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INHIBITION OF DEHYDROGENASES OF HUMAN LEUKOCYTES BY HEXETIDINE

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Abstract—The inhibition of dehydrogenases of human leukocytes was estimated by measuring the percentage of cells which reduced neotetrazolium in the presence of the inhibitor. The mode of inhibition suggests a mechanism involving pyrophosphate-linked coenzymes, divalent cations, and sulfhydryl groups. Dehydrogenase activity was also found to be influenced by a variety of other substrates and inhibitors.

ENZYMATIC reductions of tetrazolium salts have been shown in a variety of tissues. The activity has been related to DPN-* and TPN-linked flavoproteins called diaphorases,[1] and to the succinic dehydrogenase complex.[2, 3] The ability of human neutrophilic leukocytes to reduce neotetrazolium and thus to demonstrate dehydrogenase activity has been reported by Wachstein,[4] Vercauteren[5] and by DeSouza and Kothare using nitro blue tetrazolium.[6] Neotetrazolium, water soluble and colorless, produced cytoplasmic purple granules (formazan) when incubated with leukocytes, indicative of cytoplasmic sites of reducing activity. Hexetidine, a synthetic antibacterial agent with a broad antibacterial and antifungal spectrum, has previously been shown to inhibit the methylene blue coupled oxygen consumption in human erythrocytes.[7] The inhibition was competitively antagonized by TPN and to a small extent by thiamine pyrophosphate (TPP). In contrast, glycolytic reactions were found to be much less sensitive to the inhibitor. TPP has also been found to be effective in reversing the inhibition of pyruvate oxidation by a particulate fraction of spores of *B. cereus var terminalis*.[8] Hypothetically, therefore, inhibition of respiratory enzymes may be a selective mode of action of hexetidine. In substantiation of this hypothesis, in this report hexetidine was observed to inhibit neotetrazolium-reducing dehydrogenases when incubated with leukocytes, as demonstrated by a diminished number of cells containing microscopically visible cytoplasmic formazan granules. The activity could be restored by DPN and by several divalent cations, notably zinc ions. The effects of a group of substrates and inhibitors on the endogenous reaction were also observed.

EXPERIMENTAL

Collection of cells

Leukocytes were obtained by centrifugation of heparinized human venous blood in Cushman tubes. Two tubes of from 8 to 10 ml capacity provided sufficient cells

*Abbreviations used are: hexetidine (1 : 3-bis-β-ethylhexyl-5-methyl-5-aminohexahydropyrimidine) obtained from the Warner Lambert Research Institute, Morris Plains, New Jersey: DPN, TPN, di- and tri-phosphopyridine nucleotide, respectively; TPP, thiamine pyrophosphate; F-1 : 6-di-P, fructose-1 : 6-diphosphate; F-6-P, fructose-6-phosphate; G-6-P, glucose-6-phosphate.

* Fabian Lionetti unpublished observations.

for a typical experiment. Plasma was aspirated, discarded, and the "buffy coat" of leukocytes extending into the capillary part of the tube carefully removed with a small syringe and a long (no. 15) needle. The cells from 10 ml of blood were suspended in 7·0 ml of 0·11 M phosphate buffer at pH 7·2.

Reagents

Saturated neotetrazolium chloride solution (about 0·3 per cent w/v) was made by adding an excess of the salt to the buffer and shaking mechanically for from 5 to 10 min followed by filtration through medium porosity paper. Substrates, cofactors and inhibitors were also dissolved in buffer to give the concentrations shown in the tables. Hexetidine solutions were prepared as described previously.[7] Phosphorylated carbohydrate esters were obtained as the barium salts, barium ion was removed by treatment with a slight excess of saturated potassium sulfate and the $BaSO_4$ removed by centrifugation. The supernatant solutions were diluted with buffer to yield the concentrations used.

