tion, one group of mice were injected i.p. with 2.5 μCi (administered in 3 equal doses in the first 3 days) and the other with 10 μCi (administered in 5 equal doses in the first 5 days) of labelled compound in 0.1 ml of an isotonic saline solution for each animal. 13 days after the first injection, the 2 groups of mice were sacrificed, the melanomas removed and the melanin was isolated according to NICOLAUS et al.[8]. As a control the melanin from melanoma of untreated animals was also isolated.

The radioactivity was measured in a liquid scintillation spectrometer Beckman LS-150, suspending 2 mg samples of melanin in 6 ml of Insta-Gel emulsifier (Packard Instrument Company, Inc.) and 4 ml of water. Efficiency was determined by using *n*-hexadecane-1-^{14}C as an internal standard. The Table summarizes our results.

It appears that tryptophan is incorporated into melanin of Harding-Passey mouse melanoma, either by administration of D, L-tryptophan-benzene ring-^{14}C, or D, L-tryptophan-methylene-^{14}C. In both cases the incorporated radioactivity per mg melanin (see the last column of Table) is related to the radioactivity administered and it is almost equal among the group of animals treated with the same amount of the two substances. This shows that tryptophan is incorporated into melanin not only with its benzene ring, but also with alanine chain or at least with a part of it.

Taking all this into consideration, there seem to be two pathways through which tryptophan is involved in the melaninic synthesis: the 'via tryptophan → kynurenine → 3-hydroxykynurenine'[5-7] and the 'via 5-hydroxytryptophan'[3,4]. We found (see Table) that the administration of 5-hydroxytryptophan and 5-hydroxytryptamine gives an incorporation of radioactivity into melanin significantly lower than tryptophan, whether in total value or expressed as radioactivity per mg melanin. Besides we did not note any relationship between the amount of radioactive 5-hydroxytryptophan derivatives administered and the amount of radioactivity incorporated into melanin, as observed with tryptophan.

These results seem to show that the tryptophan does not follow the 'via 5-hydroxytryptophan' for the formation of melanin in Harding-Passey mouse melanoma, but that it follows its metabolic pathway through the kynurenine.

We were also able to show (see Table) that the administration of D, L-tyrosine-2-^{14}C to Harding-Passey melanoma mice gives an incorporation of radioactivity into melanin (expressed as dpm/mg pigment) almost equal to that obtained with tryptophan. Therefore from the biogenetic comparison with tyrosine, tryptophan too must be considered as an important precursor in the biogenesis of melanins.

Riassunto. Il triptofano ^{14}C- uniformemente marcato nell'anello benzenico e il triptofano marcato ^{14}C nel metilene sono incorporati in modo significativo nella melanina del melanoma di Harding-Passey del topo. 5-ossitriptofano e 5-ossitriptamina non mostrano invece una significativa incorporazione nella stessa melanina. L'incorporazione ottenuta con il triptofano marcato sia nel nucleo che nel metilene è all'incirca uguale a quella ottenuta per somministrazione di tirosina-2-C^{14}, per cui anche il triptofano deve essere considerato un precursore delle melanine.

A. DE ANTONI, G. ALLEGRI, C. COSTA and F. BORDIN

Istituto di Chimica Farmaceutica e Tossicologica dell'Università di Padova, Via Marzolo 5, Padova (Italy), 28 December 1973.

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Effect of Methylene Blue on P_{50} and 2,3DPG of Human Blood in vitro

The redox dye, methylene blue, is used in the treatment of methemoglobinemia[1] and it has been proposed for the treatment of lactic acidosis[2]. More recently, KOCHOLATY and DAWSON[3,4] have suggested the use of inosine and methylene blue (which exert a cooperative effect in the maintenance of 2, 3DPG) in the preservation of human red cells for transfusion purposes.

