reversible inhibitors can occur.[4] Koster[11] found that diisopropylfluorophosphate (DFP) given before physostigmine made animals more *sensitive* to the latter, while injections of the latter protected animals against doses of DFP that were several-fold greater than lethal. These data can be explained in terms of interference between the two inhibitors plus the fact that the DFP can be destroyed in the body as shown by Mazur.[12] In other words, physostigmine would lower the concentration of free enzyme and protect it against DFP for a time sufficient to allow for the destruction of DFP, while DFP initially would lower the effective concentration of enzyme thereby sensitizing the animal against physostigmine.

Summary. 1. The effect of enzyme concentration on the inhibition produced by certain inhibitors of the succinic dehydrogenase system has been experimentally determined. The inhibitors studied were malonate, itaconate, oxalacetate, quinone and cupric ion.

2. The reaction between succinic dehydrogenase and copper or quinone was not immediate but required 30 to 40 minutes when the amount of inhibitor was just sufficient to produce complete inhibition.

3. The effect of the strengths of binding of the inhibitor with the enzyme on the per cent inhibition produced is shown to be related to the enzyme concentration.

4. An expression has been developed which relates the velocity of reaction to enzyme concentration in enzyme-inhibitor systems.

5. The results are discussed in relation to the chemotherapy of cancer. It is pointed out that the selective inhibition of an enzyme not unique to cancer tissue is theoretically possible.

The authors gratefully acknowledge the helpful suggestions of Dr. J. A. Bain, whose data on cholinesterase inhibition were made available to us prior to publication.

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Mechanism of the Inhibition of Rat Brain Cholinesterase by Diisopropylfluorophosphate, Tetraethylpyrophosphate, and Eserine.* (17314)

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Kinetic analysis of the inhibition of enzyme systems has generally been carried out by methods which assume a reversible combination of the inhibitor and enzyme to form an inactive complex.[1-4] Ordinarily, dissociation constants for the enzyme-inhibitor complex are calculated according to the classical Michaelis-Menten treatment[1] from the concentration of inhibitor at which enzymatic activity is reduced 50 per cent. Goldstein[5] and Ackermann and Potter[6†] have pointed out that situations may exist when the assumptions used in the Michaelis-Menten derivation are no longer valid. Such a case arises when

* Supported in part by grants from the Miller Epilepsy Fund, the Rockefeller Foundation, and the Research Council of the Scottish Rite Masons.

[1] Michaelis, L., and Menten, M. L., *Biochem. Z.*, 1913, **49**, 1333.

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† The author is indebted to Dr. V. R. Potter for making available a manuscript of this paper[6] prior to publication and for much correspondence and discussion regarding its contents.

the enzyme-inhibitor complex formation is irreversible (*i.e.*, the dissociation constant approaches zero) in which case the actual concentration of the enzyme may no longer be ignored. However, such irreversibility is not always plainly apparent and when independent evidence of such a state of affairs is lacking an investigator may analyze his data by the classical methods referred to above and arrive at values for dissociation constants that will be theoretically unsound. It is not always easy to devise unequivocal tests for irreversibility particularly when working with unpurified enzyme preparations such as serum, homogenates, minces, slices or tissue extracts. Ackermann and Potter[6] have recently proposed a method which offers a simple, graphical solution to this difficulty. During the course of a study of the effects of convulsant and anticonvulsant drugs on the cholinesterase system of rat brain tissue[7] we have had occasion to test the method of Ackermann and Potter using the newly discovered cholinesterase inhibitors diisopropylfluorophosphate and tetraethylpyrophosphate for which independent evidence for irreversibility exists[8] and compared their mechanism of action to that of the classical inhibitor, eserine (physostigmine).

Experimental methods and results. The general characteristics of the cholinesterase system in rat brain homogenates and the methods for its study have been previously described.[7] The specific conditions used in this investigation are given in table headings and figure legends.

The degree of inhibition by a given concentration of DFP or TEPP‡ is dependent upon the time of incubation of the inhibitor with the enzyme before addition of the substrate, as may be seen from the curves given in Fig. 1. In subsequent experiments we have arbitrarily adopted the time of 40 minutes incubation of the enzyme with inhibitor before addition of substrate. The enzyme may be protected from TEPP by addition of substrate simultaneous to, or before, the addition of the inhibitor (Table I). This effect is not so marked using DFP (Table I).

