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Enzyme Inhibition in Relation to Chemotherapy.* (17313)

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Studies on enzyme inhibitors are frequently reported in terms of the percent inhibition in comparison with a control containing no inhibitor. It is the purpose of the present paper to show that this approach is frequently in danger of giving misleading results for the reason that with some inhibitors the percent inhibition is a function of the enzyme concentration. As will be pointed out in the discussion, the existence of such inhibitors is of considerable importance from the standpoint of pharmacology, chemotherapy and related fields, and it is therefore of some importance to have an effective experimental method for their recognition.

The phenomenon which we propose to describe is essentially the outcome of an "irreversible" reaction between enzyme and inhibitor, so that for all practical purposes the enzyme is effectively titrated or stoichiometrically combined with a definite amount of inhibitor. From the theoretical standpoint the "irreversible" reaction between enzyme and inhibitor may occur in a variety of ways; the simplest case is one in which the enzyme-inhibitor complex is theoretically reversible but has a dissociation constant so small that the combination seems irreversible. We shall therefore refer to this type of inhibition as *pseudo-irreversible*. Equally possible is a situation in which the enzyme reacts with an inhibitor in a truly irreversible manner, that is to say, the enzyme is converted to a form which cannot be converted back into active enzyme. In either case, the amount of enzyme inactivated will depend not only upon the amount of inhibitor but upon the amount of enzyme present. Regardless of the exact nature of the irreversibility, it can be recognized very simply by means of an experimental test: by determining the rate of reaction at different enzyme concentrations plus or minus inhibitor, it should be found that in the case of the controls, the rate is proportional to the enzyme concentration, so that a straight line through the origin is obtained when rate is plotted against enzyme amount. In the presence of a reversible inhibitor a straight line through the origin also results, but the slope of the line is less than in the case of the control. In the case of an irreversible inhibitor, the slope of the line is the same as that of the control but it will pass through the X-axis to the right of the origin by an amount that is proportional to the amount of inhibitor (see Bain[1]). This graphic test is not de-

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A preliminary report of this work was given at the Annual Meeting of the American Association for Cancer Research, April 16 and 17, 1949, (*Cancer Research*, 1949, **9**, 602).

[1] Bain, J. A., PROC. SOC. EXP. BIOL. AND MED., 1949, **72**, 9.

pendent upon any theoretical assumptions as to the nature of the irreversibility and is of considerable value in the interpretation of inhibitor data whether obtained with pure enzymes, homogenates, minces, slices or whole cells. It is of interest that the curve for the pseudo-irreversible type of inhibitor is simply one of a family of curves, and that the curve for the reversible type can be obtained from the same general equation that is derived from the familiar Michaelis-Menten equation in the theoretical part of this paper. With the same equation it can be shown that when the dissociation constant of the enzyme-inhibitor complex has an intermediate value, the plot of activity against enzyme amount yields a line that is intermediate between the two types described above. Since many of the recently discovered enzyme inhibitors appear to fall in this category the test may be of value in understanding their action, since in these cases the per cent inhibition also depends upon the enzyme concentration.

The fact that inhibitors may fall into 3 broad categories depending upon the relationship between the dissociation constant of the enzyme-inhibitor complex and the concentration of enzyme has been emphasized previously by Straus and Goldstein[2] and by Goldstein.[3] They pointed out that previous mathematical treatments based on the Michaelis-Menten equation assumed "that the concentration of enzyme centers is constant and so small compared with the concentration of any substance with which it may combine that it may be neglected." They called attention to the fallacy in this assumption and developed the Michaelis-Menten equation along lines that include the factor of enzyme concentration.†