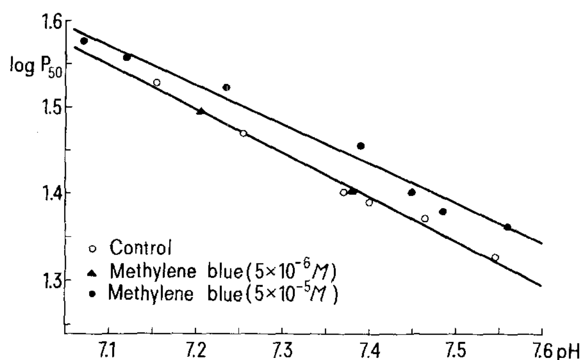

Fig. 1. Effect of different concentration of methylene blue on the oxygen affinity of human blood at various plasma pH.

The present work deals with the addition of varying concentrations of methylene blue at different pH to fresh human blood and its effect on the erythrocyte content of 2, 3DPG, H^+, K^+ and Na^+.

Material and methods. Heparinized venous blood was collected in plastic syringes from 3 normal non-smoking subjects and placed in an ice bath. The P_{50} values were determined with a direct method: i.e. equilibrating the blood samples with a gas mixture containing a partial pressure of oxygen at which hemoglobin was 50% saturated. This was obtained by using a gas mixing Control Module I.L. 208/1 composed of a flowmeter system mixing the content of 2 gas cylindres respectively containing 5.7% of CO_2 in N_2, and 5.7% of CO_2 in air. The outlet composition of the gas mixture was continuously checked by an Oxygen Monitor I.L. 208/2. Then, the gas flowed into an 'open-type' equilibrating vessel

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(I.L. 237), and the P_{50} values were obtained according to the technique of BRENNA et al.[5].

Hemoglobin, HbO_2, and HbCO were measured in an I.L. Cooximeter 182. Whole blood pH and pCO_2 were measured on a Gas-pH Analyzer I.L. 113. Either 0.2 M sodium bicarbonate or 0.2 M hydrocloric acid was used to adjust the blood pH to the desired value. Methylene blue solutions (5×10^{-2} M, 1×10^{-2} M, 5×10^{-3} M, 1×10^{-3} M, 5×10^{-4} M) were added to whole blood in proportion 1:100. All the measurements were made 25 min after the addition of methylene blue. Intracellular pH was measured as follows. After blood equilibration at P_{50}, the plasma and the red cells were separated anaerobically in about 30 sec in an Eppendorf microcentrifuge (model 3200). Lysis of the red cells was obtained by freezing and thawing. The hemolyzed solution was then re-equilibrated with the same gas phase as before, and pH was measured on the hemolyzed red cell content. 2,3DPG was measured according to the method of ROSE and LIEBOWITZ[6]. The sodium and potassium concentrations were determined by an Eppendorf flame photometer.

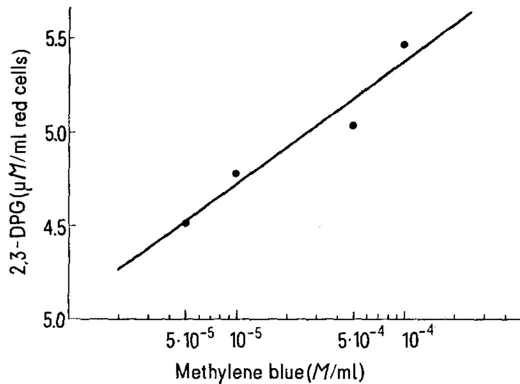

Fig. 2. The relationship between methylene blue concentrations and 2,3DPG levels.

Results. Figure 1 shows the effect of methylene blue on log P_{50} at various plasma pH values. When methylene blue was added to fresh human blood at a concentration of 5×10^{-6} M, the values of P_{50} did not differ from those of the controls. By contrast, with a concentration of methylene blue of 5×10^{-5} M, the P_{50} increased. This increase was constantly present at different pH values. With a greater concentration of methylene blue (5×10^{-4} M), a minor increase of P_{50} values was observed (data not reported in the figure).

A correlation graph of methylene blue concentrations and 2,3DPG levels is shown in Figure 2. The plot indicates a linear relation between the methylene blue concentrations (log scale) and 2,3DPG content of red cells, even at high concentrations of methylene blue.

