Reactivation of phosphorylated acetocholinesterase by pyridine-2-aldoxime methiodide

Pyridine-2-aldoxime methiodide (P-2-AM) is the most active reactivator, *in vitro*, of phosphorylated acetocholinesterase found so far. Recently it has also been shown that this oxime reactivates diethylphosphoryl-acetocholinesterase *in vivo* (KEWITZ[1]; HOBBIGER[2,3]; RUTLAND[4]) and that it acts as an antidote against poisoning by certain organophosphates, especially when used in combination with atropine (ASKEW, DAVIS AND GREEN[5]; KEWITZ AND WILSON[6]; KEWITZ, WILSON AND NACHMANSOHN[7]; HOBBIGER[2,3]). Since conditions *in vivo* and *in vitro* are not always identical, a more detailed study of the reactivation of phosphorylated acetocholinesterases by P-2-AM in concentrations which can be obtained *in vivo* was undertaken.

Samples of heparinised human blood were incubated for 15 min at 37° C with either 10^{-6} *M* tetraethyl pyrophosphate or 10^{-5} *M* di*iso*propyl fluorophosphonate. To remove the free organophosphates, which would interfere with the reactivation process (HOBBIGER[8]), the blood samples were then incubated for another 15 min with rabbit plasma (final concentration, 10%); the red cells were then separated from the plasma by centrifugation and washed 4 times with 0.9% NaCl. At least 90% of the acetocholinesterase in the red cell preparations obtained by this technique is present in a phosphorylated form which is enzymically inactive. The reactivation of the phosphorylated acetocholinesterase was studied in the Warburg apparatus at 37° C in a medium of 0.025 *M* $NaHCO_3$ and an atmosphere of 95% N_2 and 5% CO_2. Reactivation was allowed to proceed in the sidearm of the manometric vessels (fluid volume, 0.3 ml; red cell dilution, 1:9) and enzyme activity was determined by measuring the volume of CO_2 produced between 5 and 35 min after addition of the contents of the sidearm to the main compartment of the manometric vessel which held 2.7 ml acetylcholine chloride (final concentration, 0.01 *M*) in 0.025 *M* $NaHCO_3$.

Diethylphosphoryl-acetocholinesterase

The rate of reactivation of diethylphosphoryl-acetocholinesterase by P-2-AM is independent of the concentration of the phosphorylated enzyme and for a tenfold range of concentrations of the oxime the time required for 50% reactivation of the phosphorylated enzyme is inversely proportional to the concentration of the oxime (Fig. 1). This indicates that the rate determining step in the reactivation process is the rate of association of the oxime with the phosphorylated enzyme and any complex which might be formed between the oxime and the phosphorylated enzyme can only be of a transient nature. These findings might not be applicable to higher concentrations of the oxime (the effect of which cannot be studied with sufficient accuracy by the Warburg technique) or to experiments which are carried out at lower temperatures. Experiments in which enzyme activities were recorded at 2.5 min intervals, starting 2.5 min after addition of the contents of the sidearms to the main compartments of the manometric vessels, showed that enzyme reactivation is fully arrested when the oxime concentration falls below an effective level as the result of dilution.

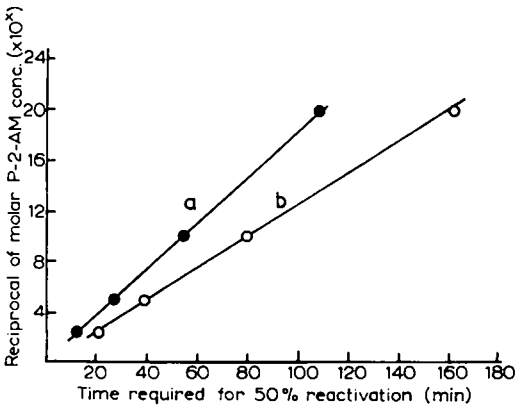

Fig. 1. x is 4 for diethylphosphoryl-acetocholinesterase (curve a) and 5 for di-*iso*propylphosphoryl-acetocholinesterase (curve b). Reactivation is expressed in % of the maximum reactivation obtained on incubation with $5 \cdot 10^{-3}$ *M* P-2-AM. The activity of all blood samples which were reactivated with $5 \cdot 10^{-3}$ *M* P-2-AM was corrected for inhibition by P-2-AM (on average 7%) and corrections were also applied for residual reactivation (resulting from insufficient dilution) during measurement of enzyme activity.