Our own observations and those of Bain[1] are in accord with the conclusions of Straus and Goldstein[2,3] and are believed to supplement their work by presenting a simple graphic method for recognizing the situations in which the older mathematical treatments are more or less inadequate. The graphic method is accompanied by a mathematical analysis in which one of the components of the graph (rate) is obtained as a function of the other component (enzyme amount) in an equation involving other variables (substrate amount, inhibitor amount, enzyme-substrate dissociation constant, and enzyme-inhibitor dissociation constant). The graphic method involves no assumptions as to the mechanism of inhibitor action, but in conjunction with the equation given it permits one to obtain an apparent dissociation constant for the enzyme-inhibitor complex. The word "apparent" is used here because in the case of an inhibitor which gives a line that intercepts the X-axis it would not be possible to decide from the graph whether the inhibitor was pseudo-irreversible (*i.e.*, dissociation constant of enzyme-inhibitor complex very small) or truly irreversible. In addition, the apparent dissociation constant will be affected by the specificity of the inhibitor and the presence of other compounds with which it may combine. It must be emphasized that the main purpose of the graph is not to determine the dissociation constants but to distinguish between the reversible type of inhibitor and the "irreversible" inhibitors. It is believed that the graph will permit this distinction regardless of the presence of interfering compounds and no evidence to the contrary has been obtained.

The data given are for illustrative purposes only, and have no intrinsic interest except insofar as they illustrate the principles described above. For this work, succinoxidase was employed as the test system because previous experience[4] showed that it could be

[2] Straus, O. H., and Goldstein, A., *J. Gen. Physiol.*, 1943, **26**, 559.

[3] Goldstein, A., *J. Gen. Physiol.*, 1944, **27**, 529.

† The important contributions by Michaelis and Menten, by Haldane, and by Lineweaver and Burk (referred to by Straus and Goldstein[2,3]) provide the foundation for the further expansion of the basic equations to include the amount of enzyme as a variable. The older equations are still very useful as well as appropriate in situations in which competitive inhibition between reversible inhibitors and substrates occurs since in these situations variation in enzyme amount does not affect the per cent inhibition. The newer treatment is more appropriate in the case of the pseudo-irreversible inhibitors.

inhibited by two types of inhibitors, the malonate-type and the type that combines with sulfhydryl groups. Whole homogenates were used as in the previous work, in which it was shown that the sulfhydryl inhibitors acted upon an essential group that could be protected by malonate, although malonate would not protect other enzymes against the sulfhydryl inhibitors. It thus seems clear that the sulfhydryl reagents used in this study inactivate succinoxidase by direct interaction with the enzyme.

Experimental. Test System. In these studies the succinoxidase system was employed. In all experiments a 10% water homogenate of liver was prepared in the usual manner and used as a source of the succinic dehydrogenase after diluting to 2.5%. The basal reaction mixture used was the same in all cases and prepared as previously described.[5] The concentration of inhibitors and homogenate are in the corresponding plots of the experimental data. The total volume of material in each flask was 3 ml and the temperature of the reaction 38°C. The rate of oxygen uptake per 10 minutes was determined on the basis of 4 successive 10-minute periods. With all inhibitors except oxalacetate in experiments reported in Fig. 1, the homogenate was incubated for 30 minutes at room temperature with the inhibitor before the addition of succinate, since some inhibitors do not react instantly with the enzyme, and are affected by the presence of the substrate.

Compounds Tested. The itaconic acid was obtained from the Chas. Pfizer Company while the malonic acid and quinone were obtained from the Eastman Kodak Company. Oxalacetic acid was prepared from sodium ethyl oxalacetate.

Results. Effect of Enzyme Concentration. The reaction velocity for the enzymatic dehydrogenation of succinate to fumarate could be measured in terms of the oxygen taken up in successive 10-minute intervals, since the succinic dehydrogenase is the limiting factor in the succinoxidase system when both cytochrome c and cytochrome oxidase are present in excess. It was established that the reaction rate in the absence of inhibitors was proportional to the amount of homogenate. Since a different liver homogenate was used for each experiment, it was necessary to include controls each time an inhibitor was tested. It may be seen in Fig. 1 that the control data in each instance (open circles) yielded a satisfactorily straight line that passes through the origin.

However, when such data were obtained with various inhibitors present and were plotted in the same manner, significant differences between the inhibitors were revealed insofar as they were affected by enzyme concentration.

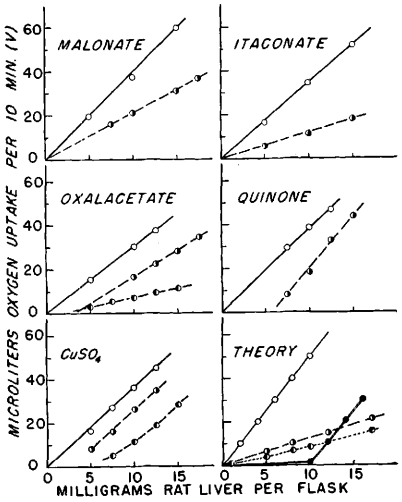

FIG. 1.

