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STUDIES ON INHIBITORS OF THE INSULIN PROTEASE OF RAT LIVER

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Abstract—Much evidence supports the proposal that a soluble protease from liver and muscle is a major agent for insulin destruction in those organs. In the present study a considerable number of low molecular weight compounds have been examined for their ability to inhibit the destruction of the hormone by the enzyme from rat liver. These include amino acid derivatives, small polypeptides, indole and quinoline derivatives, and a diverse group of dyes and dye derivatives. In addition a comparative study was made of the potency of a number of the compounds to inhibit both the insulin protease and chymotrypsin. A partially purified preparation of the insulin protease was analyzed by electrophoretic separation on polyacrylamide gels and the activity shown to be due to essentially a single enzyme. This preparation appeared to destroy insulin at initial rates which were linear by either of two methods. The first, which measured the rate of loss of immunoassayable insulin, was twice as fast as the second, which measured the rate of appearance of radioactive trichloroacetic-acid-soluble peptides from ^{125}I labelled insulin. The latter method was chosen to investigate the mode of inhibition using enzyme kinetic methods of three of the most potent of the inhibitory compounds. The nonapeptide, bradykinin, demonstrated a mixed competitive–noncompetitive pattern of inhibition as also possibly did another inhibitor quinoline-2-thiol. The compound 2,6-dichloroindophenol had a pattern of uncompetitive inhibition.

A number of investigators have shown that the integrity of the insulin molecule is rapidly destroyed by enzymes present in a variety of tissues from the rat [1–4]. From evidence obtained using the rat diaphragm it has been proposed that this inactivation of the hormone by proteolysis is carried out by a soluble enzyme after its entry into the cytoplasm of the cell [5]. Recent reports using isolated liver cells have provided evidence which may support this postulate [6, 7]. The soluble enzyme responsible for this cleavage has been partially purified from both muscle [8] and liver [9] and shown to be a protease which degrades insulin much more rapidly than proinsulin. Both liver perfusion [10] and *in vivo* studies [11] have demonstrated that insulin disappears from the circulation much faster than proinsulin. These latter observations further support the above postulate and argue for the importance of the enzyme in the removal of insulin from the circulation.

Another enzyme of somewhat similar properties has been described in liver plasma membranes [12]. It is probable from quantitative measurements of the degradation of physiological concentrations of insulin [9], however, that the latter activity is of negligible importance.

Polypeptide inhibitors of the soluble enzyme have been described in human plasma [13]. Their presence suggests physiological control of the insulin protease which may enter the blood in small amounts normally from cells that are being replaced and whose action is blocked by the inhibitors. If the activity of the enzyme within the intact cell could be inhibited by low molecular weight compounds (if permeable to the cell membrane) then the possibility exists that circulating insulin levels could thereby be controlled. In the present study a number of such compounds have been investigated together with the enzyme kinetic pattern of inhibition produced by those which are active at the lowest concentrations. In addition certain other properties of the enzyme have been investigated shedding some further light upon its mode of action.

MATERIALS AND METHODS

(^{125}I) Iodoinsulin, containing 1 mole of iodine or less per mole of insulin, was obtained from Cambridge Nuclear Corporation, Billerica, MA.

Insulin (24–25 U/mg), the amino acid and dipeptide derivatives, quinine, chloroquine, DCIP*, indole and its derivatives, diphenyl amine-4-sulfonate, bovine serum albumin (fraction V), dithiothreitol and crystalline chymotrypsin were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. Quinoline-2-thiol and 4-hydroxyquinoline-2-carboxylic acid were obtained from the Aldrich Chemical Co., Milwaukee, WI, U.S.A.

Immunoassay of insulin was carried out by the method of Morgan and Lazarow [14], as modified [15].