Procedure

The incubation medium contained 3·0 ml of buffered neotetrazolium (0·11 M phosphate pH 7·2), 1·0 ml of leukocyte suspension containing approximately 1×10^7 mixed granulocytes and 2·0 ml of buffer containing various substances in 15·0 ml conical-shaped centrifuge tubes. Controls contained buffer, neotetrazolium and cell suspensions. The total volume was 6·0 ml. Six tubes were used in most experiments and were incubated for 1 hr at 37 °C in a water bath. They were then centrifuged at $500 \times g$ for 10 min and nearly all (about 5·5 ml) of the supernatant solution was decanted. A drop of the concentrated cell suspension at the bottom of the tube was transferred to a slide and mounted as a wet preparation, using a coverslip with greased edges. The slides were further incubated at room temperature for a second hour. They were then observed microscopically ($\times$ 1000) under an oil immersion lens. Of the mature neutrophiles 300–400 were counted and the percentage which contained one or more distinct formazan granules was recorded. Clumped or disrupted cells were not included.

RESULTS AND DISCUSSION

Whereas several di-tetrazolium salts (blue tetrazolium and a nitro derivative, nitro-BT) have been shown to possess advantages for histochemical application,[1, 2] good diformazan deposition was obtained from neotetrazolium with simple media in aerobic incubations. Diformazan granules produced were well formed, very dark and not noticeably diffuse when substrates were present. However, the cytoplasm of the cells always contained sufficient endogenous substrates for a visible reaction. The leukocytes in the control tubes of a large (approximately fifty) number of experiments contained from 30 to 90 per cent of stained neutrophiles, depending on conditions. In a single sample of cell suspension the endogenous reaction could be measured with a precision as indicated by the mean deviation $\pm$ 9·0 per cent. Erythrocytes were unstained. Platelets and lymphocytes, however, did contain visible granules. The endogenous staining reaction could be diminished and occasionally abolished by several conditions. Storage of the cell suspension overnight at 4 °C, or maintaining a sterile whole blood sample at 25 °C for approximately 8–10 hr prior to the collection

and pre-incubation of the cells depleted the endogenous substrate and reduced the production of formazan granules from 50 to 60 per cent. Furthermore, washing the cells three times in isotonic (0·15M) NaCl and then pre-incubating them at 25 °C for 4 hr further reduced the dye reduction, but disruption of much of the cells invalidated quantitation. Whereas the formazan granules in unfortified preparations became diffuse after 2–3 hr and usually disappeared in about 5 hr, those produced when substrates such as succinate were present (Table 1) remained sharp and distinctly visible for 5 hr. The morphological characteristics of the reaction have been well characterized in drawings by Vercauteren.[5]

The persistence of a high endogenous dye reduction defeated most of the attempts to define substrate and cofactor dependencies of the reaction. The cells frequently clumped or lysed when allowed to pre-incubate at 25 °C sufficiently long (4–5 hr) to make the enzyme reaction dependent on exogenous substrate. In spite of this limitation, it was still possible to detect and estimate the effects of a number of substrates and cofactors for dehydrogenases (Table 1). While in some instances noticeable

TABLE 1. EFFECTS OF VARIOUS SUBSTANCES ON DYE REDUCTION IN LEUKOCYTES

Substance added	Number of experiments	Molarity	Mean % change in cells with formazan granules (+ increase, − decrease)
Succinate	6	0·012	+126
Succinate + malonate	5	0·020 0·012	−33
Malate	5	0·018	+3·0
Pyruvate	5	0·019	+5·4
*iso*Citrate	5	0·013	−3·0
Glucose	8	0·018	+51
G-6-P	7	0·016	−5·0
F-6-P	5	0·016	−7·0
F-1 : 6-di-P	8	0·008	−13
Iodoacetate	4	1×10^{-4}	−64
p-Chloromercuribenzoate	2	1×10^{-4}	−57
Fluoride	9	0·020	+73
Versenate (Na_2)	6	0·037	+21
Hexetidine	6	$2·4 \times 10^{-5}$	−55
DPN	5	$1·7 \times 10^{-4}$	−14
TPN	5	$9·0 \times 10^{-5}$	+1·0

The basic system contained only neotetrazolium, phosphate buffer and leukocytes, as described in the text.