The addition of methylene blue had no effect on the intra- and extracellular concentrations of H^+, Na^+, and K^+, as indicated in the experiment reported in the Table.

H^+, Na^+, and K^+ concentrations in the plasma and in the red cells in the absence and in the presence of methylene blue

	Control	Methylene blue (5×10^{-5} M)
Hb g/100 ml	14.0	13.5
HbCO % of Hb	1.9	1.9
P_{50}	26.1	28.4
log P_{50}	1.419	1.454
pH (plasma)	7.40	7.41
pH (erythrocyte)	7.21	7.21
K^+ (plasma)	4.77	4.57
K^+ (erythrocyte)	102.3	96.5
Na^+ (plasma)	140.5	140.5
Na^+ (erythrocyte)	21.5	23.2

Discussion. So far experiments concerning the effect of methylene blue on red cell metabolism have been done only with incubation of long duration (5 h in the experiments of MILLS[7], and some weeks in those of DAWSON and KOCHOLATY[3,4]), and always in presence of phosphate. In the present investigation the incubation times were

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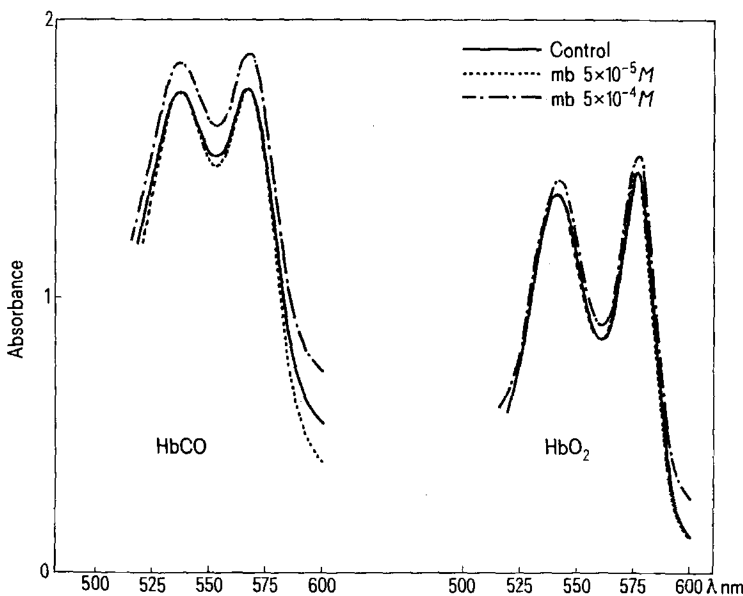

Fig. 3. Effect of the addition of methylene blue at 2 different concentrations on the absorption spectra of HbCO and HbO_2 of human blood.

limited to 25 min and no other substances were added but methylene blue. The effect of methylene blue only appeared when a concentration of 5×10^{-5} *M* was reached. The increase of P_{50} values seems to be the consequence of a rapid increase of 2,3DPG levels, because the red cell concentration of H^+ and cations was not affected by methylene blue, as happens for other drugs (like propranolol[8]).

To the observation that there is an optimal concentration of methylene blue for affecting P_{50} values (as indicated by DAWSON and KOCHOLATY[3,4] and as appeared in our experiments), this must be considered an error. In fact, when P_{50} is measured by means of spectrophotometric techniques, the methylene blue at high concentration (5×10^{-4} *M*) interferes with the absorption spectrum of HbO_2 and HbCO (Figure 3).

Riassunto. L'aggiunta a sangue umano di blu di metilene riduce l'affinità dell'Hb per l'O_2. Tale effetto sembra la conseguenza di un incremento dei livelli intraeritrocitari di 2,3DPG. La concentrazione intra- ed extracellulare di H^+, Na^+ e K^+ non è influenzata dal blu di metilene.

C. BERNASCONI, G. C. GERLI, R. STABILINI and A. AGOSTONI

Istituto di Patologia Medica dell'Università, Via Pace 15, I-20122 Milano (Italy), 3 December 1973.