Enzyme assay method. The assay was carried out in a similar fashion to the method previously described [8, 9] as follows: The incubation mixture contained the enzyme, 0.1 M Tris–HCl or other specified

* *Abbreviations*: DCIP (2,6 Dichlorophenol indophenol), *t*-BOC (*t*-butyloxy carbonyl-radical), CBZ (carbobenzoxy-radical), DTT (dithiothreitol).

buffer (whose pH is 7.5 at 37°), 5 mM EDTA (pH 7.5) 0.3% bovine serum albumin, approximately 0.1 nM (^{125}I) iodoinsulin together with, where noted, the compounds used as inhibitors in a total volume of 1 ml. After an appropriate period of incubation, usually 10 min, the reaction was stopped by the addition of 1.5 ml of 10% trichloroacetic acid. After centrifugation the supernatant was decanted into another test tube and counted separately from the precipitate as previously described [8, 9] in a Packard Autogamma spectrometer. In the enzyme kinetic studies the quantities of enzyme used were adjusted after a preliminary experiment to give amounts of degradation less than 10 per cent of the total substrate present.

Enzyme preparation. The enzyme used for qualitative assay of inhibition was the crude, dialyzed 100,000 *g* supernatant from liver homogenate [9]. For the study of the mode of inhibition of the most inhibitory compounds, the enzyme was further purified 7-fold by adsorption and elution from calcium phosphate gel as previously described [9].

Polyacrylamide gel electrophoresis of the enzyme preparation. Polyacrylamide gel electrophoresis at pH 8.9 was carried out according to the method outlined in the brochure "Research Disc Electrophoresis Instructions", 1969 (Canalco Co., Rockville, MD) except for the following: electrophoresis was performed at 4° for 4 hr after a pre-run of 4 hr in the presence of 1 mM dithiothreitol while the system was constantly purged with nitrogen to prevent dithiothreitol oxidation. No sample or stacking gel was used as 0.2 ml of calcium phosphate gel purified enzyme in 20% sucrose was carefully layered directly on top of the running gel. After completion of a run the gels were extruded from each of the tubes and placed in capped tubes overnight at 4°. In the morning one gel from the run was frozen on a block of dry ice or with liquid nitrogen. The gel was sliced with an array of razor blades with a uniform spacing between blades of 0.9 mm. Each slice was placed in 0.9 ml of solution containing 3 mg bovine serum albumin, 5 μmoles EDTA and 100 μmoles of Tris-buffer, pH 7.8 (at 23°). The reaction was initiated by the addition of 0.1 ml of approximately 1 nM ^{125}I (iodoinsulin) and terminated 45 min later by the addition of 1.5 ml of 10% trichloroacetic acid. Processing of the samples thereafter was the same as in the assay method described above. Solutions containing dithiothreitol were analyzed for SH content using the method of Stadtman [16], except that it was necessary to extrapolate a plot of the logarithm of the A_{200} readings to zero time because *p*-hydroxymercuribenzoate apparently catalyzes the oxidation of the compound.

RESULTS

Survey of inhibitors. A number of amino acid and dipeptide derivatives (all of the L-configuration), tested for their ability to inhibit insulin degradation, were without effect upon enzyme activity at a concentration of 1 mM. Included were alanyllysine, alanylglutamate, alanyltryptophan, *N*-benzoylarginine, *N*-*t*-BOC-aspartic acid benzyl ester, *N*-acetylphenylalanine ethyl ester, *N*-CBZ phenylalanine-*p*-nitrophenyl ester, N-CBZ-glycylphenylalanine amide and *N*-acetyltryptophan. Though the data are not shown the compounds *N*-acetyltryptophanamide and alanine benzyl ester were slightly inhibitory at the same concentration, 10 and 5 per cent respectively, whereas the naturally occurring nonapeptide, bradykinin, markedly inhibited enzyme activity even at low concentration (73.5 per cent inhibition at a concentration of 0.16 mM).

It was reported much earlier [1] that tryptophan and several other indole derivatives inhibited the degradation of insulin in crude liver homogenates. Those observations have now been extended using the dialyzed 100,000 *g* supernatant solution from liver homogenates. Compounds tested were tryptophan, *N*-acetyltryptophan, tryptophol, indole-3-acetamide, indole-3-acetate, indole-2-carboxylate and indole. Of these only indole, tryptophol and indole-3-acetamide were inhibitory at a concentration of 1 mM (30, 12 and 10 per cent inhibition respectively). The latter compounds were more inhibitory at a concentration of 10 mM (38, 14 and 30 per cent inhibition, respectively) with indole-2-carboxylate being the next most important inhibitor at this concentration (19 per cent inhibition).