The reaction velocity as measured by oxygen uptake for the enzymatic dehydrogenation of succinate to fumarate in the presence and absence of inhibitors. The concentration of malonate was 1×10^{-3}M; oxalacetate was 6.7×10^{-5}M and 3.4×10^{-5}M; copper sulfate was 1.3×10^{-4}M and 2.6×10^{-4}M; itaconate was 5×10^{-2}M; quinone was 3.3×10^{-4}M. In all cases the concentration of succinate was 5×10^{-2}M. In the curve systems labeled malonate, oxalacetate, Cu SO_4, itaconate and quinone, the broken lines represent activity in the presence of the inhibitors. The system of curves labeled theory were calculated from equation (13) assuming for all cases $S = 5 \times 10^{-2}$M and $K_s = 10^{-3}$: for the solid curve $I_t = 0$; for the broken curve $K_i = 10^{-4}$, $I_t = 1 \times 10^{-3}$M; for the dashed curve $K_i = 10^{-6}$, $I_t = 5 \times 10^{-5}$M; for the double lined curve $K_i = 10^{-9}$, $I_t = 1 \times 10^{-5}$M.

[4] Potter, V. R., and Du Bois, K. P., *J. Gen. Physiol.*, 1943, **26**, 391.

[5] Schneider, W. C., and Potter, V. R., *J. Biol. Chem.*, 1943, **149**, 217.

In the presence of malonate or itaconate (Fig. 1), which are structurally similar to succinate, the rate of dehydrogenation was also proportional to the enzyme concentration, but the slope of the line was less than in the case of the controls. The per cent inhibition was thus the same at any concentration of enzyme and depended solely upon the concentration of inhibitor since the succinate concentration was constant.

In contrast to the data obtained with malonate and itaconate, oxalacetate, which also bears a close structural relationship to succinate, yielded data that do not fall on a line through the origin, but rather on a line which appears to intercept the enzyme axis (Fig. 1).

Even more striking are the results with quinone and copper (Fig. 1). Here the data fell on lines that tend to be straight and parallel to the control. With these inhibitors the per cent inhibition is clearly related not only to the inhibitor concentration but also to the enzyme concentration.

Interference Phenomenon with Irreversible Inhibitors. In earlier studies on succinoxidase[4] it was shown that the inhibition produced by malonate was instantaneous and independent of the order of addition of substrate and inhibitor. This was in contrast to results obtained with agents like quinone and copper, which produced a slowly increasing per cent inhibition that was strongly affected by the presence or absence of succinate or, indeed, of malonate. In the presence of succinate or malonate, the sulfhydryl inhibitors react more slowly with the enzyme than they do when both of these compounds are absent. A more detailed experiment on this point is reported in Fig. 2. In this experiment a series of Warburg flasks was prepared with the usual reaction mixture but no succinate, and with an equal amount of tissue in each flask. At zero time copper in the form of $CuSO_4$ was added to all of the flasks, and at successive intervals succinate was added from the side arm and oxygen uptake was measured. The continuous lines represent the rate of oxygen uptake during successive 10-minute time intervals after the

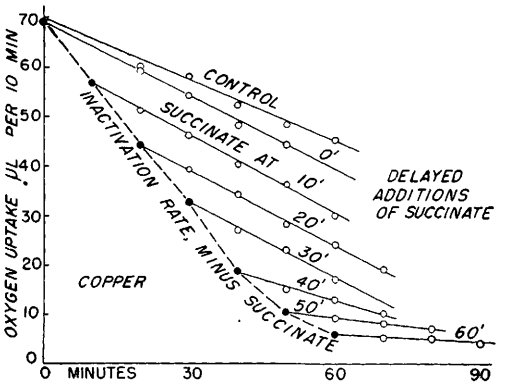

FIG. 2.

