Biochemical Pharmacology, 1959, Vol. 2, pp. 6–231. Pergamon Press Ltd., London

INHIBITION OF OXYGEN CONSUMPTION IN HUMAN ERYTHROCYTES BY HEXETIDINE*

F. J. LIONETTI, W. L. MCLELLAN and F. COMUNALE
Department of Biochemistry, Boston University School of Medicine

(*Received* 31 *January* 1959)

Abstract—Hexetidine was found to inhibit the oxygen consumption of methylene blue-stimulated erythrocytes when inosine or glucose-6-phosphate were substrates. At amounts of inhibitor insufficient to cause extensive lysis of cells, TPN competitively reversed the inhibition; a small effect of TPP was observed. Comparable quantities of inhibitor did not influence the amount of glucose catabolized or of lactate produced by the cells. The exchange of phosphate between plasma and cells was likewise not affected.

HEXETIDINE (1:3-bis-β-ethylhexyl-5-methyl-5-aminohexahydro-pyrimidine, abbreviated BEP†), is a "synthobiotic" drug possessing broad antibacterial and antifungal properties.[1] In a preliminary report,[2] growth of *E. coli-B* was inhibited by hexetidine and completely reversed by thiamine. In spores of *B. cereus var. terminalis* and also a particulate fraction derived from these, the oxidation of pyruvate was inhibited by hexetidine and reversed by thiamine.[3] Another report[4] has indicated that the inhibition by hexetidine of the growth of *Psuedomonas aeruginosa* could be reversed singly and to varying degrees by a number of cofactors, namely pyridoxal phosphate, TPN, TPP and biotin. DNA and RNA were also effective. However, little is yet known of the mode of inhibition of this drug in mammalian tissues.

The formed elements of human blood offer several advantages for the elucidation of biochemical pharmacological mechanisms. The cells comprise several kilograms of tissue which come intimately in contact with administered drugs. Furthermore, they are readily obtained and enumerated, and may be maintained in simulated physiological environments, i.e. in plasma under O_2–CO_2 gas tensions. Metabolically, erythrocytes are restricted mainly to glycolysis and certain aspects of the hexosemonophosphate[5, 6] and pentose phosphate[7] pathways. There is no functioning tricarboxylic acid cycle and little, if any, anabolism of simple substrates to fat or glycogen. Some properties of leukocytes especially suited for inhibition studies will be detailed separately. In these tissues, the biochemical and morphological effects of inhibitors may be evaluated by a variety of well developed chemical and microscopic procedures. Data are presented here with erythrocytes which show hexetidine to inhibit the oxygen-consuming ability in preference to the glycolytic pathway. Competitive inhibition is

* A preliminary report containing part of the data described here was presented to the Division of Medicinal Chemistry, American Chemical Society, April 1957.

† Abbreviations used are: BEP, hexetidine (1:3-bis-β-ethylhexyl-5-methyl-5-aminohexahydro-pyrimidine); R-1-P, ribose-1-phosphate; G-6-P, glucose-6-phosphate; 6-P-G, 6-phosphogluconate; MeB, methylene blue; FMN, flavin mononucleotide; DPN, diphosphopyridinenucleotide; TPN, triphosphopyridinenucleotide; TPP, thiamine pyrophosphate; DNA, desoxyribonucleic acid; RNA, ribonucleic acid.

shown with the coenzymes TPN and TPP on the methylene blue–coupled oxygen consumption using inosine as substrate.

EXPERIMENTAL

Erythrocyte suspensions. Blood from normal male medical students was collected in heparinized, non-wettable plastic bags. The cells were obtained by centrifugation (900 × *g* for 15 min); the supernatant plasma was discarded, and the buffy coat of leukocytes was removed with a cotton swab. The erythrocytes were washed twice with 0·11 M Na_2HPO_4–NaH_2PO_4 buffer (pH 7·4) and resuspended with buffer to a volume fraction of cells (hematocrit) of 0·6 for the oxygen consumption studies. White cell-free whole blood for glycolysis measurements was prepared by resuspending cells with plasma containing substrates to a volume fraction of cells of 0·4.