The results obtained with indole and its derivatives suggested the testing of quinoline and several of its derivatives. As shown in Table 1 quinoline, itself, was not significantly inhibitory, but a number of its derivatives were. Quinine, chloroquine, and 4-hydroxyquinoline-2-carboxylate were of about equal effectiveness. Quinoline-2-thiol produced the maximum inhi-

Table 1. Quinoline, quinoline derivatives and various dyes as inhibitors of insulin proteolysis

Compound	Concentration (mM)	Percent inhibition
1. Quinoline	2.5	3
2. Quinoline-2-thiol	0.50	36
3. Quinine	2.5	19
4. Chloroquine	2.5	21
5. 5-Hydroxyquinoline-2-carboxylate	2.5	21
6. Methyl orange	1	15
7. Ethyl orange	1	29
8. Methyl red	1	19
9. Methylene blue	1	3
10. DCIP	1	45
11. Diphenylamine-4-sulfonate	5	9

Table 2. Effect of various inhibitors on the proteolysis of insulin by chymotrypsin and insulin protease

Compound	Concentration	Percent inhibition	
		Chymotrypsin	Insulin protease
Tryptophan	1 mM	8	0
	20 mM	40	30
Indole	1 mM	46	30
	2.5 mM	64	—
	10 mM	—	38
Quinine	1 mM	0	—
	2.5 mM	15	19
Methyl orange	1 mM	20	15
Methyl red	1 mM	18	19
DCIP	1 mM	0	45

bition of protease activity since it reduced this activity by 36 per cent.

The effectiveness of the indole and quinoline derivatives suggested enzymic sensitivity to aromatic compounds containing nitrogen. This possibility prompted the investigation of DCIP and several azo dyes as potential inhibitors as shown in Table 1. All of the dyes tested were partially effective, but DCIP possessed the highest degree of potency. The compound diphenylamine-4-sulfonate, which is related in structure to the latter dye, was of borderline effectiveness as an inhibitor even at a concentration five times that of the indophenol dye.

Effect of inhibitors upon chymotrypsin. The possibility exists that the inhibition by the compounds tested might not be specific for the insulin protease from the liver. Therefore, the effect of a number of the compounds upon chymotrypsin was also tested. The results of these experiments are shown in Table 2. The corresponding inhibition of insulin protease taken partially from the text and Table 1 are presented for comparison. Tryptophan and indole possessed greater inhibitory capacity against chymotrypsin than against the insulin protease, whereas in the case of DCIP this effectiveness was very much reversed.

Polyacrylamide gel electrophoretic analysis of degradative activity in the enzyme preparation. The mode of inhibition by selected inhibitors was next examined using an enzyme preparation from rat liver partially

