isolated from the udder fat fell progressively with increasing chain length up to stearic and oleic acids. The short-chain acids could therefore not have been formed directly by degradation of long-chain acids.

4. The mean specific activities of fatty acid fractions isolated from udder fat from the 'bicarbonate' half were very low, indicating negligible incorporation of $^{14}\text{CO}_2$. The deuterium content of these fractions was also low, probably due to dilution of newly formed fat.

5. Cholesterol isolated from milk and udder fat from the 'acetate' half was radioactive, the specific activity of the udder cholesterol being higher than that from the milk. This confirms previous work on

the goat indicating that cholesterol is synthesized in the udder.

6. Lactose isolated from milk from the 'acetate' half was appreciably radioactive, while milk lactose from the 'bicarbonate' half was not. The udder tissue must therefore incorporate acetate carbon into lactose by some mechanism other than carbon dioxide fixation.

7. The application of the udder perfusion technique to biochemical studies of milk formation is discussed in the light of the results of this experiment, and it is concluded that the perfusion conditions must be carefully controlled if valid comparisons are to be made with the lactating udder *in vivo*.

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The Turnover Number of Cholinesterase

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(Received 21 March 1951)

Many problems in cholinesterase kinetics could be greatly simplified by a knowledge of the concentration of enzyme in the system being studied. It would, for instance, be of value to know the concentration of enzyme in various tissues, the blood in particular.

The concentration of enzyme in any system can be calculated from the activity if the turnover number (i.e. the number of molecules of substrate transformed in unit time/mol. of enzyme) is known. The turnover number (T) cannot be determined unless the enzyme is obtainable in a high degree of purity. Cholinesterase is not easily purified; in fact no pure preparation has been isolated from mammalian sources.

Easson & Stedman (1936), in discussing the fact

that certain drugs, such as physostigmine, inhibit cholinesterase in very minute amounts, assumed that 'one molecule of drug will inhibit the activity of one unit or active centre of enzyme', and based their calculation of active-centre concentration on this assumption, together with the demonstration that these drugs behaved non-competitively. They found that horse serum cholinesterase had a T of 1490 mol. of acetylcholine/sec./active centre.

The theory of inhibition by means of a bimolecular reaction between active centre and inhibitor was furthered by Straus & Goldstein (1943), but the validity of the turnover number found, 450 mol./sec., was later denied by Goldstein (1944), since physostigmine behaved competitively. Augustinsson & Nachmansohn (1949) confirmed Goldstein's

observation that the inhibitors in question are competitive, although with a comparatively low dissociation constant.

The discovery of certain irreversible inhibitors of the phosphate ester type has enabled a fresh attack to be made on the problem. These inhibitors appear to be highly specific for cholinesterase (Mackworth & Webb, 1948; Mazur, 1946; Mazur & Bodansky, 1946). There is strong evidence that they react bimolecularly with enzyme active centres (Bournsnel & Webb, 1949; Jansen, Nutting & Balls, 1948, 1949). If the assumptions are made that these inhibitors are in effect specific reagents for cholinesterase, and that an irreversible bimolecular reaction occurs, they may be used to titrate the active centres by means of an appropriate technique.

THEORETICAL

Determination of T

In this paper the turnover number will be given simply as a number, the number of molecules of acetylcholine hydrolysed/min./active centre at 38° and pH between 7.2 and 7.4 in the presence of Krebs-Henseleit saline. The acetylcholine concentrations are, for acetylcholinesterases, 3×10^{-3} M; for butyrylcholinesterases, 6×10^{-2} M. The types of enzyme will be designated as AChE and BChE respectively.