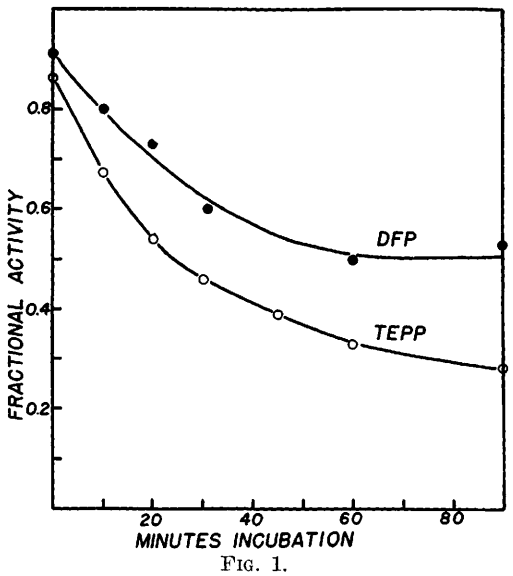

FIG. 1.

Effect of Incubation of the Enzyme with Inhibitor Before Addition of Substrate. Total volume 2.2 ml containing final concentrations as follows: 0.025 M $NaHCO_3$, 0.075 M KCl, 0.075 M NaCl, 0.04 M $MgCl_2$, 0.01 M acetylcholine-bromide (added from sidearm after incubation times indicated), 2.3×10^{-7} M DFP or 8×10^{-9} M TEPP, 25 mg (wet weight) per flask rat brain tissue added as 5% homogenate in buffer (see 7)). 5% CO_2—95% N_2 gas phase, temperature 37.5°C. Each point average of 2 experiments in duplicate. Activities calculated as in 7.

The inhibition of rat brain cholinesterase by DFP, TEPP and eserine under specified conditions is shown in the curves of Fig. 2 where enzyme activity is plotted against inhibitor concentration. It may be seen that the general shape of the curves is the same and without closer analysis it might be inferred that the inhibitors differed only in potency.

The results obtained using the method of Ackermann and Potter[6] are shown in Fig. 3. Here both tissue concentration and inhibitor concentration were varied. In the case of

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‡ The abbreviations DFP—diisopropylfluorophosphate, and TEPP—tetraethylpyrophosphate, will be used throughout this paper. These compounds were obtained through the courtesy of Dr. H. E. Himwich of the Medical Division, Army Chemical Center, Edgewood, Md., and Dr. K. P. DuBois of the University of Chicago Toxicity Laboratory, Chicago, Ill.

TABLE I.
Effect of Order of Addition of Substrate and Inhibitor on Rat Brain Cholinesterase Activity. Basic conditions as in Fig. 1.

Order of addition	% inhibition	
	TEPP (8×10^{-7} M)	DFP (4×10^{-6} M)
Simultaneous	9	40
Substrate 10′ before inhibitor	2	—
Inhibitor 30′ before substrate	47	80

TEPP, when tissue concentration was plotted against rate for various concentrations of inhibitor a series of curves resulted, the control curve intercepting the x-axis at zero and the curves to which various amounts of TEPP had been added intercepting the x-axis at progressively larger values of tissue concentration. When these intercepts were plotted against the concentration of inhibitor (inset Fig. 3) a straight line through the origin was obtained. The same result was found with DFP as with TEPP, the former requiring higher concentrations, *e.g.*, the molarity at an x-intercept of 0.25 ml being 11.5×10^{-7} M DFP compared to 4.5×10^{-9} M TEPP. However, when the same procedure was applied to eserine a different result was obtained. It may be seen from Fig. 3, that in the case of eserine, a series of curves of different slopes results all intercepting the x-axis at zero in contrast to the DFP and TEPP curves.

Neither the eserine, the DFP, nor the TEPP inhibition of brain cholinesterase was reversed by cysteine as were the effects of the nitrogen mustards.[7] Atropine, which is a specific antidote for eserine and DFP *in vivo*, does not reverse their effects upon cholinesterase. Atropine itself does not affect the enzyme.[7] Eserine has been reported to protect brain cholinesterase against irreversible inactivation by DFP[9] and we have confirmed this finding.