Rate of inactivation of succinic dehydrogenase by copper in the presence and absence of succinate. The final concentrations of succinate and of Cu SO_4 were 0.067 M and 3.3 × 10-5M, respectively. The solid circles represent extrapolated points. The open circles are experimental points.

addition of succinate, and show that when succinate was present no inactivation of the enzyme by the copper occurred, since the decline in rate was no greater than in the controls. The broken line indicates the rate of enzyme inactivation by copper when no succinate was present, and shows that a progressive decrease in active enzyme occurred as long as succinate was absent, but as soon as succinate was added the process of inactivation ceased abruptly. This experiment shows that succinate *interferes* with the reaction between copper and succinic dehydrogenase, while copper once combined with the enzyme cannot be effectively displaced by succinate. When larger amounts of sulfhydryl inhibitors were added to the succinoxidase system, it was possible to progressively inhibit the enzyme even in the presence of succinate, although not as rapidly as in the absence of succinate. It is clear that copper and succinate compete for the enzyme and that their reactions with it are *mutually exclusive* as suggested earlier in the case of quinone.[4] For the present we shall refer to this type of effect as an "interference phenomenon" and will not attempt to describe it as competitive inhibition. But it must be pointed out that the latter term is a purely operational concept that is at present restricted to reversible inhibitors. The experimental approach indi-

cated in Fig. 2 provides a means of studying competition between substrates and irreversible inhibitors during the course of the reaction between the inhibitor and the enzyme as opposed to competition during the measurement of enzyme activity. It is proposed that this kind of competition be called *interference* in order to avoid needless confusion in terminology.

Theoretical. To derive an expression in which ES is expressed as a function of E_t, S, I_t, K_s, and K_i, the symbols listed below were used:

E_t = Total Enzyme Concentration.
E_f = Uncombined Enzyme Concentration.
EI = Enzyme-Inhibitor Complex Concentration.
ES = Enzyme-Substrate Complex Concentration.
S = Total Substrate Concentration.
S_f = Uncombined Substrate Concentration.
P = Product Concentration.
K_i = Enzyme-Inhibitor Complex Dissociation Constant.
K_s = Enzyme-Substrate Complex Dissociation Constant.
I_t = Total Inhibitor Concentration.
I_f = Uncombined Inhibitor Concentration.
k = Velocity Constant.

The equation was developed along lines similar to the familiar Michaelis-Menten equation but includes more variables.† The following assumptions were made:

(a) The reaction between enzyme and substrate or inhibitor can be formulated as in equations (1) and (2):

$$E_f + S_f \rightleftharpoons ES \longrightarrow P + E_f \qquad (1)$$
$$E_f + I_f \rightleftharpoons EI \qquad (2)$$

(b) Where (S) is very large, (S_f) is assumed to equal (S) and (E_f) approaches zero.

(c) The total inhibitor concentration is equal to the sum of the uncombined and combined forms.

$$(I_t) = (I_f) + (EI) \qquad (3)$$

(d) The total enzyme concentration is the sum of the uncombined enzyme, that combined with the inhibitor and that combined with the substrate

$$(E_t) = (E_f) + (EI) + (ES) \qquad (4)$$

An equation in which ES is expressed as a function of E_t, S, I_t, K_s and K_i may now be derived.

From expressions (1) and (2) and the mass law one can write:

$$\frac{(E_f)\quad(S_f)}{(ES)} = K_s \qquad (5)$$

$$\frac{(E_f)\quad(I_f)}{(EI)} = K_i \qquad (6)$$

By substituting the value of (EI) from (6) into (3) and rearranging one obtains expression (7).

$$(I_f) = (I_t)/\left[1 + \frac{(E_f)}{K_i}\right] \qquad (7)$$

If one substitutes (I_f) of expression (7) into (6), solves the resulting expression for (EI) and substitutes what is then equivalent to (EI) into (4) one arrives at expression (8).

$$(E_t) = (E_f) + \frac{\dfrac{(E_f)\quad(I_t)}{K_i}}{1 + \dfrac{(E_f)}{K_i}} + (ES) \qquad (8)$$

Since from (b), (E_f) is small, (8) reduces to (9)

$$(E_t) = \frac{\dfrac{(E_f)\quad(I_t)}{K_i}}{1 + \dfrac{(E_f)}{K_i}} + (ES) \qquad (9)$$