Haemolysates. Haemolysates were prepared by a threefold freezing and thawing of washed and centrifuged erythrocytes.

BEP. Hexetidine solutions were prepared from the pure liquid (an oil) by adding 0·2 ml to diluted HCl at pH 3·0 in a 50 ml volumetric flask. The resultant clear solution was diluted to volume with 0·15 M NaCl after adjusting the pH to 3·85 just prior to final dilution to 50 ml. The concentration of hexetidine was 10·2 μmoles/ml.

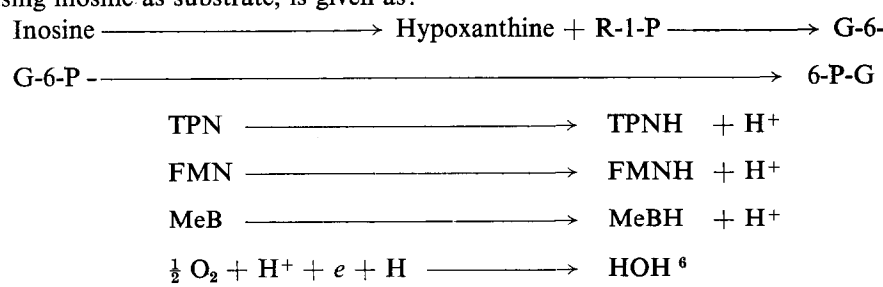
Cofactors. TPN and TPP of commercial purity were dissolved in a minimum volume of 0·15 M NaCl prior to use.

Equilibration technique. Respiration experiments were undertaken in Warburg vessels using standard manometric technique. Glycolytic experiments were carried out in 50 ml silicone-treated flasks in which the erythrocyte suspensions were maintained under one atmosphere of 5 per cent CO_2–95 per cent air and shaken at a rate of 50–60 oscillations per minute. Aliquots were removed at intervals for analysis and deproteinized with $Ba(OH)_2$ (1 : 20) or with 10 per cent trichloroacetic acid (1 : 10).

Methods. Glucose was measured using the Nelson modification of the Somogyi technique,[8] lactate by the Barker–Summerson[9] method, and inorganic phosphate by the method of Fiske and Subbarow[10]. In one experiment with radioactive phosphate, $NaH_2P^{32}O_4$ was added to give a concentration of 0·29 μc/ml of cell suspension. Aliquots were withdrawn at the beginning and at hourly intervals up to 5 hr. Plasma was separated quickly at 2°C and rendered protein-free with 10 per cent trichloroacetic acid (1 : 10). Aliquots (1 ml) of these were taken to dryness and counted with an end window Geiger counter.

RESULTS AND DISCUSSION

Inhibition of oxygen consumption by hexetidine. The inhibition of oxygen consumption produced by hexetidine (BEP) is shown in Fig. 1. The theory of the reaction, using inosine as substrate, is given as:



The phosphorolysis of inosine in erythrocytes yields R-I-P.[11] This is metabolized by an as yet unknown pathway to G-6-P. Dische[12] had previously shown an accumulation of hexosephosphate in adenosine fortified hemolysates of human blood. It is now known[7] that one of the products of the metabolism of ribose-1-phosphate produced from inosine in erythrocyte ghosts is G-6-P. The chief advantage of using inosine in this study was its apparent rapid penetrability into the cells and prompt metabolism to G-6-P, thus permitting use of intact cells for the inhibition study. However, it was later found possible to use G-6-P as substrate, although rates of oxygen uptake were less than for comparable amounts of substrates, suggesting that permeability of phosphorylated hexose is a rate-limiting factor when using intact cells. However, only in the experiments with thiamine pyrophosphate was it found necessary

