Substrate inhibition of hydrogenase enhanced by sodium chloride

Inhibition of soluble hydrogenase by methylene blue was first reported by GEST[1] who attributed it to heavy metal impurities in the dyestuff. CURTIS AND ORDAL[2] demonstrated inhibition by methylene blue in preparations from *Micrococcus aerogenes*, and KING AND WINFIELD[3] reported similar results with *Desulphovibrio desulphuricans* hydrogenase and an inorganic catalyst. During a study of the salt tolerance of *D. desulphuricans*[4] we encountered a similar phenomenon and observed that the inhibitory effect of dyes was enhanced by sodium chloride. These results will not be published elsewhere, but, since they point to an additional technical hazard in the assay of hydrogenase, we feel that a brief report of the phenomenon is desirable.

Cells of *D. desulphuricans*, strain Wandle (National Collection of Industrial Bacteria No. 8305) were grown at 30° in a lactate-peptone-yeast extract-sulphate medium. Intact cells, particulate and soluble preparations were tested for hydrogenase activity manometrically using methylene blue, benzyl viologen or methyl violet as hydrogen acceptor. Manometer cups contained 1.3–1.5 ml KH_2PO_4 (0.5 % w/v; pH 6.30 ± 0.02), $CdCl_2$ (10 % w/v) in the centre well to absorb any H_2S produced by side reactions, 0.2–0.5 ml cells or enzyme preparation and atmosphere of H_2 which had not been specially purified; since hydrogenase is reversibly inactivated by aeration, the vessels were left to incubate for 1–2 hours before adding substrate. All experiments were run at 37°. The following preparations were examined: (a) washed fresh cells 0.3–0.4 mg dry wt/vessel; (b) cells treated with cetyltrimethylammoniumchloride (CTAC: 100 μg/mg dry wt cells) 0.3–0.4 mg dry wt/vessel, which correspond to a particulate hydrogenase preparation[5]; (c) vacuum dried cells, 0.24–0.3 mg/vessel; (d) soluble extract of vacuum dried cells: 2 ml/vessel of supernatant fluid from 50 mg vacuum dried cells/ml phosphate buffer (e) acetone-dried cells 0.24–0.3 mg/vessel; (f) soluble extract of acetone-dried cells: 0.2–0.4 ml/vessel of supernatant fluid from 50 mg acetone-dried cells/ml phosphate buffer.

Table I gives a qualitative record of the results obtained. Minor quantitative differences, such as partial inhibition by dyes at higher salt concentrations, are not recorded. All preparations tested showed hydrogenase activity towards methylene blue or benzyl viologen if salt were absent. If it were present, however, the soluble preparations (d) and (f) did not reduce methylene blue except in the one instance quoted. Hydrogenase must nevertheless have been present since the preparations both reduced methyl violet in hydrogen at all salt concentrations examined. Hydrogenase was detectable in preparation (d) with benzyl viologen but methyl violet was the only substrate that detected it in preparation (f).

Hence inhibition of hydrogenase by dyestuffs such as methylene blue, whatever its mechanism, is increased by NaCl. Results not recorded in the table indicate that this is true even with particulate preparations, though complete abolition of hydrogenase activity was then rarely

TABLE I

MANOMETRIC TESTS FOR HYDROGENASE IN PREPARATIONS OF *D. desulphuricans*

Type of preparation	*Hydrogen acceptor*	*Apparent presence or absence of hydrogenase*						
	NaCl(%)	*0*	*1*	*2*	*3*	*4*	*5*	*6*
(a)	methylene blue	+	+	+	+	+	+	+
(intact cells)	benzyl viologen	+	+	+	+	+	+	+
(b)	methylene blue	+	+	+	+	+	+	+
(CTAC-treated cells)	benzyl viologen	+	+	+	+	+	+	+
(c)	methylene blue	+	+	+	+	+	+	+
(vacuum-dried cells)	benzyl viologen	+	+	+	+	+	+	+
(d)	methylene blue	+	—	—	—	—	—	—
(extract of (c))	benzyl viologen	+	···	+	···	+	···	+
	methyl violet	+	···	+	···	+	···	+
(e)	methylene blue	+	···	+	···	+	···	—
(acetone-dried cells)	benzyl viologen	+	···	+	···	+	···	+
(f)	methylene blue	+	+	—	—	—	—	—
(extract of (e))	benzyl viologen	+	—	—	—	—	—	—
	methyl violet	+	···	+	···	+	···	+

obtained. In common with other workers we find that methyl violet is the least inhibitory of the three dyestuffs.

This note is published by permission of the Director, Chemical Research Laboratory.

