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.

[8] A. AGOSTONI, C. BERNASCONI, G. C. GERLI, M. LUZZANA and L. ROSSI-BERNARDI, Science *182*, 300 (1973).

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 non-specific with respect to the oxidant, the possibility was suggested that it might also occur in vivo with an intracellular 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.

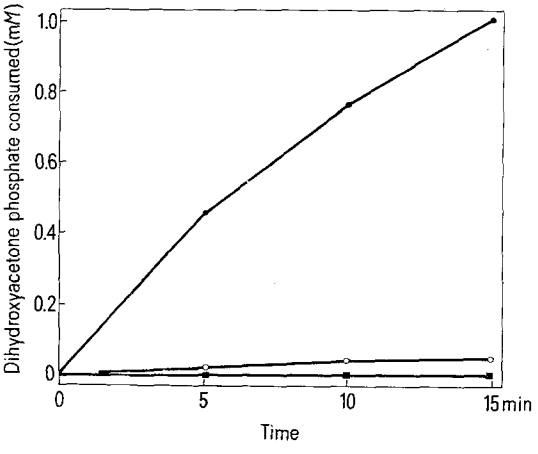

Fig. 1. Aldolase-catalyzed oxidation of dihydroxyacetone phosphate by H_2O_2. The reaction mixture contained 3.9 m*M* dihydroxyacetone phosphate, 40 m*M* H_2O_2, 5.6 U/ml aldolase in 0.05 M *Tris* chloride (pH 7.5) at 25 °C (●); the controls contained no aldolase (○) or no H_2O_2 (■), respectively.

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 ($\varepsilon_{240} = 0.036$ 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

[1] B. L. HORECKER, O. TSOLAS and C. Y. LAI, Enzymes *7*, 213 (1972).
[2] P. CHRISTEN and J. F. RIORDAN, Biochemistry *7*, 1531 (1968).
[3] M. J. HEALY and P. CHRISTEN, J. Am. chem. Soc. *94*, 7911 (1972).
[4] M. J. HEALY and P. CHRISTEN, Biochemistry *12*, 35 (1973).
[5] M. J. HEALY, Thesis, University of Zürich, 1973.
[6] J. F. RIORDAN and P. CHRISTEN, Biochemistry *8*, 2381 (1969).
[7] C. E. BALLOU and H. O. L. FISCHER, J. Am. chem. Soc. *78*, 1659 (1956).
[8] T. BÜCHER and H.-J. HOHORST, in *Methoden der enzymatischen* Analyse (Ed. H. U. BERGMEYER; Verlag Chemie, Weinheim 1970), vol. 2, p. 1282.
[9] R. H. WEAVER and H. A. LARDY, J. biol. Chem. *236*, 313 (1961).
[10] H. U. BERGMEYER, K. GAWEHN and M. GRASSL, in *Methoden der enzymatischen Analyse* (Ed. H. U. BERGMEYER; Verlag Chemie, Weinheim 1970), vol. 1, p. 440.
[11] H. U. BERGMEYER, K. GAWEHN and M. GRASSL, in *Methoden der enzymatischen Analyse* (Ed. H. U. BERGMEYER; Verlag Chemie, Weinheim 1970), vol. 1, p. 391.
[12] T. BARANOWSKI and T. R. NIEDERLAND, J. biol. Chem. *180*, 543 (1949).