Reactivation by P-2-AM is not additive with the reactivation by choline (Table I) and we can, therefore, assume that P-2-AM approaches the phosphorylated enzyme via the anionic site of the latter; it is possible that interaction of the quaternary ammonium structure of the oxime with the anionic site promotes the activity of P-2-AM; this has been suggested by WILSON[9] for other reactivators which possess a cationic centre.

TABLE I

Medium for reactivation	Changes in the effectiveness of P-2-AM as a reactivator	
	Diethylphosphoryl-acetocholinesterase 1	Diisopropylphosphoryl-acetocholinesterase 2
0.025 M $NaHCO_3$	100	100
0.025 M $NaHCO_3$ + 0.1 M NaCl	63	87
0.025 M $NaHCO_3$ + 0.1 M KCl	59	80
0.025 M $NaHCO_3$ + 0.05 M $MgCl_2$	37	49
0.025 M $NaHCO_3$ + 0.1 M NaCl + 0.1 M KCl	48	70
0.025 M $NaHCO_3$ + 0.1 M NaCl + 0.1 M KCl + 0.05 M $MgCl_2$	26	44
0.025 M $NaHCO_3$ + 0.1 M choline chloride	80*	66
0.025 M $NaHCO_3$ + 0.1 M acetylcholine chloride	38	61

Reactivation obtained after 60 min incubation of P-2-AM with the phosphorylated enzyme was used as measure of the effect of different media on reactivation. Changes in the effectiveness of P-2-AM were calculated from the slope obtained by plotting the log. of the concn. of P-2-AM against % reactivation and all figures in columns 1 and 2 represent the effectiveness of P-2-AM in % of its effectiveness in 0.025 M $NaHCO_3$. Results given in columns 1 and 2 were obtained using $1.5 \cdot 10^{-5}$ M and $2 \cdot 10^{-4}$ M P-2-AM respectively.

* 0.1 M choline itself produces 50% reactivation in 90 min and if its effect were additive with that of P-2-AM a 1.45 fold increase in the potency of P-2-AM should be observed.

The rate of reactivation of the phosphorylated enzyme by a given concentration of P-2-AM is determined by the medium in which reactivation is carried out. NaCl, KCl and $MgCl_2$ reduce the effectiveness of P-2-AM (Table I). Comparative studies of the effects of P-2-AM on phosphorylated acetocholinesterases obtained from different species will only be useful if the experiments are carried out in the same medium. Since P-2-AM might possess complexing properties the reduction in the effectiveness of P-2-AM by Mg^{++} could at least be partly due to a reduction in the effective concentration of the oxime.

Acetylcholine, another possible source of interference *in vivo*, also reduces the effectiveness of P-2-AM (Table I). The results with NaCl, KCl and acetylcholine and previous findings with trimethylamine (HOBBIGER[8]) support the interpretation that P-2-AM interacts with the anionic site of the phosphorylated enzyme during reactivation. It is possible that NaCl, KCl, choline and acetylcholine also interfere to some extent with the reactivation process *in vivo*, but it will be difficult to assess this from the rates of reactivation obtained at different sites since the concentrations of these substances and ions in the vicinity of cholinesterase are unknown.

Ethanol which has previously been shown to reduce the effectiveness of the combined treatment with atropine and P-2-AM in organophosphate poisoning (HOBBIGER[3]) has no effect on the reactivation *in vitro* if used in concentrations up to 5% v/v.

Diisopropylphosphoryl-acetocholinesterase

The rate-determining step in the reactivation of diisopropylphosphoryl-acetocholinesterase by P-2-AM also appears to be the rate of association of the oxime with the phosphorylated enzyme (Fig. 1). NaCl, KCl, choline and acetylcholine retard the reactivation (Table I), but their effect is less marked than in the case of diethylphosphoryl-acetocholinesterase. If attachment of the oxime to the anionic site plays a part in the reactivation process it must, therefore, be less important than with diethylphosphoryl-acetocholinesterase.