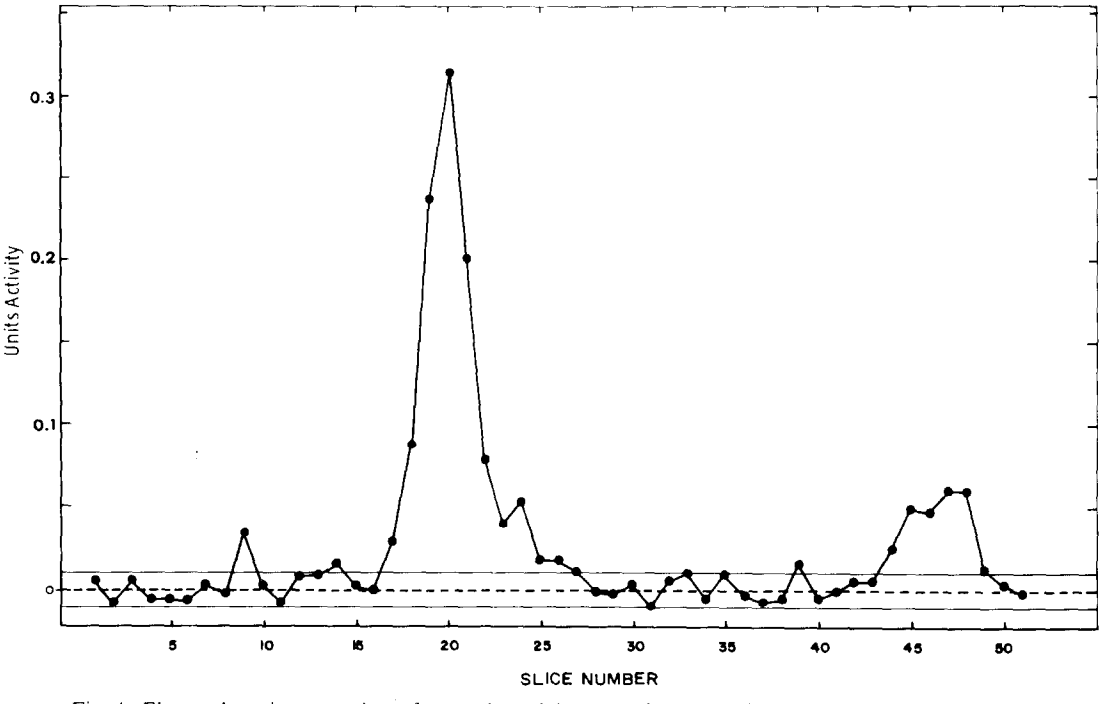

Fig. 1. Electrophoretic separation of enzymic activity on polyacrylamide gel. Calcium phosphate gel purified enzyme was utilized in the study. Ordinate values have had subtracted from them the average value of the blank (5.13%) obtained from control gel slices run under identical conditions, but to which no enzyme had been added. The lines just above and below the ordinate origin represent a measure of the error in the determination of the base line and are two standard deviation units on each side of it. The apparent activity on gel slice 9 is not reproducible and being present on only one slice is considered artifactual.

purified on calcium phosphate gel [9]. First, however, it was necessary to determine if insulin degradation by the preparation was due to a single enzyme species. This question was examined using polyacrylamide gel electrophoresis with analysis of the separated activity on thin sections of the gel. The results of a representative experiment are shown in Fig. 1. The largest peak of activity contained 83 per cent of the total activity with the second peak near the lower end of the gel possessing 17 per cent of the total. The small peak may be an isozyme rather than a contaminating non-specific protease since there is a five million-fold excess of other protein (bovine serum albumin) in the assay media whose digestion would most probably prevent attack by non-specific proteases. In addition the isozyme probably does not possess substantially different kinetics from that of the major peak, or else the kinetic data plotted in double reciprocal form and shown in Figs 3–5 would depart from linearity.

Enzymatic degradation of insulin by immunoassay and by solubilization in trichloroacetic acid. In Fig. 2 is presented a further study to determine the validity of initial rate measurements in enzyme kinetic studies