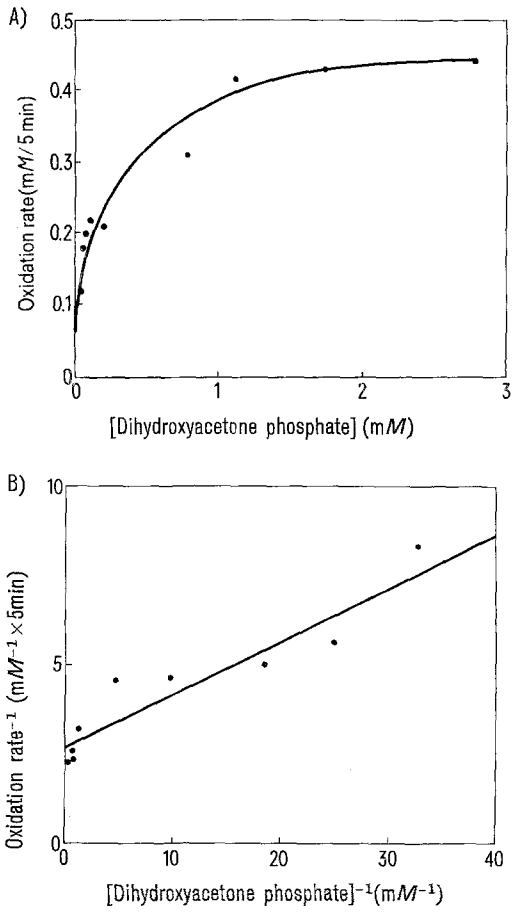

Fig. 2. Dependence of the oxidation rate on the concentration of dihydroxyacetone phosphate. A) The oxidation rate is given as the decrease in concentration of dihydroxyacetone phosphate in the first 5 min of the reaction. The concentration of dihydroxyacetone phosphate indicated on the abscissa is the mean of the concentration at zero and 5 min reaction time. H_2O_2 concentration was 150 mM. The aldolase concentration was adjusted between 0.42 and 2.8 U/ml to keep the substrate consumed to $< 65\%$ of the initial concentration; the rates indicated are normalized to an aldolase concentration of 2.8 U/ml. Other conditions were as in Figure 1. B) Double reciprocal plot of oxidation rate vs. concentration of dihydroxyacetone phosphate.

Aldolase-catalyzed oxidation of dihydroxyacetone phosphate to hydroxymethylglyoxal phosphate

Reaction time (min)	Dihydroxyacetone phosphate (mM)	Hydroxypyruvaldehyde phosphate (mM)
0	7.5	0
5	7.1	0.4
30	5.5	0.5
80	4.1	0.7
160	2.5	0.8

The reaction mixture contained 67 mM H_2O_2, 4.3 U/ml aldolase in 0.05 M *Tris* chloride (pH 7.5) at 25 °C. At the indicated times an aliquot was analysed for dihydroxyacetone phosphate and hydroxymethylglyoxal phosphate.

required to induce the disappearance of dihydroxyacetone phosphate; in the control experiments, when either one is absent the substrate concentration remains virtually unchanged. The kinetic features of the reaction are consistent with an oxidation of an enzyme-bound intermediate of dihydroxyacetone phosphate. The initial rate of substrate oxidation is proportional to the concentration both of the enzyme and of H_2O_2 and follows saturation kinetics with respect to the concentration of dihydroxyacetone phosphate (Figure 2A). The double reciprocal plot of the initial rate of dihydroxyacetone phosphate oxidation, vs. the concentration of dihydroxyacetone phosphate, yields a straight line (Figure 2B). The dihydroxyacetone phosphate concentration resulting in half-maximum rate of oxidation (7.4×10^{-5} M) agrees with the values determined for the reactions with other oxidizing agents[2,4]. The primary product of oxidation of aldolase-activated dihydroxyacetone phosphate is apparently the corresponding ketoaldehyde, hydroxymethylglyoxal phosphate (Table). However, at the high H_2O_2 concentrations used in the present experiments, only a fraction of the total dihydroxyacetone phosphate consumed was recovered as hydroxymethylglyoxal phosphate. The rest apparently was oxidized further by H_2O_2 in a non-enzymatic reaction. It has been reported that α-ketoaldehydes are readily oxidized by H_2O_2 at neutral pH; in the present case the expected products are formic acid and phosphoglycolic acid[13].

Discussion. Recent reports have demonstrated the existence of a cytosolic steady state concentration of H_2O_2 in living tissues which may amount to 10^{-7} M[14,15]. The extent of intracellular aldolase-catalyzed oxidation of dihydroxyacetone phosphate at this H_2O_2 concentration may be estimated from the values reported for the intracellular concentrations (rabbit skeletal muscle) of aldolase[16], fructose 1,6-diphosphate and dihydroxyacetone phosphate[17]. Extrapolation of the experimentally determined rate of oxidation (cf. Figure 1) to the in vivo conditions yields a rate of 10–20 nM/min. In certain cells under special circumstances, e.g. phagocytizing leukocytes, where much higher H_2O_2 concentrations in the range of 1–100 mM may occur[18,19], aldolase-catalyzed oxidation may become of quantitative importance as a first step of an irreversible non-phosphorylating shunt to the Embden-Meyerhof pathway. Hydroxymethylglyoxal phosphate is a substrate for the glyoxalase system; with reduced glutathione as a cosubstrate it is transformed to D-3-phosphoglycerate in 2 consecutive reactions catalysed by glyoxalase I and glyoxalase II[9]. Hydroxymethylglyoxal phosphate thus represents one of the long-sought natural substrates for the glyoxalases, an ubiquitous and very active enzyme system[20]. Ketoaldehydes have been reported to