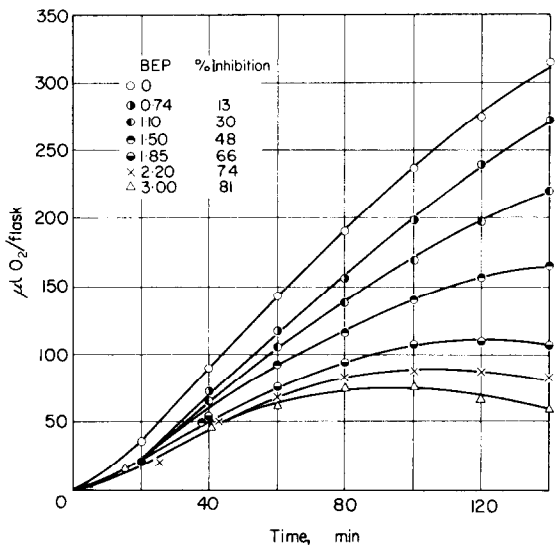


FIG. 1. The effect of BEP on oxidation of inosine by human erythrocytes. The flasks contained 1·7 ml of erythrocyte suspension and 0·2 ml of 0·1 per cent methylene blue in the main chamber. The side arm contained 37·1 μmoles of inosine (0·4 ml) and from 0 to 3 μmoles of BEP. The centre well contained 0·2 ml of 30 per cent KOH. The total volume was 2·9 ml. The columns at the right give the μmoles of BEP and the percentage inhibition.

to use haemolysates in place of intact cells. Figure 1 shows that 1·50 μmoles of BEP (0·000495 M) produced approximately 50 per cent inhibition. The amount of tissue was 1·02 ml of packed erythrocytes containing approximately $1{\cdot}3 \times 10^{10}$ cells. On a numerical basis 50 per cent inhibition was evoked by 1 μg of BEP per 4×10^8 cells. With other blood samples, values from 40–60 per cent were found. Except for the extremes of the concentrations of inhibitor, a linear increase in inhibition was observed with increasing concentration of BEP. In Fig. 1 the slopes of the uptake curves changed continuously at the high concentrations of inhibitor. The initial rates were not as reliable as the net oxygen uptake in 2 hr, since they were less reproducible in the first hour. Preincubation of cells with inhibitor was tried but not employed, as haemolysis (20 per cent) was observed with 3·0 μmoles of inhibitor per flask. Therefore, the inhibitor concentration used was limited to 1·5 μmoles. The surface active nature of hexetidine at the high concentrations probably contributes a component to the

total inhibition observed. This was not further characterized, but was assumed to be not simply linked to cellular metabolic events. It was possible, however, with the unit of oxygen uptake employed and inhibitor concentrations up to 1·48 μmoles per flask to describe the inhibition as competitive for cofactors.

Relative lack of inhibition of glycolysis by hexetidine

Glycolytic experiments were undertaken in which 35·0 ml of erythrocytes were suspended in plasma to a volume fraction of 0·4. The flasks contained heparin (sodium) $NaH_2P^{32}O_4$, and 20 μmoles of glucose per ml of packed cells. Inhibitor was varied from 1·84–5·52 μmoles per ml of packed cells. Controls contained all components except inhibitor. In 4 hr of equilibration at 37°C there were no singificant differences observed in the glucose used or the lactate produced by the cells. Kinetic curves for these, with and without inhibitor, were superimposable and thus showed no inhibition. The stoichiometry of the reaction expressed as a ratio of moles of lactate produced to moles of glucose utilized (theoretically equal to 2·0) was 2·4 in the control flask, 2·4 with 1·84 μmoles of inhibitor per ml of packed cells, and 2·0 with three times as much (5·52 μmoles) inhibitor. In the last instance, the lower value is not significantly different from the others and may have resulted from the moderate haemolysis (24 per cent) caused by the higher concentration of hexetidine. The time variation of inorganic phosphate was the same in all flasks, as was the specific activity of plasma inorganic phosphate. The maintenance of the cells in an approximately steady state of ^{32}P-exchange, and the lack of an increase of inorganic phosphate in the plasma of blood containing inhibitor, attest to the relative lack of influence of hexetidine on the cellular pool of phosphate esters. These observations support the conclusions that hexetidine in concentrations up to three times that used in the oxygen consumption inhibition experiments was without influence on the initial and terminal reactions of glycolysis and on the phosphorylated pool of intermediates between these extremes.