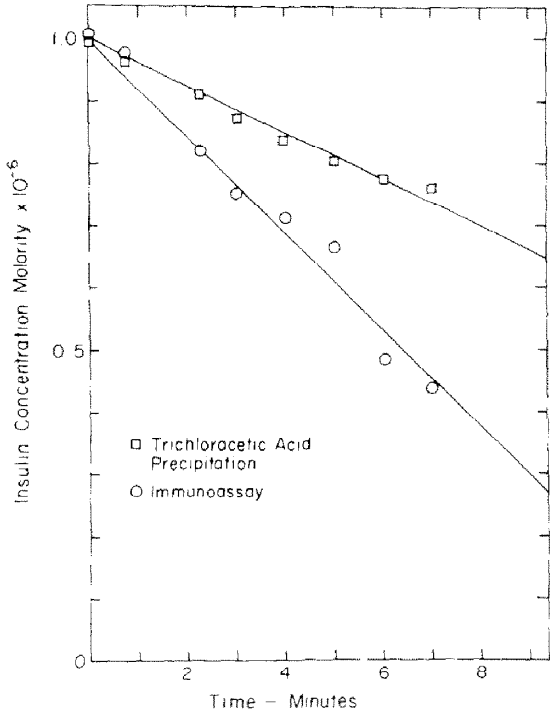

Fig. 2. Rate of proteolysis of insulin as measured by trichloroacetic acid precipitation and by insulin immunoassay. The incubation mixture contained Tris HCl buffer, EDTA, bovine serum albumin and (^{125}I) iodoinsulin as described in Methods. In addition it contained 10^{-6} M insulin and 3 ml of calcium phosphate gel purified enzyme [7] in a total volume of 10 ml. At the indicated times 1.0 ml of the mixture was transferred to 1.5 ml cold 10% trichloroacetic acid and centrifuged at 4°. The supernatant was decanted and counted as described. The precipitates in each case were counted while still cold. Each of the precipitates was then dissolved in 1.0 ml of 0.1 M Tris, diluted 1600 times in the borate-bovine serum albumin mixture and immuno-assay performed as described [17].

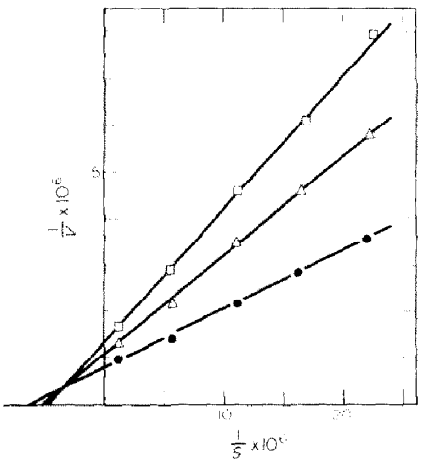

Fig. 3. Effect of bradykinin upon enzyme activity. (●) No bradykinin; (△) + bradykinin, 52.5 μM; (□) + bradykinin, 105 μM. Units of S are moles/l and of v are moles/l/10 min/ml enzyme. See Methods for assay.

of inhibitor action. In that study the relative rate of the degradation of insulin is measured by immunoassay and by the trichloroacetic acid precipitation method. As can be seen the rate is linear by both methods for at least 6 min during which time approximately 24 per cent has become trichloroacetic acid soluble and 48 per cent has been converted to non-immunoassayable products. Therefore, the latter rate is twice that of the former. The trichloroacetic acid method is, however, less complex. In addition it measures the initial rate of product formation instead of the inherently less accurate rate of substrate disappearance.

Mode of enzyme inhibition. Of the compounds which were found to be inhibitory, three of the most potent were selected for the study of their mode of inhibition, namely bradykinin, DCIP, and quinoline-2-thiol.

In Fig. 3 are presented Lineweaver Burk plots of the data obtained for bradykinin as inhibitor with the slope and intercept determined by least squares regression fit of the data in double reciprocal form. The inhibition constant determined from comparison of the slopes of the two curves in the presence of the inhibitor to that in its absence yields an average value and standard error for the two determinations of 0.082 ± 0.004 mM. The corresponding constant obtained from the two intercept values is 0.153 ± 0.003 mM. Thus, these two constants are significantly different from each other.

In Fig. 4 are presented the results of the kinetic study obtained in the presence of DCIP. A series of lines parallel to the line obtained in the absence of inhibitor is evident. The slopes of lines were obtained by a least squares fit of the data in double reciprocal form as in Fig. 3. The slopes were then averaged and this value then used to provide a least squares fit to the data with the assumed average slope. In a similar manner to that described above this yielded intercept values from which a dissociation constant and standard error of 0.19 ± 0.02 mM was obtained.

In Fig. 5 are presented kinetic results obtained with the inhibitor, quinoline-2-thiol. The lines drawn according to the method of fitting the data described

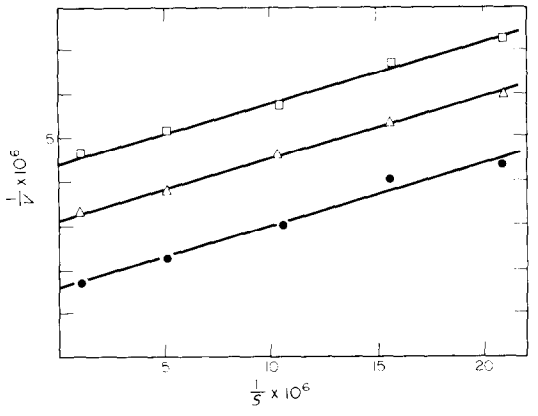

Fig. 4. Effect of DCIP upon enzyme activity. (●) No DCIP; (△) + 0.17 mM DCIP; (□) + 0.35 mM DCIP. Units of S and v are the same as in Fig. 3. See Methods for assay.