The unit of enzyme activity is 1 μ l. carbon dioxide/30 min. A preparation of activity a units therefore hydrolyses $1.49a \times 10^{-9}$ mol. monobasic ester/min. Assuming that 1 mol. inhibitor reacts with 1 mol. active centre, then on completion of the reaction, loss of activity L is equivalent to the quantity of inhibitor added. Therefore, if I is the molar concentration of inhibitor and v the volume in ml. of the system, L is equivalent to $Iv \times 10^{-3}$ mol. of enzyme. This quantity of enzyme hydrolyses $L \times 1.49 \times 10^{-9}$ mol. of ester/min. Therefore 1 mol. active centre hydrolyses

$$\frac{1.49L \times 10^{-6}}{Iv} \text{ mol. of ester/min.} = T \text{ (turnover no.).} \quad (1)$$

Experimental results can be expressed as the ratio of L to I such that $b = L/I$. Substituting in equation (1) this becomes

$$T = \frac{1.49b \times 10^{-6}}{v}.$$

The calculation of b and its error is particularly simple, since on hypothesis the slope passes through the origin, and the complication of calculating the regression about an origin whose coordinates are the mean values of L and I is not necessary (Snedecor, 1946).

Determination of the active centre concentration

Once T is known, the active centre concentration may be calculated from the activity.

Let m be the molar concentration of active centre. The system of volume v thus contains $mv \times 10^{-3}$ mol. As before, the activity is $1.49a \times 10^{-9}$ mol. ester/min. 1 mol. active centre hydrolyses T mol. ester/min. Therefore system hydrolyses $mvT \times 10^{-3}$ mol. ester/min.

Therefore

$$mvT \times 10^{-3} = 1.49a \times 10^{-9},$$

whence

$$m = \frac{1.49a \times 10^{-6}}{vT}. \quad (2)$$

If the substrate concentration used is not that at which T was determined, the activity may be adjusted by means of a standard activity-substrate concentration curve.

This treatment involves the assumption, in addition to those made, that the inhibitor is not involved in side reactions such as spontaneous hydrolysis, or hydrolysis by an enzyme as described by Mazur (1946). The inhibitor dicyclohexyl fluorophosphonate (DCFP) is extremely resistant to spontaneous hydrolysis (Chapman & Saunders, 1948). It has, therefore, been used to determine the turnover number of the cholinesterase in the erythrocytes and plasma of various species.

EXPERIMENTAL

Preparation of enzymes from blood

Blood was collected in tubes containing solid oxalate. Plasma was separated by centrifuging, and the cells were washed twice with isotonic saline. Plasma or cells were diluted to the extent required to give activities of about 100–150 μ l. CO₂/30 min., usually with distilled water, but for some experiments with Krebs-Henseleit saline.

Purification was effected by one of two methods. In the first, due to Davies & Smellie (1949), the cells were lysed, the stroma precipitated at pH about 6, and washed with distilled water and phosphate buffer till the liquor was colourless. The precipitate was then dispersed at pH 8 in Krebs-Henseleit saline. The purification was about 12-fold, and the separation of aliesterase (measured with tributyrin) poor. In the second method, the stroma, purified in a similar manner, was further purified by treatment with kieselguhr. For details see Moulder & Whittaker (1950).

The final suspension was in Krebs-Henseleit saline. The purification was about 30-fold, and aliesterase was completely removed.

Horse serum was purified by the first stage of Strelitz's (1944) method.

Estimation of enzyme activity and effect of inhibitors

Enzyme activities were measured in the Warburg apparatus, and calculated from four readings (Aldridge, Berry & Davies, 1949). In the case of acetylcholinesterase the ACh concentration was 3×10^{-3} M. Since at this concentration gas output is not linear after 15 min. from the time of mixing enzyme and substrate, readings were taken 5, 8, 11 and 14 min. after mixing. In the case of butyrylcholinesterases the ACh concentration was 6×10^{-2} M. Linear out-

puts were seldom observed, but the departure from linearity was slight and was ignored. Readings were taken at 10 min. intervals.