If one solves equation (5) for (E_f) and substitutes the result into (9) one arrives at (10).

$$(E_t) = \left[\frac{(I_t)}{1 + \dfrac{K_s\ (ES)}{K_i\ (S)}}\right]\left[\frac{K_s\ (ES)}{K_i\ (S)}\right] + (ES) \qquad (10)$$

By multiplying through equation (10) by the term $1+[K_s\ (ES)]/[K_i(S)]$ and rearranging and solving for (ES) by the quadratic formula one obtains expression (11), in which (ES) is expressed as a function of (E_t).

$$(ES) = -\left[\frac{K_i\ (S)}{2K_s} + \frac{(I_t)}{2} - \frac{(E_t)}{2}\right] \pm \sqrt{\left[\frac{K_i\ (S)}{2K_s} + \frac{(I_t)}{2} - \frac{(E_t)}{2}\right]^2 + \frac{K_i\ (S)\quad(E_t)}{K_s}} \qquad (11)$$

Since the velocity of reaction (V) is equal to the product of the concentration of the enzyme-substrate complex and the velocity constant for its conversion to the product, then:

$$V = k\,(ES) \qquad (12)$$

or if one multiplies through expression (11) by k and substitutes $V = k\,(ES)$ one obtains‡

$$V = -k\left[\frac{K_i\,(S)}{2(K_s)} + \frac{(I_t)}{2} - \frac{(E_t)}{2}\right] \pm k\sqrt{\left[\frac{K_i\,(S)}{2K_s} + \frac{(I_t)}{2} - \frac{(E_t)}{2}\right]^2 + \frac{K_i\,(S)\,(E_t)}{K_s}} \qquad (13)$$

The velocity is thus expressed in terms which can be experimentally determined.

We may now consider various tests of the equation. If one lets (I_t) equal O, then equation (13) reduces to the following form:

$$V = \frac{-k(S)\,K_i}{2K_s} + \frac{k(E_t)}{2} + \frac{k(S)\,K_i}{2K_s} + \frac{k(E_t)}{2}$$

$$V = k(E_t) \qquad (14)$$

For large values of (S) and no inhibitor, it follows from expression (4) that (E_t) equals (E_s); hence expression (14) is in agreement with (12). Expression (14) is the equation of a straight line of slope k passing through the origin. It is also what is experimentally observed when values of V are plotted against corresponding values of (E_t) in the absence of inhibitor (Fig. 1). The slope is the velocity constant for conversion of the enzyme-substrate complex to the product.

For small values of K_i, *i.e.* where the binding of inhibitor with enzyme is great compared to that of the substrate with enzyme, the terms of equation (13) containing K_i approach zero and the equation approaches the form of a straight line with a slope k and an intercept on the enzyme axis. Such an inhibitor virtually "titrates" the enzyme.

$$V = \frac{-k}{2}\left[(I_t) - (E_t)\right] \pm k\sqrt{\left[\frac{(I_t) - (E_t)}{2}\right]^2}$$

$$V = k(E_t) - k(I_t) \qquad (15)$$

However equation (14) only approaches the form of (15) since the term containing K_i only approaches zero. When equation (14) is plotted using various values of K_i, a system of curves is obtained as shown in Fig. 1, theoretical curves. For values of K_i that approach the magnitude of K_s, *i.e.* 10^{-4} compared to 10^{-2}, a straight line through the origin is obtained. For smaller K_i values a curved line is obtained and for very small values, *i.e.*, 10^{-9}, a curve is obtained which rapidly approaches a straight line at a point equal to kI_t and has a slope equal to k.

These plots may assist in the interpretation of the experimental data. Malonate with a K_i equal to 10^{-4} gives a straight line through the origin. Itaconate, a less effective inhibitor, is of the same nature. Copper and quinone which combine with sulfhydryl groups probably have very small K_i values, while oxalacetate,[6] an intermediate case (K_i near 10^{-6}), occupies an intermediate position.

Thus it is possible to explain the nature of the experimental curves relating velocity of reaction to enzyme concentration on the basis of the degree of binding of the enzyme-inhibitor complex relative to the enzyme-substrate complex. This approach also provides an experimental method for determining when one is justified in using the Michaelis-Menten equation to calculate values of K_i or when a more exact expression is necessary.