[13] T. E. FRIEDEMANN, J. biol. Chem. *73*, 331 (1927).
[14] A. BOVERIS, N. OSHINO and B. CHANCE, Biochem. J. *128*, 617 (1972).
[15] N. OSHINO, B. CHANCE, H. SIES and T. BÜCHER, Arch. Biochem. Biophys. *154*, 117 (1973).
[16] W. E. KNOX, *Enzyme Patterns in Fetal, Adult and Neoplastic Rat Tissues* (Karger, Basel 1972), p. 302.
[17] D. H. WILLIAMSON and J. T. BROSNAN, in *Methoden der enzymatischen Analyse* (Ed. H. U. BERGMEYER; Verlag Chemie, Weinheim 1970), vol. 2, p. 2187.
[18] G. Y. N. IYER, D. M. F. ISLAM and J. H. QUASTEL, Nature, Lond. *192*, 535 (1961).
[19] B. PAUL and A. J. SBARRA, Biochim. biophys. Acta *156*, 168 (1968).
[20] W. E. KNOX, Enzymes *2*, 253 (1960).

react readily with both proteins[21] and nucleic acids[22,23], to possess antiviral activity[24], to inhibit protein synthesis[25] and cell division[26], and have been postulated to be involved in the regulation of these processes[27]. The role of the glyoxalases in preventing an accumulation of hydroxymethylglyoxal phosphate would therefore seem to be essential for normal cell function. The oxidation of enzyme-substrate carbanions by H_2O_2 might also be considered as a metabolic feature mediating the effect of an increased intracellular H_2O_2 concentration in biological processes such as oxygen poisoning[28], radiation damage[29], or phagocytosis by leukocytes[18,19].

Zusammenfassung. H_2O_2 oxydiert eine Enzym-Substrat-Zwischenverbindung der Fructose-1,6-diphosphat-Aldolase. Aus Dihydroxyacetonphosphat wird dabei Hydroxymethylglyoxalphosphat gebildet. Dieser Ketoaldehyd dürfte eines der lange gesuchten Substrate für das Glyoxalase-System sein.

P. Christen, T. K. Anderson, and M. J. Healy

Biochemisches Institut der Universität Zürich, Zürichbergstrasse 4, CH-8032 Zürich (Switzerland), 21 December 1973.

[21] E. Racker, in *Glutathione* (Eds. S. Colowick, A. Lazarow, E. Racker, D. R. Schwartz, E. Stadtman and H. Waelch; Academic Press, New York 1954), p. 171.
[22] M. Staehelin, Biochim. biophys. Acta *31*, 448 (1959).
[23] N. Krymkiewicz, FEBS Lett. *29*, 51 (1973).
[24] B. D. Tiffany, J. B. Wright, R. B. Moffett, R. V. Heinzelman, R. E. Strube, B. D. Aspergren, E. H. Lincoln and J. L. White, J. Am. chem. Soc. *79*, 1682 (1957).
[25] L. G. Együd and A. Szent-Györgyi, Proc. natn. Acad. Sci. USA *56*, 203 (1966).
[26] L. G. Együd and A. Szent-Györgyi, Proc. natn. Acad. Sci. USA 55, 388 (1966).
[27] A. Szent-Györgyi, *The Living State. With Observations on Cancer* (Academic Press, New York 1972), p. 88.
[28] R. Gerschman, in *Oxygen in the Animal Organism* (Eds. F. Dickens and E. Neil; Pergamon Press, Oxford 1964), p. 475.
[29] D. B. Menzel, A. Rev. Pharmac. *10*, 379 (1970).