above indicate a pattern similar to that obtained for bradykinin. The K_i from the slopes of the two inhibited lines yields a value of 0.88 ± 0.07 mM. Determination of this constant from the intercept values is 1.16 ± 0.10 mM. The two values are not significantly different from each other, and therefore, the mechanism can equally well be interpreted in terms of non-competitive inhibition.

The K_m for insulin as measured by the data of Figs 3–5 is 0.12 ± 0.02 μM.

DISCUSSION

In a previous study [9] it was shown that the two compounds, 1-butyl-3 (*p*-tolylsulfonylurea (tolbutamide) and 1-phenylethylbiguanide (phenformin) were inhibitors of the insulin protease of rat liver. In the present study a much larger group of compounds have been tested in a search for ones which are effective at lower concentrations. Of the amino acid and dipeptide derivatives none were inhibitory except those containing tryptophan, the latter effect having been reported much earlier [1]. The nonapeptide bradykinin, however, was quite potent even though there is no tryptophan in the molecule. Other derivatives of indole related to tryptophan but possessing no charge were effective at concentrations of 1 mM where tryptophan is not inhibitory. This may suggest an interaction with hydrophobic regions of the molecule. Addition of one more carbon atom to the indole ring containing nitrogen to form quinoline, however, nearly abolishes activity whereas addition of other functional groups to the quinoline nucleus enhances inhibitory potency especially in the case of quinoline-2-thiol. The azo dyes tested also were somewhat effective but a stronger inhibitor is the redox dye DCIP.

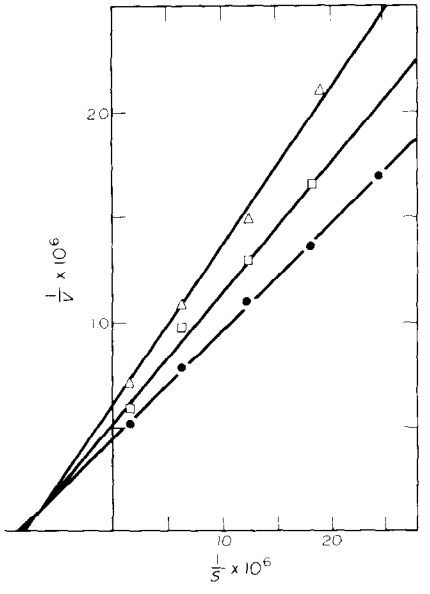

Fig. 5. Effect of Quinoline-2-thiol upon enzyme activity. (●) No quinoline-2-thiol; (□) + 0.2 mM quinolinethiol; (△) + 0.4 mM quinolinethiol. Units of S and v are the same as in Fig. 3. See Methods for assay.

Recent studies with proteinase inhibitors from the snail, *Helix pomatia* [17] have suggested the insulin protease possesses a strength of interaction with inhibitors more similar to chymotrypsin than trypsin [18]. The comparison of a number of inhibitors tested herein against both enzymes also indicates some similarity to chymotrypsin. The two enzymes are distinctly different, however, with respect to DCIP which inhibits only the insulin protease and benzylsulfonylfluoride which inhibits only chymotrypsin [8, 19]. A third essential difference is of course that the insulin protease is inactivated by the sulfhydryl reagents, *N*-ethylmaleimide and *p*-hydroxy-mercuribenzoate [8, 9], whereas chymotrypsin is not.

The data of Fig. 1 indicate that the hydrolytic reaction is probably carried out by a single enzyme. The results presented in Fig. 2 demonstrating a difference in the rate of the enzymic reaction by the two different methods of assay are difficult to interpret mechanistically. Recent results [20] have demonstrated dispersion, recovery losses and diminution of specific activity in individual fractions upon ion exchange chromatographic separation of the enzyme. Restoration of the total and specific activity occurred upon recombining the active fractions strongly suggesting the presence of dissociable subunits in the enzyme. The latter aspect of enzyme structure may be related in some manner to the observed rate differences presented in Fig. 1.