Chemical Research Laboratory, Teddington (England) DOROTHY LITTLEWOOD
JOHN R. POSTGATE

[1] H. GEST, *J. Bacteriol.*, 63 (1952) 111.
[2] W. CURTIS AND E. J. ORDAL, *J. Bacteriol.*, 68 (1954) 351.
[3] N. K. KING AND M. E. WINFIELD, *Biochim. Biophys. Acta*, 18 (1955) 431.
[4] D. LITTLEWOOD AND J. R. POSTGATE, (in preparation).
[5] K. R. BUTLIN AND J. R. POSTGATE in *Microbial Metabolism*, supplement to *Rend. ist. sup. sanità*, (1953) p. 131.

Received January 27th, 1956

The action of carboxypeptidase on different human haemoglobins

Most of the investigations of the eleven different human haemoglobins (A, B, C, D, E, F, G, H, I, J and K) described at the present have been carried out on normal adult (Hb-A), sickle-cell (Hb-B), Hb-C Hb-E and foetal haemoglobin (Hb-F). The results of these studies and especially of those concerning the N-terminal group analyses[1-4] have revealed that the molecules of these haemoglobins are built up by more than one polypeptide chain. According to the end-group analyses using Sanger's DNP method, Hb-A, Hb-B, Hb-C and Hb-E have 5 N-terminal valine residues and Hb-F two N-terminal valine residues. In the present paper some investigations are reported concerning the influence of carboxypeptidase on four different human haemoglobins (A, B, C and F) and what it reveals of the number, nature and distributions of C-terminal groups in these proteins.

The Hb-A was obtained from normal adults (laboratory workers); the Hb-B from patients with sickle-cell anemia and the Hb-C from patients with the homozygous Hb-C disease. These patients are described elsewhere[5,6]. Haemoglobin solutions were prepared and purified in the way described earlier[7]. The different haemoglobins were homogeneous in electrophoretic and chromatographic experiments[8]. The foetal haemoglobin was derived from samples of cord blood. A haemoglobin sample containing 96% Hb-F estimated by the alkali denaturation method[9] was used. Two samples of purified foetal haemoglobin, prepared by the alkali denaturation method of CHERNOFF[10], were also investigated. The time of denaturation was one minute (sample 1) and two minutes (sample 2). In both cases only foetal haemoglobin was present after this purification procedure.

The enzyme used during the course of this work was a preparation six times recrystallized obtained from Armour and Company. For qualitative studies 2.5 mg haemoglobin was incubated at 37° C for different periods (0; 6; 18; 27; 36; 72 hours) with 0.1 mg carboxypeptidase (enzyme-substrate mole ratio, 2:25) in 0.05 *M* phosphate buffer at pH 8.0 and 0.5% lithium chloride (final concentrations). An excess of di*iso*propyl fluorphosphate ($1.2 \cdot 10^{-5}$ m*M* of pure DFP per ml of solution) was added to destroy the last traces of endopeptidase activity in the carboxypeptidase preparation[11]. The final volume was 0.8 ml. The addition of 50 mg of Dowex-50 (H form, 20–50 mesh, 8% cross-linked) served to terminate the enzyme reaction by lowering the pH of the reaction solution to 2 to 3. The reaction products which were absorbed on the Dowex-50 resin were eluted with 5 *M* ammonia (0.2 ml) according to the molecular sieve method of PARTRIDGE AND THOMPSON[12,13] and subjected to paper chromatography. One-dimensional ascending paper chromatography (Whatman No. 1) was used with butanol-acetic acid-water (40:6:15) as solvent.

For quantitative analyses a hundred-fold enzyme substrate incubation mixture was prepared. The only difference was a lowered amount of the carboxypeptidase preparation (5 mg carboxypeptidase in 250 mg haemoglobin; enzyme-substrate mole ratio 1:25) and a corresponding lowered amount of DFP ($0.6 \cdot 10^{-5}$ m*M* per ml of solution). In general the same procedure was followed as described for the qualitative analyses. The mixture of amino acids finally obtained was freed from ammonia *in vacuo* and subjected to the quantitative column chromatographic method of STEIN AND MOORE[14] in the way described previously[7].

Using one-dimensional paper chromatography, two intensive spots and some faint but definite ones were obtained when haemoglobin A was incubated for six hours with carboxypeptidase. The two intensive spots corresponded to tyrosine and histidine, while the other ones were identical with leucine, alanine, glycine or glutamic acid. After longer periods of incubation (18, 36, and 72 hours) these and some other amino acids (phenylalanine, valine and lysine) were released in increased amounts. Approximately the same pictures were obtained when the haemoglobin from cord blood, Hb-B and Hb-C, was studied, while in similar experiments carried out with foetal haemoglobin, purified by the alkali denaturation method, the same amino acids were