Reversal of inhibition by TPN and TPP

Several cofactors were found to effect the inhibition of hexetidine in the oxygen consuming system. Both TPN and TPP, alone and in combination, were found to reverse partially the inhibited oxygen consumption. TPN was more effective than TPP in systems using either inosine (Table 1) or G-6-P (Table 2) as substrates. The relatively

TABLE 1. ANTAGONISM OF HEXETIDINE BY TPN

Hexetidine (μmoles)	TPN (moles)	Q_{O_2} (μl/flask per hr)
0·00	0·00	150
0·00	0·50	162
0·59	0·50	135
1·18	0·00	48
1·18	0·50	84
1·18	1·0	111
1·18	2·0	122
0·00	1·0*	16

The substrate was 37·1 μmoles of inosine per flask.
* In the absence of substrate, an insignificant consumption of oxygen occurred.

TABLE 2. ANTAGONISM OF HEXETIDINE BY TPN

Hexetidine (μmoles)	TPN (μmoles)	Q_{O_2} (μl/flask per hr)
0·0	0·0	141
0·0	0·5	152
0·0	1·0	169
0·0	1·5	236
1·48	0·0	10
1·48	0·5	36
1·48	1·0	168
1·48	1·5	299
1·48	2·0	370

The substrate was 57 μmoles of G-6-P per flask. Less than 20μl of oxygen were consumed when washed cells without susbtrate were incubated with 1·0 μmole of TPN.

large effect of TPN in the inhibited system, as compared with the uninhibited, is consistent with the postulate that a small percentage of haemolysis disrupted the cell

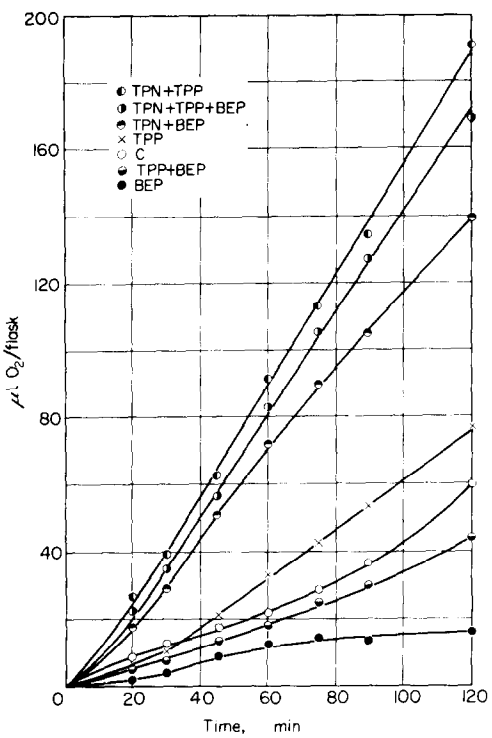


FIG. 2. The reversal of inhibition of oxygen consumption by TPN and TPP in haemolysates of human blood. The flasks contained 1·6 ml of frozen–thawed haemolysate, 0·2 ml of 0·1 per cent methylene blue and either, TPN (0·5 μmoles) or TPP (6·2 μmoles), or both, were present in the centre chamber. The side arm contained 37·1 μmoles (in 0·4 ml) of inosine and BEP (1·48 μmoles). The centre well contained 0·2 ml of 30 per cent KOH; the total volume was 2·9 ml. The curves are labelled for the cofactors and inhibitor composition. *C* is the control (2·9 ml) containing haemolysate, inosine, MeB, and KOH (centre well), but no cofactors or BEP. No significant oxygen uptake was observed in flasks incubated with either TPN or TPP in the absence of inosine.