The results of Fig. 3 utilizing the nonapeptide, bradykinin, as an inhibitor gave an unusual pattern of inhibition similar to that obtained earlier with phenylethylbiguanide [9]. This pattern has been termed mixed competitive and noncompetitive inhibition [21]. A similar interpretation may also be applicable to the data of Fig. 5 for quinoline-2-thiol but the data is not conclusive.

Using DCIP a pattern of inhibition characteristic of uncompetitive inhibition was obtained. In deriving the equation for this mechanism it must be assumed that the inhibitor can combine only with the enzyme-substrate complex or perhaps with an enzyme-product complex derived from it [22]. Its meaning on a molecular level in the present case is, however, unclear.

Thus, the studies presented offer some newer results shedding additional light upon the enzyme's mode of action and suggesting possible inhibitory compounds of potential usefulness in controlling the enzyme's activity *in vivo*.

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REFERENCES

1. I. A. Mirsky, *Recent Prog. Horm. Res.* **13,** 429 (1957).
2. M. Vaughan, *Biochim. biophys. Acta* **15,** 432 (1954).
3. H. H. Tomizawa, M. L. Nutley, H. T. Narahara and R. H. Williams, *J. biol. Chem.* **214,** 285 (1955).
4. J. L. Izzo, A. Roncone, M. J. Izzo, R. Foley and J. W. Bartlett, *J. biol. Chem.* **247,** 1219 (1972).
5. J. S. Brush and A. E. Kitabchi, *Biochim. biophys. Acta* **215,** 134 (1970).
6. A. Le Cam, P. Freychet and P. Lenoir, *Diabetes* **24,** 566 (1975).
7. S. Terris and D. F. Steiner, *J. biol. Chem.* **250,** 8389 (1975).
8. J. S. Brush, *Diabetes* **20,** 140 (1971).
9. G. A. Burghen, A. E. Kitabchi and J. S. Brush, *Endocrinology* **91,** 633 (1972).
10. A. H. Rubenstein, L. A. Pottenger, M. Mako, G. S. Getz and D. F. Steiner, *J. clin. Invest.* **51,** 912 (1972).
11. R. W. Stoll, J. L. Touber, L. C. Winterscheid, J. W. Ensinck and R. H. Williams, *Endocrinology* **88,** 714 (1971).
12. P. Freychet, R. Kahn and D. M. Neville, Jr., *J. biol. Chem.* **247,** 3953 (1972).
13. J. S. Brush, *Biochem. biophys. Res. Commun.* **53,** 894 (1973).
14. C. R. Morgan and A. Lazarow, *Diabetes* **12,** 115 (1973).
15. A. E. Kitabchi, W. C. Duckworth, J. S. Brush and M. A. Heinemann, *J. clin. Invest.* **50,** 1792 (1971).
16. E. R. Stadtman, in *Methods in Enzymology*, Vol. III, (Eds S. P. Colowick and N. O. Kaplan) p. 940. Academic Press, New York (1957).
17. J. S. Brush and H. Tschesche, *Proteinase Inhibitors. Proceedings of the Second International Research Conference, Bayer Symposium V.* (Eds H. Fritz, H. Tschesche, L. J. Greene and E. Truscheit) p. 581. Springer Verlag, Berlin (1974).
18. H. Tschesche and T. Dietl, *Eur. J. Biochem.* **30,** 560 (1972).
19. E. E. Fahrney and A. M. Gold, *J. Am. chem. Soc.* **85,** 997 (1963).
20. J. S. Brush, *Abstracts of the Tenth International Congress of Biochemistry, Hamburg, Germany*, p. 417 (1976).
21. M. Dixon and E. C. Webb, *Enzymes*, 2nd edition, p. 324. Academic Press, New York (1964).
22. M. Dixon and E. C. Webb, *Enzymes*, 2nd edition, p. 318. Academic Press, New York (1964).