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H_2O_2 Oxidizes an Aldolase Dihydroxyacetone Phosphate Intermediate to Hydroxymethylglyoxal Phosphate

The aldol cleavage-condensation reaction catalysed by fructose 1,6-diphosphate aldolase proceeds via a carbanionic enzyme-bound intermediate of dihydroxyacetone phosphate ($^-CHOH \cdot C = NH^+R \cdot CH_2OPO_3^{2-}$, H_2NR = active site lysyl residue of aldolase)[1]. Recently, we have shown that th is intermediate is readily oxidized to hydroxymethylglyoxal phosphate (= hydroxypyruvaldehyde phosphate, $CHO \cdot CO \cdot CH_2OPO_3^{2-}$) by a number of oxidants such as tetranitromethane[2] and the oxidation-reduction indicators hexacyanoferrate (III), porphyrindin, porphyrexide, 2,6-dichlorophenol-indophenol and N-methylphenazinium methosulfate[3,4]. The oxidation of the intermediate proceeds with about equal facility using fructose 1,6-diphosphate, fructose 1-phosphate, or dihydroxyacetone phosphate as the substrate[2]. Whereas the substrate is consumed stoichiometrically by the reaction, the enzyme serves solely as a catalyst. Oxidative activity in the presence of suitable electron acceptors is exhibited both by the class I Schiff base forming aldolases (rabbit muscle, liver and brain[5]) and the class II metalloaldolase from yeast[6]. Since the reaction apparently is nonspecific with respect to the oxidant, the possibility was suggested that it might also occur in vivo with an intra cellular oxidant. The present communication reports the reaction of one of the possible candidates, H_2O_2, with the aldolase substrate intermediate. In analogy to the reactions of the oxidizing agents examined previously, the reaction product was identified as hydroxymethylglyoxal phosphate. This ketoaldehyde, a potentially biologically active compound, thus represents one of the long-sought natural substrates of the glyoxalase system.

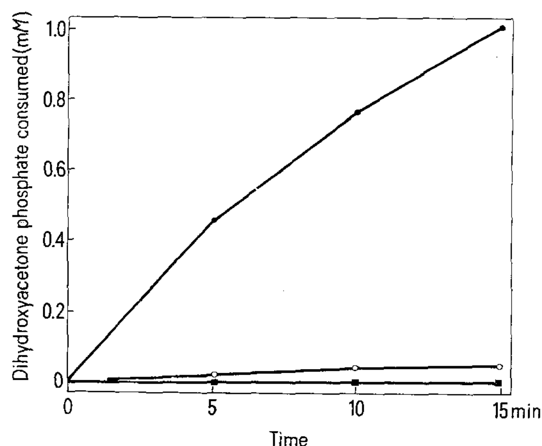

Fig. 1. Aldolase-catalyzed oxidation of dihydroxyacetone phosphate by H_2O_2. The reaction mixture contained 3.9 m*M* dihydroxyacetone phosphate, 40 m*M* H_2O_2, 5.6 U/ml aldolase in 0.05 M *Tris* chloride (pH 7.5) at 25 °C (●); the controls contained no aldolase (○) or no H_2O_2 (■), respectively.

Experimental. Fructose 1,6-diphosphate aldolase from rabbit muscle (specific activity 6 U/mg), glycerol phosphate dehydrogenase, and glyoxalase I (E.C.4.4.1.5) were obtained from Boehringer. The dicyclohexylammonium salt of the dimethyl ketal of dihydroxyacetone phosphate was chemically synthesized and converted into free dihydroxyacetone phosphate as reported by BALLOU and FISCHER[7]. H_2O_2 (Perhydrol ® 30%) was from Merck.

Dihydroxyacetone phosphate was determined with glycerol phosphate dehydrogenase and NADH[8], hydroxymethylglyoxal phosphate with glyoxalase I and reduced glutathione[9]. H_2O_2 (ε_{240} = 0.036 mM^{-1}cm^{-1})[10], aldolase activity[11] and concentration[12] were determined spectrophotometrically.

Results. Addition of H_2O_2 to a mixture of dihydroxyacetone phosphate and aldolase initiates a substrate-consuming reaction (Figure 1). Both H_2O_2 and aldolase are

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