentioned previously, the net effluxes of cellular potassium shown in Fig. 6, with a few exceptions, represent a linear increase in extracellular potassium during the experimental period of 3–4 h. Furthermore, no change in cell volume took place, consistent with the finding that in this period cellular sodium concentration increased in magnitude. Thus the pump was not only confronted with a decreasing concentration of cellular potassium but also with an increase in the concentration of cellular sodium, which is the dominant regulator of the pump. All the same, according to the above-mentioned estimate, no increase in pump activity seems to take place and the pump therefore cannot be ruled out as one of the primary targets of picrate. Direct determinations of the influence of picrate on the ouabain-sensitive Na and K fluxes are at present in progress in our laboratory.

Since Dunn (5) has shown that TNC at a concentration of 10 mM can be used to load red cells with Ca, it was possible that the K permeability increase found with picrate resulted from a weak Ca-ionophoric action of this compound. In an experiment with 4.4 mM picrate added to cells suspended in a Ca-free Ringer (normally the extracellular concentration of Ca was 2.0 mM) containing 2 mM EGTA, we found the same increase in the rate of glycolysis and the same net efflux of cellular potassium as in an experiment with 4.4 mM picrate and the normal extracellular concentration of Ca (see Figs. 3, 5, and 6). We conclude therefore that picrate under the present experimental conditions does not act as a Ca ionophore to produce these changes. The apparent difference between TNC and picrate with respect to ionophoric ability towards Ca and Mg ions needs further investigations.

In the controls we found an average lactate production of 3.4 mmol/liter cells × h. If one assumed a Rapoport-cycle percentage of 10 (see footnote 2), the net production of ATP per mole lactate produced will be 0.9 mmol, corresponding to an ATP production in the controls of 3.1 mmol/liter cells × h. In the presence of 0.5–7 mM picrate, the lactate production was 1.75 × 3.4 = 5.95 mmol/liter cells × h (see Fig. 3). In addition, phosphate was released at a rate of 0.3–0.9 mmol/liter cells × h (Fig. 5) which should result in an ATP production of 0.15–0.45 mmol/liter cells × h. In the presence of picrate the Rapoport-cycle percentage can be taken as almost zero and accordingly we can calculate the ATP production to be 5.95 plus 0.15–0.45 mmol, that is 6.1–6.4 mmol/liter cells × h. This leaves us with an extra production of ATP of 3.0–3.3

mmol per liter cells × h in the presence of picrate, that is a doubling of ATP production.

If we assume that the Na-K-ATPase consumes 1 mmol of ATP per 3 mmol Na and 2 mmol K transported (33), and that the plump fluxes across the membrane of the red cell corrected for exchange fluxes normally amount to 2.5 mmol Na and 1.7 mmol K or possibly less (10), then the pump in the controls consumed about 0.8 mmol ATP/liter cells × h.

An increased permeability of the membrane to K ions by a factor of ~2, as indicated by the [42]K experiments, should cause the pump to consume extra ATP up to a maximum of 1 mmol/liter cells × h, if steady state is to be maintained. However, Fig. 6 shows that the net effluxes of K ions were generally so large that the extra pump flux induced should be less than 1 mmol/liter cells × h, or equivalent to an extra consumption of ATP smaller than 0.5 mmol/liter cells × h.

This leaves us with two choices, the first of which is to postulate a system with a consonant high ATP production and consumption. The increased consumption is probably not the result of an increased transport activity of the Na-K-ATPase or the Ca-ATPase. In view of the strong influence picrate may exert on various configurations in the membrane, an increased consumption of ATP by other membrane-bound ATPases or protein kinases involved in maintaining shape, fragility, etc. of the cells could very well take place (cf. reference 15). Or perhaps the Na-K-pump is partly uncoupled so that an increased consumption of ATP takes place without a simultaneously increased transport.

The alternative explanation is that ATP production is much lower than calculated because of an uncoupling of one or both of the ATP-generating reactions, that is the PGK and the PK steps. That picrate exerts an effect on PGK is indicated by the observed displacement of the concentration ratio [1,3-DPG][ADP]:[ATP][3-PG] away from the normally existing equilibrium ratio.

It is at present impossible to exclude with certainty one of these alternatives, but work is in progress in our laboratory to make such an exclusion possible. It is of interest in this connection that our preliminary experiments showed that phloretin (also a substituted phenol but, in comparison with picric acid, a very weak acid) also accelerated glycolysis in human red cells. Phloretin profoundly inhibits exchange as well as net fluxes of chloride across *Amphiuma* red cell membranes but induces only a slight increase in cation permeability and apparently little or no change in the active transport of Na and K (30).

In the preceding Discussion we have indirectly assumed that no compartmentation exists for, as an example, the ATP of the pump. Such compartmentations are possible and have been suggested previously (16, 24), but the results obtained in the type of experiments discussed here allow no decision with regard to compartmentation of some glycolytic intermediates.

Picrate and other substituted phenols such as phloretin are membrane-active agents. Their effects on anion, cation, and glucose transport are well known. Our demonstration that these compounds influence glycolysis supports the concept that the various enzymes in the glycolytic pathway are organized and ordered by the cell membrane, rather than being a chaotic group of proteins in the cytosol.

A crucial finding may be that the so-called band 3 polypeptide, which spans the membrane and which is generally considered to represent the anion carrier (3), may be closely associated with some of the glycolytic enzymes. Thus evidence has been presented that the enzymes glyceraldehyde-3-P dehydrogenase (34) and aldolase (26) both are bound to an intracellular part of this peptide. If inhibitors of anion exchange, such as picrate and phloretin, cause conformational changes in the carrier-polypeptide, then such changes may affect the binding of some glycolytic enzymes on the cytoplasmic side.

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