METHODS TO ESTIMATE THE TURNOVER NUMBER OF PREPARATIONS OF OX RED CELL CHOLINESTERASE

by

J. A. COHEN AND MIA G. P. J. WARRINGA

Medical Biological Institute of the National Defence Research Council T.N.O., Leyden (Netherlands)

INTRODUCTION

Acetylcholinesterase (true cholinesterase) has been indicated as the enzyme, which is normally responsible for the breakdown of acetylcholine in the organism.

It is generally believed that in this capacity it is essential for nervous function. It is obvious that investigations aiming at establishing the exact role played by the enzyme in nervous function should be based on a thorough knowledge of the kinetics of this enzyme. These enzyme kinetics involve primarily means of assessing the enzyme concentration or rather the concentration of the active centres, occurring in the preparations studied. When this concentration is known, a value for the turnover number (t.o.n.) can usually be given expressing in the case of cholinesterase the number of molecules of acetylcholine hydrolysed under the experimental conditions per active centre per minute. Because our primary interest lay in the significance of the enzyme in mammalian nervous function, it was considered appropriate to choose mammalian tissue as a source of true cholinesterase; ox red cells served this purpose.

No reliable data are available on the t.o.n. of mammalian true cholinesterase. This is due to the difficulty of assessing this value in even the best preparations described, because, in spite of manyfold purification, these still possess only a limited purity.

The best method for the estimation of the concentration of enzyme active centres (and consequently of t.o.n.) is that described by JANSEN *et al.*[1] for chymotrypsin. The method involves the coupling of radioactive DF^{32}P (di*iso*propylfluorophosphonate labelled with ^{32}P) to the enzyme active centres of crystalline chymotrypsin. DF^{32}P combines irreversibly with the enzyme and, assuming a combination of one molecule of DFP per active centre, measurement of the radioactivity of the combination product should enable the calculation of the number of active centres combining with DFP; this value can then be related to the loss of original enzyme activity due to the combination. From these data the initial concentration of enzyme active centres and the t.o.n. may be computed. This method can only be applied to enzyme preparations of a purity which has not been achieved for most esterases known, including mammalian true cholinesterase. This is due to the fact that these preparations of esterase contain non-enzymic groups (impurities and perhaps fractions of the esterase molecule itself), which also combine with DFP. The combination product, therefore, contains more DFP than enzyme-active groups since groups other than the ones associated with enzymic activity are labelled.

References p. 58.

The present paper presents two methods (henceforth to be referred to as method A and B) that have been employed successfully to overcome these difficulties and that enabled us to arrive at reliable figures for molar concentrations of active centres and for the t.o.n. in crude and partly purified preparations of ox red cell cholinesterase.

The methods will be described in some detail because it is believed that the principle involved will prove generally applicable in the determination of the molar concentration of enzyme-active centres and t.o.n.'s in studies on enzyme kinetics where preparations of only limited purity are available. This will be elaborated in the discussion.

METHODS AND MATERIALS

1. *Cholinesterase assay*

The cholinesterase activity was assayed manometrically according to AMMON's method[2] applied as follows: 0.25 ml of the solution to be assayed is brought in the reaction vessel of a Warburg manometer and 2.5 ml of Krebs bicarbonate Ringer solution are added. 5 mg acetylcholine chloride dissolved in 0.25 ml of the medium are placed in the side arm. The atmosphere consisted of N_2 95% and CO_2 5%. The temperature of the bath was kept at 37° C. The reaction was started by tipping the substrate into the main vessel after equilibration for 7 minutes.

Activity is expressed in units *i.e.* the number of μl CO_2 released per hour under the experimental conditions. The purity of the enzyme preparations is expressed as the number of units per mg nitrogen. Nitrogen was estimated according to DEKKER[3]. (Where ammonium sulfate was present, this was first removed by thoroughly washing the trichloroacetic acid precipitate.)

2. *Enzyme preparations*

Initial experiments were carried out on a commercial ox red cell preparation (WINTHROP[4]). Most experiments were performed using a preparation purified, according to a method previously described[5], from ox red cells (butanol extraction method).

