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Paraoxin degradation *in vitro*: A contribution to the methodology of assay procedures

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IN ORDER to select the proper methods for studying metabolism–toxicity relationships Lauwerys and Murphy[1] compared various procedures used in the past to investigate paraoxon inactivation *in vitro*. Their findings demonstrated that the results obtained with an anticholinesterase system (low substrate concentration) greatly differed from those obtained with a spectrophotometric and manometric assay (high substrate concentration).

The methodological approach followed in this laboratory was different from that mentioned above. Instead of comparing paraoxon degradation in non-identical enzymatic preparations with widely different substrate concentrations, a single incubation mixture was used from which subsamples were taken for anticholinesterase and spectrophotometric assays.

The spectrophotometric method was based on the direct measurement of *p*-nitrophenol produced in an incubation mixture which contained buffered (Soerensen phosphate buffer, pH 8·0, 0·07 M) solutions of human, rabbit and rat blood plasma, and 7×10^{-5}M paraoxon. The incubation temperature was 37°. Colour formation was followed at intervals of 1 or 2 min using a Klett–Summerson photoelectric colorimeter equipped with a 420 nm filter.

The principle and the possibilities of our automated cholinesterase inhibition method, applied to a wide variety of analytical and biochemical problems associated with organophosphates and carbamates, have been described in detail.[2,3] Recently a technical modification was developed[4] which also served as the basis of the present studies on paraoxon degradation. At appropriate time intervals a subsample is removed from the incubation mixture at 37° by means of a microsyringe and immediately injected into a glass chamber downstream from the proportioning pump. Since the volume of the cholinesterase solution flowing through the injection chamber is constant the accuracy of the dilution depends only on an error due to manipulating the microsyringe. The inhibition peaks recorded were compared with those obtained from known paraoxon standards. As outlined earlier[2] it is possible to cover a wide range of inhibitor concentrations by using a highly sensitive or less sensitive source of cholinesterase and different types of inhibition (simultaneous addition of inhibitor and substrate, pre-inhibition).

The results presented in Fig. 1 demonstrate that both methods, the spectrophotometric determination of *p*-nitrophenol and the automated cholinesterase inhibition technique gave identical curves and are thus equivalent tools for determining paraoxon degradation *in vitro* at relatively high substrate concentrations (7×10^{-5} M).

Investigations at low substrate concentrations require the use of a sensitive anticholinesterase method. Lauwerys and Murphy[1] observed that under such conditions the inactivation of paraoxon "did not exhibit the time response relationship expected for an enzyme catalyzed reaction". They incubated rat plasma

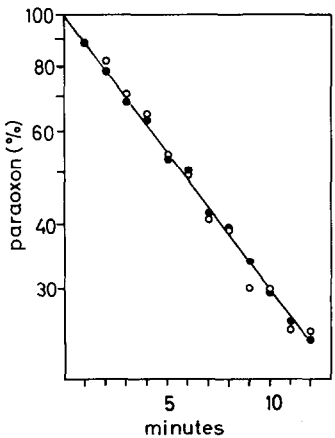

FIG. 1. Rate of degradation of paraoxon as measured by *p*-nitrophenol formation (closed circles), and decrease of anticholinesterase activity (open circles). Incubation mixture: rabbit plasma, 20-fold diluted with Soerensen phosphate buffer pH 8·0, 7×10^{-5} M paraoxon (= 100%), 37°. Human plasma cholinesterase was used as enzyme source for the anticholinesterase test.

(100-fold diluted) with 10^{-7} M paraoxon and found that a certain fraction of the compound was inactivated immediately after addition followed by almost no further degradation. This finding was confirmed in our own studies when incubating rat plasma (20-fold diluted) with 3×10^{-7} M paraoxon. A less diluted sample of rat plasma (8-fold) inactivated the entire amount of paraoxon (3×10^{-7} M) within one minute after addition to the incubation mixture (Fig. 2, left part). After refortification with 3×10^{-7} M paraoxon a further portion of the organophosphate disappeared rapidly, but then the remaining substrate was hydrolyzed according to first order reaction kinetics (Fig. 2, middle part). This same rate of degradation was also observed after adding a third portion of paraoxon (Fig. 2, right part). The initial velocity of approximately 0·08 nmole ml plasma^{-1} min^{-1} was calculated from these experiments which is close to the value of 0·1 nmole/g of liver tissue/min obtained by Lauwerys and Murphy. These authors applied the kinetic findings of their experiments with high paraoxon concentrations (spectrophotometric assay) to the conditions of the anticholinesterase test.

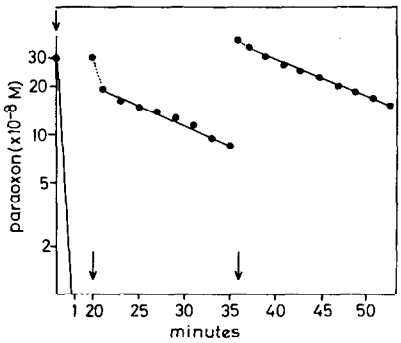

FIG. 2. Degradation of paraoxon (3×10^{-7} M initial concentration) by rat plasma (8-fold diluted with phosphate buffer pH 8·0, 37°). Arrows indicate time at which paraoxon was added to the plasma solution. Paraoxon was determined by an automated cholinesterase inhibition method using peacock plasma as a source for a particularly sensitive cholinesterase.

The major conclusions which can be drawn from the experiments described in this communication are the following: (1) Principally the anticholinesterase assay is as reliable as any other analytical procedure. The great advantage of the enzymatic method is its applicability to a very wide range of substrate concentrations. (2) At low substrate concentrations it is possible that nonspecific mechanisms, such as binding to proteins and/or inhibition of esterases[1] can contribute to the disappearance of the substrate from the test solution. However, as soon as the saturation point is reached, the rate of degradation due to enzymatic cleavage can be measured in exactly the same way as with high substrate concentrations. Nonspecific binding reactions do certainly occur also at high substrate concentrations, but they cannot be measured in such tests because of the large amount of excess substrate. (3) The nonspecific mechanism contributing to paraoxon inactivation in preparations of rat liver and rat plasma was not detected in solutions of rabbit plasma incubated with the same concentration of paraoxon (3×10^{-7} M). With rabbit plasma the anticholinesterase assay measured a first order reaction degradation from the beginning and no binding or other unspecific reaction were observed. Therefore, any generalization regarding the unreliability of the enzymatic method is not justified. (4) The author agrees with Lauwerys and Murphy that "after administration of a maximum tolerated dose the concentration of paraoxon *in vivo* is closer to the substrate concentration used in the anticholinesterase assay than to that used in the spectrophotometric technique". This indicates that further research in this area should consider the reactions and kinetic parameters prevailing at low substrate concentrations. The cholinesterase inhibition method provides a useful methodological tool for such studies.

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