surfaces sufficiently to facilitate the uptake of TPN by the cells. A progressive diminution of the inhibition was observed as TPN was increased in increments of 0·5 μmoles to 2·0 μmoles per flask. The effect of TPP alone and in combination with TPN is shown in Fig. 2. Haemolysates of erythrocytes were found to be much more dependent on TPN for oxygen consumption and also were much more sensitive to the action of hexetidine. Very little oxygen was consumed in the control reaction without the addition of TPN, probably because of the destruction of endogenous TPN during the preparation of the haemolysate. Conversely, TPP had little effect on the control reaction, but did show a small reversal of the inhibition by hexetidine (30-μl increase). The same magnitude of increase (30 μl) was observed when TPP was added to the flasks in which TPN largely overcame the inhibition by hexetidine, making it apparent that TPP exercised its limited effect through an independent mechanism. It is well known that glucose-6-phosphate dehydrogenase in human erythrocytes has several cofactor requirements, including TPN and a flavin coenzyme.[6] The vitamin constituents of these coenzymes, namely nicotinamide and riboflavin, respectively, were without effect on the inhibition caused by BEP. Participation of TPP even to a small extent implicates hexetidine as a potential inhibitor of transketolase, since TPP is known to be a cofactor for this enzyme.[13] Potentially, cofactor competition between hexetidine and TPP for transketolase in human erythrocytes may diminish the rate of production of glucose-6-phosphate from the pentose of inosine by way of a mechanism producing sedoheptulosephosphate and fructose-6-phosphate.[14] However, the methylene blue-coupled oxygen consumption which is inhibited by hexetidine and reversed to a large extent by TPN is the only reaction in the human erythrocyte which is known to be linked to this cofactor. The relative insensitivity of glycolysis to hexetidine suggests that components of respiratory pathways are specifically inhibited. Support of this view has been derived from observations with human leukocytes which indicate that respiratory dehydrogenases are inhibited by hexetidine and competitively reversed by DPN.*

* In manuscript.

REFERENCES

1. R. B. Edwards, B. D. Church, C. D. Dukes and R. G. Sanders, *Abstr. Soc. Amer. Bact.* (1956).
2. B. D. Church, R. G. Read and R. G. Sanders, *Abstr., Div. Med. Chem., Amer. Chem. Soc.* (1957).
3. H. H. Halvorson and B. D. Church, *J. Appl. Bacteriol.* **20**, 359 (1957).
4. J. F. Martin and B. D. Church, *Abstr. Soc. Amer. Bact.* (1958).
5. O. Warburg and W. Christian, *Biochem. Z.* **242**, 206 (1931).
6. O. Warburg, W. Christian and W. Griese, *Biochem. Z.* **282**, 157 (1935).
7. W. L. McLellan and F. J. Lionetti. In manuscript.
8. A. Nelson, *J. Biol. Chem.* **153**, 375 (1944).
9. S. Barker and W. H. Summerson, *J. Biol. Chem.* **138**, 535 (1941).
10. C. H. Fiske and Y. Subbarow, *J. Biol. Chem.* **66**, 375 (1925).
11. F. M. Huennekens, E. Nurk and B. W. Gabrio, *J. Biol. Chem.* **221**, 971 (1956).
12. Z. Dische, *Phosphorus Metabolism* (Edited by W. D. McElroy and B. Glass) Vol. 1, p. 171. Baltimore (1951)..
13. B. L. Horecker and P. Z. Smyrniotis, *J. Amer. Chem. Soc.* **75**, 1009 (1953).
14. B. L. Horecker, M. Gibbs, H. Klenow and P. Z. Smyrniotis, *J. Biol. Chem.* **207**, 393 (1954).