Inhibition was measured as follows: 0.4 ml. of enzyme preparation and 0.1 ml. of either distilled water or inhibitor in distilled water were placed in one side arm of a double side arm Warburg flask. In the other side arm was 0.5 ml. acetylcholine chloride in distilled water, and in the main compartment 2 ml. of Krebs-Henseleit saline. The flasks were gassed with 5% CO₂ in N₂ at 38° and incubated for 1 hr., preliminary trial having shown that no further inhibition occurred after this time, provided the extent of inhibition was not greater than 30–50%. A plot of loss of activity against concentration of inhibitor was in most cases linear, until such a degree of inhibition was reached, after which the loss of activity was less than expected from the higher concentrations. This is explicable in that the time of 1 hr. was insufficient to allow the higher concentrations of inhibitor to react to completion. In calculating *T* only the linear portion of the curve was used. At the end of the hour, both side arms were tipped, and readings taken. The reasonable assumption has been made that DCFP behaves like DFP with which, as Aldridge (1950) has shown, there is no reversal of inhibition on dilution. This procedure is therefore a legitimate device for speeding up the rate of inhibition.

The concentration of inhibitor reported is one-sixth that actually used in the side arm, because the enzyme solution was diluted to that extent in order to measure its activity. It is obvious that inhibitor concentration must also be referred to the entire volume in which the activity was measured.

Deviations from this procedure were required in two instances. (1) In order to check the possible deterioration on storage of our sample of DCFP, it was compared with a fresh sample of diethyl *p*-nitrophenyl phosphate (E600). Using the above technique, E600 inhibited to a far lesser extent. A consideration of the kinetics of the reaction, using the rate constant given by Aldridge (1950), showed that impossibly long incubation times, at least 15 hr., would be required. The enzyme preparations were not stable at 38° for more than 3 or 4 hr. Accordingly the enzyme and inhibitor were both concentrated tenfold, incubated for 2–2.5 hr. and the mixture diluted 1:10 into separate Warburg flasks for activity determinations. It was thus found that the *T* of partly purified human erythrocytes was the same for both inhibitors, which was taken as evidence that the DCFP had not deteriorated.

Table 1. *The purity of stored DCFP examined by comparison with fresh E 600*

(Turnover number of partially purified human erythrocyte ChE (Davies & Smellie).)

With DCFP	$1.61 \pm 0.09 \times 10^{-5}$	3 points on graph
With E 600	$1.63 \pm 0.18 \times 10^5$	5 points on graph

(2) Some red-cell enzymes, notably those from dog and rat, required more than 1 hr. incubation to show linearity between loss of activity and inhibitor concentration; indeed dog cells failed to show linearity even after 5 hr. This shows that the red-cell enzyme of these species is inhibited more slowly than most.

RESULTS

The *T* of a partly purified human red-cell AChE preparation was, as shown in Table 1, 162,000. The *T* of partly purified horse serum BChE was 93,000, in excellent agreement with Easson & Stedman's value of 89,400.

That there should be a difference in the *T* of these two enzymes is not surprising in view of their different properties, especially in respect of substrate specificity. There is one other value with which these may be compared, that of Nachmansohn's electric eel AChE (Rothenberg & Nachmansohn, 1947). The *T*/mol. is 20,000,000; and if, as has been suggested (Nachmansohn, Rothenberg & Feld, 1948; Augustinsson & Nachmansohn, 1949) there are some 30 active centres/mol., the *T*/active centre is 670,000, a vastly different figure from human red-cell AChE. This observation suggested that there might be species differences between the AChE's and possibly also the BChE's. On the hypothesis that DCFP is a specific reagent, partial purification of tissue extracts should be unnecessary, provided that the ChE present is mainly if not wholly of one type. Nevertheless, a comparison was made between washed human red cells and a partly purified preparation from them of activity 700 μ l. carbon dioxide/mg. protein/hr. (30-fold purification). Table 2 shows that the difference between them was small, thus indicating that a good approximation to the turnover number could be obtained without the necessity of carrying out an extensive programme of purification.