3. *Synthesis of DF^{32}P*

DF^{32}P of a specific activity of 40 μ C/mg DF^{32}P was prepared from $H_3{}^{32}PO_4$, supplied by the A.E.R.E. at Harwell, according to a method to be described elsewhere.

4. *Radioactivity measurements*

The radioactivity was estimated in a G.M. liquid counter against a standard of known DFP and ^{32}P content. The standard was prepared by alkaline hydrolysis of a known quantity of DF^{32}P in the presence of excess carrier.

EXPERIMENTAL RESULTS

The methods to be described are based on the use of butyrylcholine as a means of differentiating between enzyme-active groups combining with DFP and those groups (aspecific groups) that combine with DFP without possessing enzyme activity. Butyrylcholine, for example, combines with enzyme-active groups and by so doing protects them against inhibition by DFP[6]; consequent dialysis dissolves the enzyme-butyrylcholine combination and removes butyrylcholine and uncombined DFP from the solution, which is demonstrated by a complete return of the original enzyme activity. Assuming now that butyrylcholine does not equally protect against combination of DFP with non-enzymic aspecific sites, an assumption which will be proved to be correct in this paper, all DFP found on the dialyzed preparation must be combined with aspecific groups. This principle has been applied in two methods (A and B) which will be described in detail.

References p. 58.

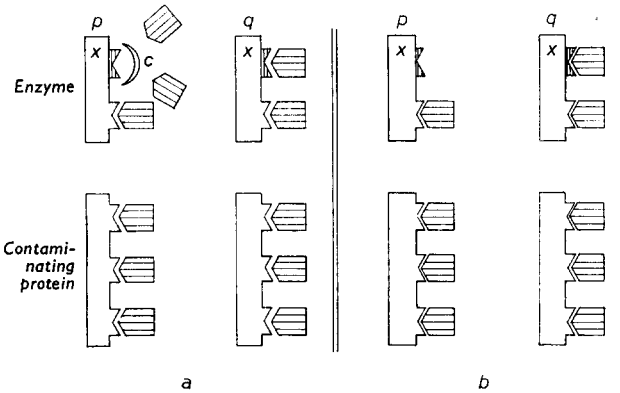

Fig. 1. Diagram of method A. *a* before dialysis, *b* after dialysis. *p* and *q* correspond to the *p*- en *q*-preparations described in the text. The drawings on the left represent receptor groups capable of reacting with DFP on the enzyme molecule (including one enzyme active (x) and one aspecific group) and on contaminating protein. $DF^{32}P$ is represented by the wedge-shaped structures to the right of the receptor groups. The crescent (c) represents a molecule of protective butyrylcholine.

Method A (Fig. 1)

10 ml of an enzyme preparation of known activity (preparation *p*) are incubated for 10 minutes with butyrylcholine (0.2 *M*) at 26° C. Under these conditions complete protection of the enzyme against combination with DFP occurs[6]. $DF^{32}P$ in a final concentration of $3\text{–}5 \cdot 10^{-10}$ *M* (giving a 40–90% inhibition) is then added and the incubation is continued for a further 40 minutes. After 18–20 hours dialysis the enzyme activity is assayed to make sure that the original activity is recovered; dialysis is then continued for six days in the cold room under continuous stirring against 1% NaCl in distilled water.

10 ml of the same enzyme preparation (preparation *q*) are treated in an identical fashion except for the omission of the butyrylcholine treatment. After dialysis both preparations are tested for radioactivity in the liquid counter.

The t.o.n. may be calculated as follows:

$$A = \frac{(q_1 - p_1)\, E}{S \times M}$$

where A = number of μmol DFP bound to enzyme-active groups
= number of μmol active groups that have reacted with DFP, assuming that per active group one molecule of DFP is bound.
S = number of counts per minute of standard preparation
E = μg DFP in standard preparation
p_1 = counts per minute in preparation *p*
q_1 = counts per minute in preparation *q*
M = molecular weight of DFP

$$B = \frac{a - b}{60} \times \frac{1}{22.4}$$

where B = total original enzyme activity expressed in μmol acetylcholine hydrolysed per minute by the number of enzyme groups corresponding with A.
a = activity in μl CO_2 per hour of preparation *p*
b = activity in μl CO_2 per hour of preparation *q*

References p. 58.

