A NONCHOLINESTERASE COMPONENT IN THE MOLECULAR MECHANISM OF ACTION OF THE CHOLINESTERASE REACTIVATOR DIPYROXIME

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UDC 615.917:547.241].015.4:616.153.93 + 616.153.96:615.917:547.241]-02:615.246.9

KEY WORDS: dimethyldichlorovinyl phosphate; dipyroxime; serum proteins; albumin; reversible complex formation.

There has recently been a steady rise in the number of papers published on reversible interaction between drugs and blood serum proteins. Toxic compounds have received much less study from this aspect. As regards binding of poisons with plasma proteins against the background of antidotes, practically no work has been done. Yet such research would be of undoubted interest.

The aim of this investigation was to study reversible binding of the organophosphorus poison dimethyldichlorovinyl phosphate (DDVP) and an antidote for this group of toxic compounds, the cholinesterase (ChE) reactivator dipyroxime, with serum proteins.

EXPERIMENTAL METHOD

Interaction of DDVP and dipyroxime with whole rat serum proteins and with a preparation of human serum albumin (HSA) (from Reanal, Hungary) was investigated by methods of equilibrium dialysis (ED), spectrofluorography, and polarography, as described by the writers previously [2-6]. Fluorescence probing of the surface layers of the HSA molecule with 1-anilinonaphthyl-8-sulfonate (ANS) also was carried out by the method in [3]. The results of ED were expressed on Scatchard plots [12] or as isotherms. Serum for ED was obtained from intact Wistar rats or after preliminary administration of sulfadimethoxine (500 mg/kg by the intragastric route). This substance was used as a compound capable of competing with other substances for binding with serum proteins [7]. Toxicity of DDVP was verified on animals of two similar groups. Calculations of the association contant (K_{assn}) and the number (N) of binding sites of DDVP with albumin, and also of mean lethal doses (LD_{50}) were done by computer.

EXPERIMENTAL RESULTS

The binding isotherms (Fig. 1) show a sufficiently high (71-73%) degree of reversible complex formation between DDVP and whole serum proteins. Practically no binding of dipyroxime was observed under these conditions. This phenomenon may be explained by the presence of a large number of polar groups in the molecule of this compound, for these groups are known [10] to inhibit reversible complex formation.

Meanwhile addition of DDVP and dipyroxime to the dialysis cell simultaneously caused a marked increase (up to 78-80%) in binding of the poison. Control experiments in which DDVP was incubated with the antidote in protein-free medium did not reveal any direct interaction between these compounds. Consequently, the increase in binding recorded by the ED method could only have been due to changes in interaction between DDVP and serum proteins taking place under the influence of dipyroxime.

According to established views [11], the intensity of biological action of a compound at any concrete moment is a function of its free concentration in the blood plasma only. Accordingly, reversible complex formation with serum proteins, for most cases, may be regarded

Kiev Research Institute of Pharmacology and Toxicology, Ministry of Health of the Ukrainian SSR. (Presented by Academician of the Academy of Medical Sciences of the USSR S. N. Golikov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 93, No. 6, pp. 66-68, June, 1982. Original article submitted November 6, 1981.

0007-4888/82/9306-0773$07.50

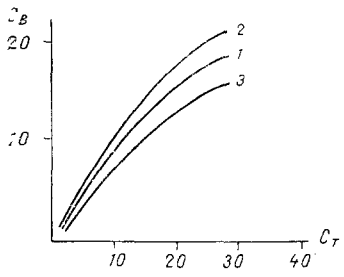

Fig. 1

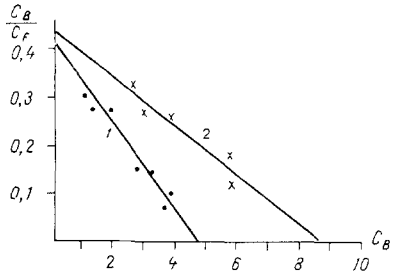

Fig. 2

Fig. 1. DDVP binding isotherms in whole serum from intact rats (1), in the same serum in the presence of equimolar concentrations of dipyroxime (2), and in serum of rats receiving sulfadimethoxine (3). C_T) Total concentration of DDVP in dialysis chamber ($\cdot 10^{-5}$ M); C_B) concentration of DDVP bound with proteins ($\cdot 10^{-5}$ M).

Fig. 2. Scatchard plots for determination of quantitative parameters of complex formation (K_{assn} and N) for DDVP with HSA (1) and for DDVP with HSA in the presence of dipyroxime (2). C_B) Concentration of DDVP bound with HSA ($\cdot 10^{-8}$ M). C_B/C_F) Ratio between concentrations of bound and free DDVP.