Table 2. *Effect of partial purification on turnover number of human erythrocyte ChE*

Enzyme	<i>T</i> $\times 10^{-5}$	No. of points
Crude	1.94 ± 0.17	4
Purified	1.67 ± 0.13	6

$P=0.3$

As a result of this observation, a survey was made of the turnover numbers of the red-cell enzyme of a number of species. Figures for human and dog plasma are included because these tissues, while

Table 3. *Turnover number of the blood ChE of different species*

Species	Plasma, BChE	Cells, AChE
Human	48,000	171,000
Horse	93,500*	13,000
Dog	99,000	50,000†
Rat	—	10,000

* Partly purified.

† Lower limit.

showing moderate to high BChE activity, have a comparatively low AChE activity, as revealed by specific substrate tests (Mendel, Mundell & Rudney, 1943).

This table shows that there are species differences among cholinesterases of the same type. This could be due to the fact that the ChE in each species is different, or it might be that the basic activity of the enzyme, which is the same in all species, is modified by its environment.

Preliminary studies on goat blood, which it was desired to study intensively, gave rather confusing results, in that each individual goat seemed to have its own T for red-cell AChE. It was not possible to keep a goat exclusively for bleeding, as Aldridge (1950) had done in earlier work, so that blood had to be drawn from such animals as were available. Accordingly, blood was drawn from ten goats, mostly females, and the following determinations carried out on the washed cells: normal ChE activity, normal aliesterase activity (as measured with tributyrin), ChE and aliesterase activities after 1 hr. inhibition by a given concentration of DCFP. This gave maximal inhibition of ChE. All samples were subject to precisely the same washing procedure, so that the results accurately reflect individual variation. The packed cells were diluted 1:4 with water. The results are given in Table 4.

There are no correlations or trends discernible in this table. On the assumption that DCFP is a specific reagent for ChE, the same loss of activity is

to be expected regardless of the normal level. The variations in T should not exceed those expected from the regression method examined earlier. Tables 1 and 2 show that the coefficient of variation of T is between 5.4 and 8.8%. (The exception, determined with E 600, involved the additional step of dilution, and may be disregarded.) Two separate 'regression' determinations of the same sample of goat cells gave coefficients of variation of 4.5 and 6.1%. By contrast, the coefficient of variation of the T of Table 4 is 51.3%. These variations are so much greater than the error of the method that it may be concluded that individual variations in susceptibility to inhibition exist. These variations are not entirely due to the fairly high sensitivity of aliesterase to inhibition, since there is no correlation between ChE loss and either normal aliesterase or loss of aliesterase. There is possibly some other component of comparable affinity for DCFP.

A similar examination was made of ten human bloods from fit young Service men. The results are given in Table 5. The mean T is 1.87×10^5 and the coefficient of variation 22.3%. Compared with the worst of the data of Tables 1 and 2, the variance ratio 6.4 with $n_1 = 9$ and $n_2 = \infty$, indicates individual variation greater than would be expected from the method of estimation.

Table 4. *Individual variations in turnover number of goat erythrocyte ChE*

Goat no.	Sex	DCFP $\times 10^{10}$	ChE activity (μ l. CO ₂ /30 min.)		$T \times 10^{-4}$	AliE activity (μ l. CO ₂ /30 min.)	
			Normal	Loss		Normal	Loss
114	F	5.97	76	23	1.91	71	2.5
410	F	5.97	73	38	3.16	67	3
123	M	5.97	67	23.5	1.95	66	7
—	—	5.23	103.5	48	4.56	67.3	5.5
161	F	6.6	70.5	47.5	3.58	80.5	5
J102	F	6.6	116.5	12	0.90	106	15
J110	F	5.47	78.5	13.5	1.23	88	15
J117	F	5.47	127.5	28.5	2.59	84.5	2
J125	F	5.47	71	15.5	1.41	91	6.5
J171	F	5.79	120.5	54.5	4.68	90	6
Mean			90.4 ± 7.5		2.62 ± 0.44	81.2 ± 4.2	