‡ On the basis of the same assumption made in deriving equation (11), Goldstein[3] has developed the following expression:

$$I' = S'\left[\frac{1-a}{a}\right] + (1-a)\,E_i'$$

wherein I', S' and E' represent the "specific concentrations" of I, S and E_t and a is the fractional activity of the enzyme. If one multiplies through the expression of Goldstein by the dissociation constant, K_i, of the enzyme-inhibitor complex, converts the specific concentrations to absolute concentrations and substitutes ES/E_t for a, then on solving the resulting equation for (ES) in terms of I_t, K_i, K_s and E_t one also arrives at expression (11) which was used in developing expression (13).

[6] Pardee, A. B., and Potter, V. R., *J. Biol. Chem.*, 1948, **176**, 1085.

If a plot of data representing the change of velocity of reaction with changes in enzyme concentration appears to intercept the enzyme axis when extrapolated to zero velocity, then the amount of inhibition will vary with enzyme concentration. In such a case the value of K_i determined from the Michaelis-Menten equation will not be a constant but will vary depending on the enzyme concentration used in the experiment and a more exact expression than the Michaelis-Menten equation is necessary for calculating K_i. We have not attempted to derive such an expression because in the case of "titration type" curves the results can be due either to high affinity or true irreversibility as explained earlier.

The analysis represented by equation (11) is incomplete insofar as it does not encompass the effect of the presence of substances other than the enzyme that can combine with the inhibitor. It is clear that the more specific is the inhibitor the less important these considerations will be.

Discussion. The main purpose of this paper is (1) to show that in the case of "irreversible" inhibitors, regardless of the nature of the irreversibility, the degree of enzyme inhibition depends upon the enzyme concentration and (2) to consider the implications of this fact. The first question that arises is whether the phenomenon of decreasing per cent inhibition with increasing enzyme amount is a general phenomenon for high affinity inhibitors, or whether the data presented here are merely due to the fact that the high affinity inhibitors are non-specific and are used in a whole homogenate. We believe that the phenomenon is a general one and that increasing numbers of examples will be found (*cf.* Bain[1]).¶ It is difficult to see how the presence of the other SH groups in the homogenate could produce the type of curve seen with copper or quinone in contrast to the malonate curve, since the ratio between succinoxidase SH groups and "other" SH groups would remain constant as the amount of homogenate was increased. The enzyme system and inhibitors chosen for this study are probably not the best possible examples for illustrative purposes, but the data and conclusions are strongly supported by the accompanying paper on cholinesterase by Bain.[1] In the case of any "irreversible" inhibitor the degree of inhibition produced at any given molarity will depend upon the concentration of enzyme *and* upon the concentration of other substances that will combine with the inhibitor. This fact applies to any inhibitor, since it can never be assumed that an inhibitor, even though it is known to be highly specific, does not react with unknown constituents in living cells or in preparations therefrom. The reaction with unknown constituents becomes of great importance if it is irreversible. However, the occurrence of side reactions does not invalidate the conclusion that copper and quinone in effect irreversibly inactivate succinoxidase. This inactivation cannot be reversed by succinate, (which has a lower affinity for the *enzyme* than copper does) but can be reversed by glutathione,[7] which has an affinity for *copper* similar to that of the enzyme (cf also Barron and Kalnitsky.[8]) This inhibition is therefore considered to be pseudo-irreversible, *i.e.* it has a dissociation constant, but the constant is so small that the percent inhibition varies with the amount of enzyme.

Oxalacetate, which is an intermediate case, is a potent inhibitor for succinoxidase and is not known to inhibit any other enzyme (its action in the malic dehydrogenase system is an equilibrium effect.)

It is thus concluded that the results are not artifacts and that their significance may be examined.

From the standpoint of studies in enzyme

¶ Note added Oct. 24, 1949: Since this paper was submitted for publication, two additional reports that the enzyme concentration may affect the degree of inhibition have appeared: E. C. Slater reported studies with a partially purified succinoxidase system in *Biochem. J.*, 1949, **45**, 130, especially page 138, and O. H. Lowry, O. A. Bessey and E. J. Crawford have reported the effect of 2-amino-4-hydroxy-6-formylpteridine upon pterine oxidase and commented that "The extremely low dissociation of the enzyme-inhibitor complex permits a virtual titration of the enzyme." (*J. Biol. Chem.*, 1949, **180**, 399).