Discussion. The values for the concentration required to give 50% inhibition of brain cholinesterase by DFP, TEPP, and eserine are of the same order of magnitude as those reported by others[10-12] using somewhat similar conditions; but it is apparent from the data in this report and that of others, particularly Nachmansohn, *et al.*,[13] that conditions of enzyme concentration, incubation time, and order of addition of inhibitor and substrate can profoundly affect the results with the fluorophosphates.

Eserine was included in this study because it and prostigmine are the only other compounds whose potency as inhibitors approaches that of the alkylated fluoro- and pyrophosphates.[14] The data presented in Fig. 3 clearly show that, under the conditions employed in this study, there is a difference in the nature of the inhibitory processes. Accepting the criteria of Ackermann and Potter,[6] DFP and TEPP are seen to fall into that category of inhibitors where analysis by the classical methods will no longer apply. Further, the irreversibility of the enzyme-inhibitor complex is demonstrated by a method which does not involve dialysis,[8] dilution,[13] nor isolation.[15] In the case of eserine, on the other hand, the data of Fig. 3 show that analysis of its inhibitory action may be successfully performed by the classical methods.

On the basis of the data in Fig. 3 and other independent criteria[8,13] the inhibition of cholinesterase by DFP and TEPP may probably be assumed to be irreversible. We may then consider that the inhibitor "titrates" a

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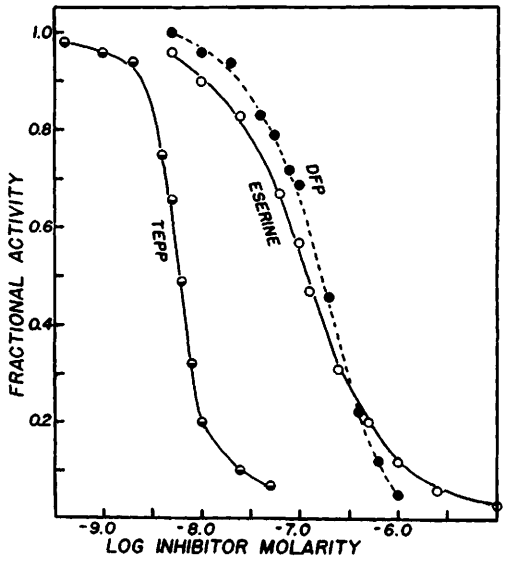

FIG. 2.

Inhibition of Rat Brain Cholinesterase by DFP, TEPP, and Eserine. Basic conditions as in Fig. 1. Inhibitors incubated with the enzyme 40 minutes before addition of substrate. Each point average of at least 2 experiments in duplicate.

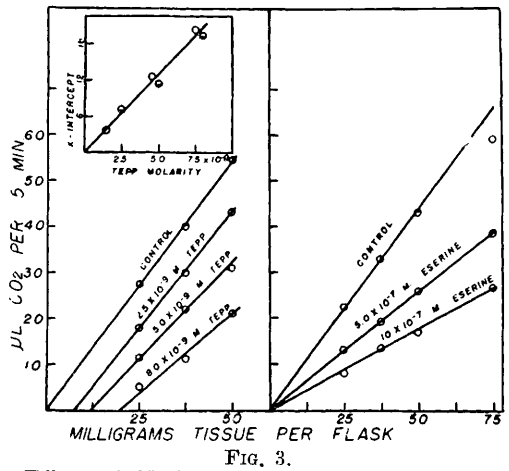

FIG. 3.

Effect of Variation in Enzyme Concentration on the Inhibition of Rat Brain Cholinesterase by TEPP and Eserine. Conditions as in Fig. 2. Inset figure: Half-solid circle - - points plotted from data given in this figure, ○ - - points plotted from another duplicate experiment using different rat brain as tissue source, included to show reproducibility.