Table 5. *Individual variations in turnover number of human erythrocyte ChE*

ChE (μ l./30 min.)		DCFP $\times 10^{10}$	$T \times 10^{-5}$	Aliesterase (μ l./30 min.)	
Normal	Loss			Normal	Loss
176	31.5	1.03	1.52	20	0
180	43.5	1.03	2.10	19	1
166	39	1.03	1.88	23	3
152	45	1.12	2.00	15	1
154	29.5	1.12	1.31	18.5	0
167	42	1.12	1.86	17	1
158	46.5	1.12	2.06	18	0.5
146	36	1.12	2.04	16	0
153	30	1.12	1.33	22	2.5
191	57.5	1.12	2.55	—	—

Table 6. *Effect of partial purification on turnover number of goat erythrocyte ChE*

Enzyme	Normal activity (μ l. CO ₂ /30 min.)	Loss of activity	DCFP $\times 10^{-10}$ M	$T \times 10^{-4}$
Crude	129	40	5.34	3.7
Purified	83	68	5.34	6.3

In the light of this evidence we must re-examine the earlier statement, based on the comparison between crude and purified human cells, that the crude sample gives a good approximation to the T . The crude sample was taken from one person, whereas the purified sample was prepared from a bulk sample from six or eight persons. This comparison is now seen to be inadmissible. By contrast, the effect of purification on goat cells was studied on one sample, which was divided, part being taken for determination of T at one concentration of DCFP, the other part purified. The degree of purification was not measured except to note that the aliesterase activity was eliminated. The result is in Table 6. Purification has resulted in a marked increase in sensitivity to inhibition. It has thus been demonstrated that DCFP is by no means as selective as had originally been supposed.

DISCUSSION

The idea that an inhibitor might be practically specific for cholinesterase was first suggested by Easson & Stedman (1936). Although such inhibitors as physostigmine also inhibit liver esterase, they inhibit cholinesterase in such high dilution that at such dilution the effect on other esterases would be negligible. However, the possibility of reaction with inert proteins was indicated by the finding of Roepke (1937) that three to four times more physostigmine was required to produce a given inhibition in crude serum than in purified serum.

The specificity of diisopropyl fluorophosphonate appeared to be of a higher order. Mackworth & Webb (1948) found that it was thirty times as active as physostigmine against ChE, and concluded that 'fluorophosphonate has a very special affinity for the active centres attacked'. Mazur & Bodansky (1946) found that, within the limits of experimental error, the concentration giving 50% inhibition was the same for purified as for crude serum. Although Webb (1948) found that DFP was not specific for ChE, his data show that the next most sensitive enzyme studied, ox-liver esterase, required about 300 times as much DFP to give 50% inhibition. It was a reasonable supposition that on adding DFP or the chemically similar DCFP to a tissue extract the greater part of it would react with ChE, so that it would be in effect a specific reagent.

It appears, however, from the present results that tissue extracts may contain substances, such as aliesterase, which, while not greatly affected by

DCFP have for it a sufficiently high affinity to interfere with its inhibition of ChE. The fact that goat red-cell aliesterase may interfere in this manner is consistent with the supposition that it is of low activity towards its substrate, but fairly reactive with DCFP.

A rather unexpected finding was the considerable individual variation in the susceptibility to inhibition of the ChE in tissue extracts. This raises the question of whether there may be individual variations in the net rate of inhibition. Both of these factors would be reflected in the commonly determined I_{50} values for irreversible inhibitors. Future kinetic studies must be designed to take account of this possibility, either by finding whether individual variations exist, or by using pooled material. Some of the data in Table 3 are unreliable, notably those for horse and dog cells, where the samples are from one animal.

The species variations noted in Table 3 are more likely to be due to the presence of substances competing with ChE for inhibitor than to substances modifying the basic activity of ChE. It should not be assumed that interfering substances have been removed by existing methods of purification, especially of red-cell enzyme, where the utmost degree of purification is only some 30-fold.