The t.o.n., *i.e.* the number of molecules acetylcholine hydrolysed per minute per active group, is given by the relation B/A. Preliminary experiments using this method gave promising results for the commercial Winthrop preparation. No definite figures, however, can be given because, by the time technical difficulties were conquered, the preparation was no longer obtainable and attempts to carry out the purification in our own laboratory following the directions of the relevant patent[4] failed. Furthermore the method was not applicable to cholinesterase prepared by the butanol method because this preparation contains many aspecific DFP combining groups. Therefore the figure for the difference in radioactivity between the preparations p and q ($q_1 - p_1$), on which the calculation of the t.o.n. is based, will be small compared with p_1 and q_1 and consequently too inaccurate to be useful.

However, such few results as were obtained with the Winthrop preparation using method A agree satisfactorily with those obtained with method B for the butanol preparation.

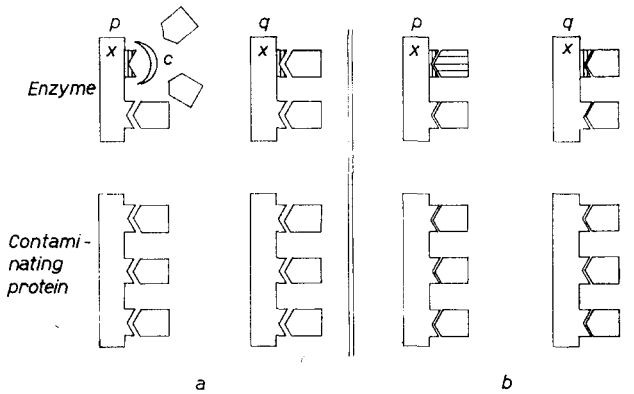

Fig. 2. Diagram of method B. Explanation as for Fig. 1. The striped wedge-shaped figure represents labelled $DF^{32}P$, the open ones unlabelled DFP.

Method B (Fig. 2)

10 ml of enzyme preparation of known activity (preparation p) are incubated during 10 minutes with butyrylcholine (0.2 M) at 26° C. Non-radioactive DFP in a final concentration of $6.4 \cdot 10^{-9}$ M is now added and the incubation is continued for a further 40 minutes. All non-enzyme-active (aspecific) groups with affinity for DFP are thus occupied. The DFP concentration used is several times the one necessary to completely inhibit the enzyme in the absence of butyrylcholine. Dialysis under the usual conditions for 18 hours is followed by spinning for 6 minutes at 2000 r.p.m. to remove small particles formed during dialysis. The remaining enzyme activity is estimated and incubation follows in the presence of radioactive $DF^{32}P$ ($4.7 \cdot 10^{-10}$ M) for 40 minutes.

Enzyme activity is again assayed and is usually found to have completely disappeared at the concentration of $DF^{32}P$ used. After 6 days dialysis with two changes of the 1% NaCl, the radioactivity is measured in the liquid counter and compared with that of a standard. 10 ml of the same enzyme preparation (preparation q) are identically treated except for the omission of the incubation with butyrylcholine. No enzyme activity tests are necessary under these circumstances because in addition to the aspecific groups all enzyme-active groups are occupied in the absence of butyrylcholine,

References p. 58.

Calculation of the t.o.n. is as follows:

$$A = \frac{(p_1 - q_1)\, E}{S \times M}$$

A expresses the number of μmol enzyme-active groups that have reacted with $DF^{32}P$. The original enzyme activity of these same groups before this combination (B) may be calculated from the equation

$$B = \frac{a - b}{60} \times \frac{1}{22.4}$$

where a = activity in μl CO_2 per hour of preparation p after 18 hours dialysis but before incubation with $DF^{32}P$.

b = activity in μl CO_2 per hour of preparation p after 18 hours dialysis but after incubation with $DF^{32}P$.

t.o.n. may be calculated from the equation: t.o.n. $= B/A$.

The t.o.n.'s obtained with method B using butanol preparations are given in Table I. The values represent experiments carried out at different times on cholinesterase preparations obtained from different animals; nevertheless the agreement in t.o.n. is striking.