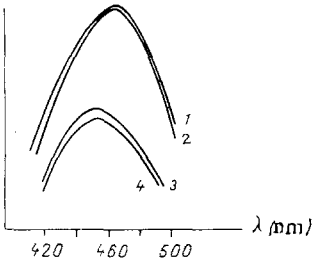

Fig. 3. Fluorescence spectra of ANS in complex with HSA in absence of test compounds (1), and in presence of DDVP (2), dipyroxime (3), and a combination of DDVP with dipyroxime (4). Concentration of HSA $1 \cdot 10^{-5}$ M, of ANS $5 \cdot 10^{-5}$ M, of DDVP and dipyroxime $16 \cdot 10^{-5}$ M.

as the factor limiting acute toxicity. Rare exceptions to this rule were encountered only in work with embichin [5]. The increase in binding of DDVP recorded in the presence of dipyroxime can therefore be interpreted as a positive effect from the point of view of antidote action.

Meanwhile the question arises how essential is the degree of increase of binding thus established. A simple calculation shows that the size of the active free fraction of poison is reduced under the influence of dipyroxime from 27-29 to 20-22% of the total concentration in the serum, i.e., is reduced on average by 1.27 times. There are good grounds for taking this value into account when predicting toxicity. However, direct experimental assessment of the contribution of this component is difficult because of predominance of the principal mechanism of action of the ChE reactivator in the intact organism.

Accordingly it was necessary to obtain evidence by an opposite approach. It was shown previously [7] that sulfonamides, with high affinity for serum proteins and, in particular, for albumin [8], can increase the toxicity of dinitrophenol poisons on account of their competitive displacement from reversible complexes. The same results were obtained in analogous experiments with DDVP. Preliminary injection of sulfadimethoxine increased the sensitivity of rats to the poison and significantly lowered LD_{50} from 71.3 ± 3.1 to 56.7 ± 4.8 mg/kg (by

the intragastric route). Binding of DDVP by the blood serum of rats receiving the sulfonamide was reduced in this case to 65-66% (Fig. 1). Consequently, changes in the degree of binding of the poison by between 5 and 8% are reflected in its toxic properties: A decrease in binding (in the case of sulfadimethoxine) increases toxicity whereas an increase in binding (as in the case of combination with dipyroxime) ought to reduce the acute toxic action of DDVP.

The molecular mechanism of the phenomenon described was revealed by experiments with serum albumin. The ED method (Fig. 2) showed that DDVP possesses an average degree of affinity for albumin ($K_{assn} = 0.85 \cdot 10^{-4}\ M^{-1}$). Each protein molecule has a single binding center capable of fixing only one ($N \approx 1.04$) DDVP molecule. In the presence of dipyroxime in an equimolar concentration the parameters of complex formation of DDVP with HSA were changed: a very small decrease of affinity ($K'_{assn} = 0.51 \cdot 10^{4} M^{-1}$) was accompanied by a significant increase in the value of N to 1.86. From the practical point of view this was evidence of doubling of the capacity of the binding center.

Physicochemical (spectrofluorometry, polarography) investigations showed that binding of DDVP was unaccompanied by any marked changes in the tertiary structure of HSA. Tryptophan fluorescence spectra of the protein were virtually unchanged. No significant effect on fluorescence of the probe (ANS) likewise could be found. This indicates that the superficial (in which ANS groups are located) and middle (in which the single tryptophanyl group [1] of albumin is located) layers of the protein molecule undergo little change in the process of complex formation. The relative increase in height of the first step and the decrease in height of the second step of the biphasic polarographic wave of HSA, according to existing views [4], must be explained by some condensation of the globular structure of the DDVP-binding protein and a change in the state of its disulfide bonds. The overwhelming majority of the latter are known [9] to be located in the central zones of the globule. The results now obtained suggest that the only molecule of DDVP which is bound with protein penetrates into the inner zones of the albumin globule through the unchanged surface layers.

Dipyroxime, which has been shown virtually not to form complexes with serum proteins, nevertheless exerts a definite influence on the conformation of albumin. This was most clearly demonstrated by quenching of ANS fluorescence (Fig. 3). This last fact indicates that the presence of dipyroxime in the medium leads to definite changes in the stearic properties of the superficial zones of the protein molecule. As a result of these changes, it must be assumed, penetration of DDVP molecules to the binding centers is facilitated, and this explains the increase in their capacity determined by the ED method.

The results indicate the existence of an additional mechanism of action of dipyroxime, unconnected with ChE reactivation, due to the ability of this compound to increase the binding capacity of albumin relative to the organophosphorus compound studied. There is no doubt that this mechanism plays a subsidiary role. However, it is interesting both from the theoretical point of view and also in connection with the search for and testing of new antidotes.

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