[7] Hopkins, F. G., Morgan, E. S., and Lutwak-Mann, C., *Biochem. J.*, 1938, **32**, 1829.

[8] Barron, E. S. G., and Kalnitsky, G., *Biochem. J.*, 1947, **41**, 346.

kinetics as carried out *in vitro*, it appears that experiments along the lines suggested by Fig. 1 and 2 would be helpful in the study of any new inhibitor. If the plot of rate against enzyme concentration is a straight line through the origin in the presence of inhibitor (*cf* malonate, Fig. 1) and if the inhibition is independent of the time of addition of substrate (in contrast to Fig. 2) then it is feasible to proceed with the determination of K_i values and tests for competitive inhibition. On the other hand, if the data are comparable to those obtained with copper, quinone, or oxalacetate, reports of inhibitor potency without showing the effect of enzyme concentration will be meaningless while the determination of K_i values and the tests for competitive inhibition by existing methods will also be of little significance.

It is from the standpoint of studies on whole animals or on tissue preparations that the results have the greatest significance. If it is established that the "irreversible" inhibitors (either pseudo-irreversible or irreversible) affect enzyme activity to an extent that depends upon the concentration of the enzyme and the concentration of inhibitor-binding compounds in the tissue, it may be anticipated that when an inhibitor (or drug or chemotherapeutic agent) is injected into an animal different tissues and tissue components will be inhibited to different extents depending on their composition. In other words, a tissue containing a small amount of a given enzyme might have its enzyme completely inactivated by an injection of an inhibitor that would inactivate only a small fraction of the enzyme in tissues containing larger amounts of enzyme and comparable amounts of other reacting components. Data which may be an illustration of this phenomenon are available in a paper by DuBois and Mangun.[9] Following injections of hexaethyl tetraphosphate at a level of 1 mg/kg in rats, they found that the per cent inhibitions of acetyl-choline esterase were 4.5, 22 and 100 in samples of brain, submaxillary gland and serum respectively. If these variations are to be explained as a result of variations in the amount of cholinesterase in the 3 tissues, one would expect the enzyme content of these tissues to vary in the way that was actually observed, *viz.* the authors found relative activity values of 100, 28 and 10 in the 3 tissues from control animals. Thus the absolute amount of enzyme destroyed was very similar in the 3 tissues, *i.e.*, 4.5, 6.2 and 10.0. Injections of higher levels of inhibitor produced 100 per cent inhibition first in submaxillary gland, then in brain. These data do not prove the point that is being made here since many factors obviously affect the data in the case of whole animals; but the viewpoint stressed here may need to be included in any future examination of similar data.

The production of alloxan-diabetes[10] may be cited as another possible example of this type of phenomenon, with the difference that in this case specific cells have been destroyed without the demonstration of inhibition of an enzyme. Alloxan is a general SH inhibitor and it seems very unlikely that it reacts with an enzyme occurring only in the insulin-producing cells. According to our interpretation, these cells would be more vulnerable to alloxan because of smaller amounts of a vital enzyme that is more plentiful in other tissues. The specific destruction of insulin-producing cells by alloxan may serve as a prototype for cancer chemotherapy. Since the enzyme pattern in cancer tissue may conceivably include no enzymes that are not also found in normal tissues (*i.e.* if it arises by a process of enzyme deletion), the chemotherapy of cancer may require the inhibition of an enzyme that is also present in normal tissues. It should be possible to completely inhibit an enzyme present in cancer tissue in small amounts, while producing only partial inactivation of the enzyme in tissues containing it in larger amounts.

The interference phenomenon described in Fig. 2 is of considerable importance in interpreting studies on whole animals, especially since interference between reversible and ir-

[9] Du Bois, K. P., and Mangun, G. H., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 137.

[10] Lukens, F. O. W., *Physiol. Rev.*, 1948, **28**, 304.

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.

[11] Koster, R., *J. Pharmacol.*, 1946, **88**, 39.

[12] Mazur, A., *J. Biol. Chem.*, 1946, **164**, 271.

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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

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