TABLE I

prep. no.	*t.o.n.*	*Units Activity per mg N.*
1	$3.10 \cdot 10^5$	14,500
2	$2.55 \cdot 10^5$	29,000
3	$2.87 \cdot 10^5$	17,500
4	$2.98 \cdot 10^5$	14,900
5	$2.68 \cdot 10^5$	19,500
6	$3.51 \cdot 10^5$	18,200
7	$2.96 \cdot 10^5$	50,000
mean	$2.95 \cdot 10^5 \pm 0.128^* \cdot 10^5$	23,370

* S.E. of mean

The methods (A and B) are based on the assumption that butyrylcholine affords no protection against combination of aspecific groups with DFP. Support for this assumption was derived from two types of experiments.

a. Initial stages of a cholinesterase purification were used for a t.o.n. determination by method B. These preparations were of poor purity containing, respectively, 4,096 and 14,000 units of cholinesterase activity per mg nitrogen. Although these preparations were approximately 5.5 and 1.7 times less pure than the average end product (see Table I) t.o.n.'s of 274,000 and 255,000 respectively were found, that is, values which are in complete agreement with those found for the purest preparations of Table I.

If butyrylcholine had protected aspecific groups, its effect would have been the more obvious the lower the purity of the preparation used. More $DF^{32}P$ would have been found combined per unit enzyme activity for impure preparations than for purer ones, leading to an apparent decrease in t.o.n. with decreasing purity. The fact that such a relationship could not be established proves that butyrylcholine only protects enzyme active and not aspecific DFP-combining groups.

References p. 58.

b. Two crude enzyme extracts were treated according to method A with DFP of very low specific radioactivity. At the end of the experiment the activity of preparation *p* stood at 987 and that of *q* at 945 counts per minute.

This means that no significant difference exists between the two preparations as to their DFP-combining power. In this experiment the difference in radioactivity to be expected on account of enzyme group protection was calculated to be only 7 counts and therefore totally negligible. The DFP combined, therefore, corresponds with aspecific groups and the absence of a difference clearly excludes the possibility of protection of aspecific groups by butyrylcholine.

DISCUSSION

Methods are described that enabled us to establish the molar concentration of enzyme active groups and the t.o.n. of purified preparations of ox red cell cholinesterase. The t.o.n. (expressed as number of molecules of acetylcholine hydrolysed per active centre) found was $2.95\ (\pm\ 0.128)\cdot 10^5$. The results were essentially obtained by the method B using cholinesterase prepared by the butanol extraction method. On this preparation the alternative method A cannot be used because it contains too many aspecific non-enzyme-active groups reacting with DFP. However preliminary results with a preparation of higher purity (WINTHROP) indicate that this method will also be applicable under many circumstances and that the results will agree satisfactorily with those obtained by the method B. Both methods depend on the assumption that butyrylcholine does not protect aspecific groups against the attack by DFP. The validity of this hypothesis could be certified experimentally. The fact that identical values for t.o.n.'s could be obtained from preparations of widely different purity supports our view that this same figure would also have been arrived at when much purer preparations had been available; it is considered to provide further evidence for the validity of the figure given.

In the literature no useful data are available on the t.o.n. of red cell cholinesterase. BERRY[7] working with the irreversible inhibitors dicyclohexylfluorophosphonate and diethyl*para*nitrophenylphosphate failed to arrive at reliable results, owing to lack of specificity of these inhibitors. This lack of specificity accounted for his widely varying figures for different species (10,000–171,000) and even within a species.

Other values mentioned in the literature refer to highly purified electric eel cholinesterase. NACHMANSOHN[8] arrived at a figure of 670,000, while MICHEL AND KROP[9] report a turnover of 420,000 for the same preparation. In a personal communication MYERS reported to us that, using reversible inhibitors and human red cells, he found a turnover number of 300,000, which of course entirely agrees with the one found by us for ox red cell cholinesterase. Starting from our t.o.n. of 295,000, a figure of $3.66\cdot 10^{12}$ for the number of active centres per ml ox blood and of 520 for that per single red cell could be computed. This would mean that one red cell contains at the most 520 molecules of cholinesterase. For electric eel cholinesterase NACHMANSOHN[8] evaluated the number of active centres per molecule enzyme at 30, while MICHEL AND KROP[9] arrived at a figure of 48. If these figures would be transferable to red cell cholinesterase this would mean that no more than 10 to 20 molecules of cholinesterase would be present on the surface of a red cell.