It is interesting in this connection that WILSON, GINSBURG AND MEISLICH[10] who studied the reactivation of diethylphosphoryl- and diisopropylphosphoryl-acetocholinesterase by nicotinhydroxamic acid methiodide and its tertiary base, also concluded from their results that the anionic site is not functional in diisopropylphosphoryl-acetocholinesterase.

Department of Pharmacology, Middlesex Hospital Medical School, London (England) F. HOBBIGER

1 H. KEWITZ, *Arch. Biochem. Biophys.*, 66 (1957) 263.
2 F. HOBBIGER, *Biochem. J.*, 66 (1957) 7P.
3 F. HOBBIGER, *Brit. J. Pharmacol.*, (1957) in the press.

[4] J. P. RUTLAND, *Biochem. J.*, 66 (1957) 43P.
[5] B. M. ASKEW, D. R. DAVIES AND A. L. GREEN, *Biochem. J.*, 66 (1957) 43P.
[6] H. KEWITZ AND I. B. WILSON, *Arch. Biochem. Biophys.*, 60 (1956) 261.
[7] H. KEWITZ, I. B. WILSON AND D. NACHMANSOHN, *Arch. Biochem. Biophys.*, 64 (1956) 456.
[8] F. HOBBIGER, *Brit. J. Pharmacol.*, 11 (1956) 295.
[9] I. B. WILSON, *Discussions Faraday Soc.*, No. 20 (1955) 119.
[10] I. B. WILSON, S. GINSBURG AND E. K. MEISLICH, *J. Am. Chem. Soc.*, 77 (1955) 4286.

Received June 22nd, 1957

Models of α-keratin structure

In simple polymers it is generally accepted that the density predicted from a plausible model structure must be greater than the observed macroscopic density, since a certain proportion of non-crystalline material is always present. Despite their considerable molecular and histological complexity, it has long been assumed that the same criterion can be applied to α-keratins. If this assumption is correct, then the average volume, $\bar{v}$, occupied by an amino acid residue in a satisfactory model structure should be less than that calculated from the macroscopic density, d, and mean residue weight, $\bar{M}$, by means of the expression $\bar{v} = 1.66\ \bar{M}/d$, where $\bar{v}$ is expressed in A^3 and d in $g \cdot cm^{-3}$.

As the macroscopic density of native α-keratins increases with crystallinity[1], it is clear that the more crystalline the specimen for which $\bar{v}$ is determined the more stringent the density criterion becomes. The most crystalline α-keratin structures recognised so far are the quill tips of various porcupines, but the calculation of $\bar{v}$ for these structures has been hampered in the past by the lack of precise analytical or density data.

On the basis of X-ray diffraction studies, we have selected the descaled quill tip from the North American porcupine (*Erethizon* sp.) as the most suitable subject for measurement and have determined both its amino acid composition and density. The analysis was carried out by ion exchange chromatography on columns of Dowex 50-X8[2] using the ninhydrin reagent described by MOORE AND STEIN[3]. Proline was determined by the method of CHINARD[4] after preliminary ion exchange chromatography[5] and cystine was estimated by amperometric titration. The results of the analysis are presented in Table I. The density was determined by flotation in a mixture of *o*-dichlorobenzene and bromobenzene, after drying at 100° C *in vacuo* for 3 h. A value of 1.317 was obtained.

TABLE I

AMINO ACID COMPOSITION OF PORCUPINE QUILL TIPS (*Erethizon* sp.)

Amino acid	*No. of Observations*	*Nitrogen as % of total nitrogen*		*g amino acid from 100 g quill tips*
		Mean	*S.E.*	
Alanine	2	4.26	0.36	4.62
Amide	2	8.99	0.70	1.75
Arginine	2	18.82	0.42	9.97
Aspartic acid	3	5.30	0.29	8.58
Cystine	1	6.77	—	9.92
Glutamic acid	3	9.52	0.56	17.05
Glycine	3	6.72	0.24	6.14
Histidine	2	1.32	0.33	0.83
Isoleucine	3	2.34	0.24	3.74
Leucine	3	5.78	0.20	9.23
Lysine	2	4.09	0.15	3.64
Phenylalanine	3	2.10	0.12	4.22
Proline	2	4.10	0.02	5.74
Serine	3	6.53	0.12	8.35
Threonine	3	3.59	0.22	5.23
Tyrosine	2	3.07	0.00	6.76
Valine	2	4.11	0.07	5.86