changes in the *amount* of formazan pigment per cell were observed with some substrates (glucose, succinate), a regular finding was an increase in the *percentage* of cells containing the formazan. In the case of inhibitors (iodoacetate, hexetidine) a decrease in the number stained was observed. Quantitative estimation of enzyme activity by observing the percentage of cells stained per unit time in a medium of specified composition was therefore possible. Succinate, glucose, fluoride and sodium versenate were observed to stimulate the reaction, whereas iodoacetate, parachloromercuribenzoate, and hexetidine were very effective inhibitors. Malonate, classically known as a competitive inhibitor of succinate, was moderately inhibitory in a ratio

of malonate to succinate of slightly greater than 2 : 1. The other substrates were without effect on the reaction. However, in cells stored for 8 hr at 25 °C prior to incubation with malate (0·012 M) the reaction was much more dependent on exogenous substrate, the stimulation by added malate increasing from a negligible effect of 3·0 per cent (Table 1) to 81 per cent. Neither DPN, TPN nor a TPNH-generating system (glucose-6-phosphate + TPN) produced significant effects on the reaction. In contrast to findings of Antopol *et al.*[9] with fibroblast cells, α-glycerophosphate plus DPN (a potential source of DPNH) had no effect.

Many similarities to the results described by Vercauteren[5] were observed. Although he incubated the cells anaerobically in Thunberg tubes and examined smears fixed in formalin, the endogenous reduction of neotetrazolium (percentage of cells stained) was of similar magnitude to that given here. The only quantitative data for intact cells were obtained with inhibitors present. These data are much like the data obtained here, allowing for certain differences in concentrations of inhibitors. It is inexplicable to us that fluoride (3×10^{-3}) should inhibit Vercauteren's system approximately 30 per cent while at 2×10^{-2} M our reaction was markedly stimulated. With homogenates of leukocytes, however, he found fluoride (3×10^{-3} M) had no effect. Vercauteren has also shown that malate, pyruvate, citrate, and TPN did not affect the reduction of the dye in intact cells. These observations are confirmed in the data of Table 1. Glucose, however, was negative in the cells but stimulated the reaction in

TABLE 2. EFFECT OF HEXETIDINE ON DEHYDROGENASES OF LEUKOCYTES

Tube	Hexetidine (μmoles)	Cells stained (%)
1	0	75
2	0	80
3	0·015	87
4	0·15	50
5	0·38	26
6	0·75	0

Reduction of neotetrazolium was dependent on endogenous substrates, as no substrate was added. Hexetidine is expressed as μmoles per 6 ml of reaction mixture as described in the text.

homogenates. In our intact cell preparation a pronounced stimulation by glucose was observed. Anaerobiasis was not a requirement in our method although the wet mounted preparations were sealed during the final hour of incubation.

The behavior of hexetidine proved interesting in several respects. The various findings with this inhibitor suggested an inhibitory mechanism which operated by interaction with the phosphorylated constituents of coenzymes.[7, 8] Some evidence which partially substantiates this type of mechanism was obtained with the leukocyte neotetrazolium method. The results of an experiment showing an increase in inhibition with increasing concentration of hexetidine are shown in Table 2. This effect could be observed regularly, although the amount of inhibitor necessary to reduce the number of cells containing formazan to 50 per cent varied appreciably in the cells from different donors. However, in most of the experiments described, the amount of hexetidine

necessary for this varied from 0·13 to 0·16 μmoles per tube. Several cofactors were found which could relieve the inhibition if incubated simultaneously with hexetidine and the cells. Although DPN alone exercised no influence on the percentage of cells containing formazan, its presence with hexetidine in molar ratios of 5 : 1 abolished the inhibition. An experiment representative of such findings is shown in Table 3. TPN was without effect. With several notable exceptions cations did not effect the inhibited reaction. Mg^{2+} and Zn^{2+} counteracted the inhibition, while Cu^{2+} was found to add to the inhibition. Zinc ion, however, in the inhibited cell preparation

TABLE 3. ANTAGONISM OF HEXETIDINE BY DPN

Hexetidine (μmoles)	DPN (μmoles)	Cells inhibited (%)
0·15	0	89
0	0	1*
0	1·0	0†
0·15	0·15	53
0·15	0·75	15
0·15	1·0	11

* 99 per cent of cells contained formazan granules.
† 100 per cent of cells contained formazan granules.
Concentrations are in μmoles per 6 ml of reaction mixture.

could repeatedly be shown to reduce the inhibition (Table 4). Other cations in amounts up to five times the amount of hexetidine had no measurable effect on the dye reduction or inhibited reaction. These included NH_4^+, Ca^{2+}, Co^{2+}, Hg^{2+}, Mn^{2+}, and Fe^{3+}.