The methods have so far only been worked out for cholinesterase. It seems not

References p. 58.

unreasonable to suppose that they possess a much wider range of applicability for the determination of the concentration of active centres and for the t.o.n. of more or less purified enzyme preparations. The methods require the availability of an irreversible enzyme inhibitor which does not have to be very specific but which can be labelled in some way. Further a reversible protecting agent will be necessary; usually the enzyme substrate(s) are capable of serving this purpose. It is felt that in many cases these requirements can be easily fulfilled.

SUMMARY

1. Two methods are described to assess molar concentration of active enzyme groups and turnover numbers for preparations of ox red cell cholinesterase of varying purity.

2. The first method (method A) requires a higher degree of purity of the enzyme preparation to be investigated than the second one (method B), which is still of value at a very low degree of purity.

3. The turnover number for ox red cell cholinesterase could be established at $2.95 (\pm 0.128) \cdot 10^5$.

4. The concentration of enzyme-active centres associated with red cells could be calculated at $3.66 \cdot 10^{12}$ per ml ox blood, the number of active groups per red cell at 520.

RÉSUMÉ

1. Les auteurs décrivent deux méthodes permettant de déterminer la concentration molaire de groupes enzymatiques actifs et les "turnover numbers" de diverses préparations de cholinestérase de globules rouges de bœuf de pureté variable.

2. La première méthode (méthode A) demande, pour la préparation d'enzyme à étudier, un degré de pureté plus élevée que la seconde (méthode B); cette dernière a de la valeur même pour des préparations de pureté médiocre.

3. Les auteurs ont trouvé, pour le turnover number de cholinestérase de globules rouges de bœuf, la valeur de $2.95 (\pm 0.128) \cdot 10^5$.

4. Pour la concentration des centres enzymatiques actifs dans les globules rouges ils ont calculé la valeur de $3.66 \cdot 10^{12}$ par ml de sang de boeuf et pour le nombre de groupes actifs par globule rouge 520.

ZUSAMMENFASSUNG

1. Es werden 2 Methoden beschrieben um die molare Konzentration aktiver Enzymgruppen und die "turnover" Nummern von verschieden reinen Präparaten von Cholinesterase aus den roten Blutkörperchen von Ochsen festzustellen.

2. Die erste Methode (Methode A) erfordert einen höheren Reinheitsgrad bei den untersuchten Enzympräparaten als die zweite (Methode B), die selbst bei einem sehr niedrigen Reinheitsgrad noch von Wert ist.

3. Es konnte eine "turnover" Nummer von $2.95 (\pm 0.128) \cdot 10^5$ für die Cholinesterase aus roten Blutkörperchen von Ochsen festgestellt werden.

4. Die Konzentration der mit den roten Blutkörperchen verbundenen aktiven Enzymzentren konnte zu $3.66 \cdot 10^{12}$ pro ml Ochsenblut, die Zahl der aktiven Gruppen pro Zelle zu 520 berechnet werden.

REFERENCES

1 E. F. JANSEN, M. D. F. NUTTING, R. JANG AND A. K. BALLS, *J. Biol. Chem.*, 179 (1949) 189.
2 R. AMMON, *Pflüger's Arch. f.d. Ges. Physiol.*, 233 (1930) 486.
3 W. A. L. DEKKER, *Klinisch Chemisch Onderzoek*, Leiden, 1940, page 195.
4 A. LESUK, U.S. Patent 2,475,793 (1949).
5 J. A. COHEN AND MIA G. P. J. WARRINGA, *Biochim. Biophys. Acta*, 10 (1953) 195.
6 J. A. COHEN, MIA G. P. J. WARRINGA AND B. R. BOVENS, *Biochim. Biophys. Acta*, 6 (1951) 469.
7 W. K. BERRY, *Biochem. J.*, 49 (1951) 615.
8 M. A. ROTHENBERG AND D. NACHMANSOHN, *J. Biol. Chem.*, 168 (1947) 223.
9 H. O. MICHEL AND S. KROP, *J. Biol. Chem.*, 190 (1951) 119.

Received November 28th, 1952