certain amount of the enzyme, evaluated by the x-intercepts in Fig. 3. If we use the most potent inhibitor, TEPP, as the limiting case, it takes of the order of 1 x 10^{-11} moles of inhibitor to titrate the enzyme in 0.25 ml of homogenate which is equivalent to 0.0125 ml of original tissue. If we further assume that 1 mole of inhibitor combines with 1 mole of enzyme§ then we may calculate that the greatest concentration of enzyme is approximately 1 x 10^{-9} moles per ml of tissue or 1 x 10^{-6} molar. The above reasoning disregards, of course, the possibility that the enzyme may be concentrated in certain regions by structural restrictions. Goldstein[5] has estimated that the concentration of cholinesterase in 4.5% dog serum has a maximum value of 2 x 10^{-8} M giving a value of approximately 1 x 10^{-7} M in 100% serum by direct extrapolation. We do not believe that the data in this paper justify any but the approximations indicated above because of considerable uncertainty as to the purity of the inhibitors used, the large dilutions of the inhibitor stock which are necessary and the possibility that some of the inhibitor is combined with proteins inert to the substrate employed.‖ The calculations do serve, however, to give an upper limit to the enzyme concentration.

Summary. The mechanism of inhibition of rat brain cholinesterase by the 3 most potent anti-cholinesterases, diisopropylfluorophosphate, tetraethylpyrophosphate, and eserine was studied by a method which involves variation in enzyme concentration as well as in-

§ A reasonable assumption considering that Goldstein[5] found 1 mole of eserine per mole of enzyme and Jansen *et al.*[15] have recently shown that 1 mole of DFP combines with 1 mole of chymotrypsin to form an inactive complex. We have been unable to estimate, in the case of TEPP, the number of molecules of inhibitor per molecule of enzyme by the method of Goldstein because the slope of the inhibition curve (Fig. 2) falls above his theoretically determined limits, possibly because we are dealing here with an irreversible combination.

‖ This last consideration is somewhat discounted by the experiments of Mazur and Bodansky,[16] who added large amounts of heat-inactivated protein to a DFP-inhibited system and found no effect. However, the very process of heat inactivation may have destroyed groupings which in the native state were inert to the substrate but might not have been inert to the inhibitor.

[16] Mazur, A., and Bodansky, O., *J. Biol. Chem.*, 1946, **163**, 261.

hibitor concentration. It was shown that the kinetics of eserine-inhibition may be analyzed by classical methods which ignore enzyme concentration but the cases of the other two inhibitors may not be so treated. Cholinesterase inhibition by the fluoro- and pyrophosphates was shown to be irreversible and to depend upon enzyme concentration and time of incubation of the enzyme with the inhibitor before the addition of substrate. The upper limit of cholinesterase concentration in rat brain was estimated to be 1×10^{-6} molar if structural restrictions are not assumed.

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Some Effects of Large Doses of Ergot Products on Rats.* (17315)

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An examination of the literature reveals much interest in various ergot products, particularly the dihydrogenated derivatives, because of their possible usefulness in the treatment of migraine,[1,2] in the prevention of cyclopropane arrhythmias,[3] and in the treatment of hypertension.[4-6]

Toxicity of the dihydrogenated derivatives is less than that of the natural alkaloids;[7] Orth and others[8] show that dihydroergocornine did not produce gangrene in the tails of rats, whereas ergotamine routinely produced gangrene. They also show that pregnant female rats receiving dihydroergocornine delivered normal litters and raised them to maturity, whereas similar rats receiving ergotamine tartrate lacked maternal instincts.

No accounts were found in the literature of experiments in which ergot products were injected over extended periods of time. Orth and others[8] made semiweekly injections during the gestation period of rats in doses up to 35 mg/kg of dihydroergocornine. Observations over extended periods were thought to be desirable since many patients would take the ergot product more or less regularly for years.

Methods. A total of 73 rats were injected subcutaneously 6 times a week for periods up to 17 weeks. This rigorous treatment contrasts with that of human therapy in which ergot products are usually injected only twice a week.

Control rats were injected 6 times a week with physiological saline.

All rats were weighed once a week.

Preliminary experiments show that doses comparable to therapeutic doses in man (for those products whose therapeutic doses have been established) produced no measurable effect on weight of rats. Therefore, it was decided to make the experiments still more rigorous by using doses comparable, on a weight basis, to those of ergotamine tartrate that produce gangrenous tails in rats. Preliminary experiments showed that a dose of about 0.5 mg/kg of ergotamine tartrate adminis-

* This study was made possible by a grant from the Sandoz Chemical Works, Inc.; the drugs were supplied by Mr. Harry Schnizer of that company.

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