A 24-Hour Rhythm in Human Serum Dopamine-β-Hydroxylase Activity

Human serum contains dopamine-β-hydroxylase (DBH), which is released from the sympathetic nerve endings and the adrenal medulla[1]. The enzyme activity in serum can be measured either by enzymatic radioassays using phenylethanolamine-N-methyltransferase[2,3], or by a micro-spectrophotometric assay[4].

The levels of DBH activity in human serum are especially high compared with various animal species[4], they showed a marked individual variation (1-100 U, μmoles/min, per 1 serum), but were very constant on successive days for a given individual[2-4]. The high enzyme activity in human serum is assumed to be caused by the increased sympathetic nerve activity due to the erect posture. Since the enzyme is secreted from the sympathetic nerves and the adrenal medulla together with norepinephrine and epinephrine, the enzyme activity in serum may be expected to have a 24-h rhythm caused by the changes in peripheral sympathetic activity. However, there has been no report on the 24-h rhythm of serum DBH activity in humans. If there is such a rhythm, it may be an important basic information for the clinical study of the enzyme activity in human serum. We have, therefore, examined changes in the enzyme activity in human serum during 24-h.

Dopamine-β-hydroxylase activity in serum was assayed by a micro-photometric method by Nagatsu and Udenfriend[4] using tyramine as substrate. 10 and 20 μl of serum were used for the duplicate assays. This assay method is highly reproducible, and the maximum velocity can be obtained under the saturated substrate

[1] J. Axelrod, Pharmac. Rev. *24*, 233 (1972).
[2] R. Weinshilboum and J. Axelrod, Circulation Res. *28*, 307 (1971).
[3] M. Goldstein, L. S. Freedman and M. Bonnay, Experientia *27*, 632 (1971).
[4] T. Nagatsu and S. Udenfriend, Clin. Chem. *18*, 980 (1972).

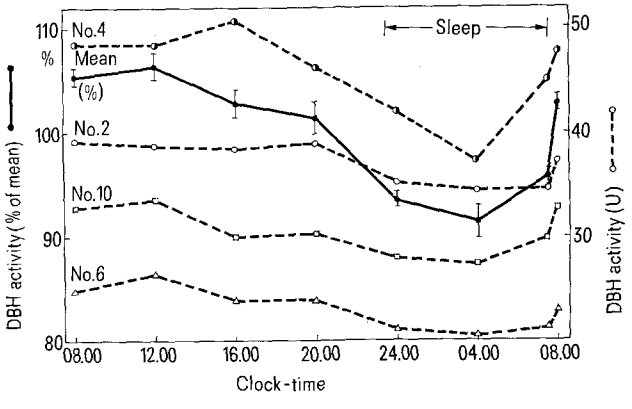

Fig. 1. Dopamine-β-hydroxylase (DBH) activities in human sera. —, Mean values (± SEM) of 10 female volunteers at various times during 24 h are expressed as the percentage of 24-h mean activity of each individual. ---, Actual DBH activities of 4 cases are shown in terms of international unit (U, μmoles/min, per 1 serum). Experiments were started at 08.00 h.

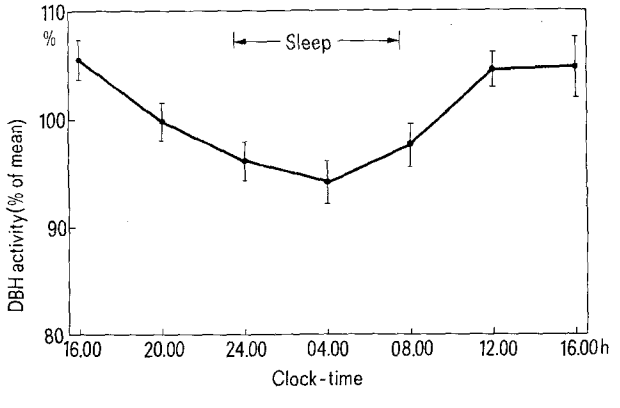

Fig. 2. DBH activities in human sera. Mean values (± SEM) of 13 male volunteers at various times during 24 h are expressed as the percentage of 24-h mean activity of each individual. Experiments were started at 16.00 h.