The relative stimulation of the enzyme reaction with succinate, in contrast to the other intermediates of the tricarboxylic cycle, is interesting in light of the pattern of inhibition displayed. That sulfhydryl active sites are involved is suggested by the high

TABLE 4. PARTIAL ANTAGONISM OF HEXETIDINE INHIBITION BY ZINC ION

Tube	Hexetidine (μmoles)	Zinc ion (μmoles)	Cells stained (%)
1	0	0	94
2	0·15	0	52
3	0·15	0·15	44
4	0·15	0·38	70
5	0·15	0·75	84

The units of concentration are similar to those of Tables 2 and 3.

inhibition shown by 1×10^{-4} iodoacetate and 1×10^{-4} *p*-chloromercuribenzoate. Circumstantially, at least, hexetidine resembles this group. The relative increase in dye reduction by endogenous substrate in the presence of versenate and fluoride suggests that the enzyme reaction is facilitated by complexing interfering cations (possibly Cu^{2+}). The reversal of the inhibition seen with hexetidine by several cations suggests that complexing of ions is one mode in which it might act biologically. In a non-cellular experiment, the formation of a complex of hexetidine and Mn^{2+}, or

Zn^{2+} (between pH 6·0 and 7·5) or Cu^{2+} (between pH 3·0 and 5·0) has been observed potentiometrically.[10] Of these, however, only Zn^{2+} could be shown to reverse the action of the inhibitor biologically. Finally, the reversal of hexetidine action by simultaneous incubation with DPN is consistent with the idea that hexetidine might act by disruption of an active sulfhydryl linked enzyme–ion–coenzyme complex such as that described by Van Eys and Kaplan[11] in explaining why DPN and DPNH competitively antagonize *o*-phenanthroline inhibition of yeast alcohol dehydrogenase.[12] Attempts to show interaction of inhibitor with either DPN or Zn^{2+}, or both, by spectrophotometric methods were unsuccessful. That reduced pyridine nucleotides do not couple directly to neotetrazolium has been shown by Farber *et al.*[1] who attribute the dye reduction to diaphorase enzymes. The leukocyte system, being most responsive to added succinate, may reduce neotetrazolium chiefly through the succinic dehydrogenase complex.

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REFERENCES

1. E. FARBER, W. H. STERNBERG and C. E. DUNLAP, *J. Histochem. Cytochem.* **4**, 254 (1956).
2. M. M. NACHLAS, K. C. TSOU, E. DESOUZA, C. S. CHENG and A. M. SELIGMAN, *J. Histochem. Cytochem.* **5**, 420 (1957).
3. E. SHELTON and W. C. SCHNEIDER, *Anat. Rec.* **112**, 61 (1952).
4. M. WACHSTEIN, *Proc. Soc. Exp. Biol., N.Y.* **73**, 306 (1950).
5. R. VERCAUTEREN, *Enzymologia* **18**, No. 2, 97 (1957).
6. E. J. DESOUZA and S. N. KOTHARE, *J. Histochem. Cytochem.* **7**, 77 (1959).
7. F. J. LIONETTI, W. L. MCLELLAN and F. C. COMUNALE, *Biochem. Pharmacol.* **2**, 226 (1959).
8. H. H. HALVORSON and B. D. CHURCH, *J. Appl. Bacteriol.* **20**, 259 (1957).
9. W. ANTOPOL, G. W. FRIED and H. KODFA, *Trans. N.Y. Acad. Sci.* **17**, 385 (1955).
10. F. LIONETTI. Unpublished observations.
11. J. VAN EYS and N. O. KAPLAN, *Biochim. Biophys. Acta* **32**, 574 (1957).
12. J. VAN EYS, M. M. CIOTTI and N. O. KAPLAN, *J. Biol. Chem.* **231**, 571 (1958).