It is clear that DCFP cannot be used in the manner originally intended, to determine the T . The meaning of the numbers labelled T is obscure. It is not feasible to use them to calculate the concentration of ChE active centres, nor to attempt to estimate the capacity of the cells to combine with DCFP because we have no information about the combination of the inhibitor with other substances in the blood.

SUMMARY

1. A mathematical expression has been developed for calculating the turnover number of an enzyme, assuming the use of a highly specific irreversible inhibitor to determine the active centre concentration.
2. The following basic assumptions are involved: (a) the inhibitor is highly specific for the enzyme; (b) inhibition is irreversible; (c) the inhibition reaction is bimolecular.
3. This expression has been applied to the study of cholinesterases in tissue extracts, using the inhibitors dicyclohexyl fluorophosphonate and diethyl *p*-nitrophenyl phosphate.
4. The turnover number of erythrocyte cholinesterase determined in this way has been found to

vary, not only among species, but also among individuals of the same species.

5. It is concluded that the inhibitors are not so highly specific as was previously believed. The variations in apparent turnover number are probably due to the presence in the tissue extracts of varying amounts of other substances competing with cholinesterase for the inhibitor.

Acknowledgement is made to the Chief Scientist, Ministry of Supply, for permission to publish this paper. [Crown Copyright reserved. Reproduced with the permission of the Controller of H.M. Stationery Office.]

I wish to express my indebtedness to Mr D. R. Davies for advice and encouragement. Thanks are also due to Dr B. C. Saunders for the sample of DCFP, to Mr S. Callaway for advice on statistical matters, and to Mr E. G. Harrold for technical assistance.

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The Preparation and Properties of β -Glucuronidase

2. CENTRIFUGAL FRACTIONATION OF WATER HOMOGENATES AND THE NATURE OF THE SEDIMENTABLE FRACTION

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(Received 15 January 1951)

In the first paper in this series (Kerr & Levvy, 1951) it was shown that buffering a water homogenate of mouse liver, kidney or uterus to a slightly acid pH led to fractionation of the β -glucuronidase activity on subsequent centrifuging at low speeds. Changes in the glucuronidase activity of these organs with the state of proliferation (Levy, Kerr & Campbell, 1948; Kerr, Campbell & Levvy, 1949) were seen to be confined to the non-sedimentable fraction. In normal adult liver the sedimentable fraction accounted for nearly half the total glucuronidase activity in the buffered water homogenate. As described below, identical fractionation of mouse-liver glucuronidase in untreated water homogenates can be achieved on the high-speed centrifuge. The partition of the enzyme has been correlated with microscopic observations in the homogenate, and methods for bringing the sedimentable fraction into solution have been studied.

METHODS

Preparation and fractionation of the enzyme. Mouse liver was ground with water for 1 min. in a glass homogenizer. We have found Veridia precision bore glass tubing (Chance Bros. Ltd.) convenient for the manufacture of homogenizers. After dilution of the homogenate to a convenient volume, samples were taken for measurement of the total enzyme activity. Other samples were fractionated by one or both of the following methods: (a) After pH adjustment, the preparation was centrifuged on the M.S.E. (Measuring and Scientific Equipment Ltd., London, S.W. 1) Minor at 1500 g for 15 min. If, as was usually the case, acetate buffer (pH 5.2) was used to adjust the pH, the terms 'acetate-soluble' and 'acetate-insoluble' are sometimes used for the enzyme in the supernatant and sediment, respectively. (b) The preparation was centrifuged without pH adjustment on the M.S.E. Major with Superspeed Attachment. This was usually done at 20,000–25,000 g for 15 min., yielding 'soluble' enzyme in the 'high-speed supernatant', and 'insoluble' enzyme in the sediment. The Superspeed centrifuge head was chilled before use.