BBA 66643

INACTIVATION OF GLYCOGEN SYNTHASE BY THE TUMOR INHIBITOR VERNOLEPIN

CARL H. SMITH*, JOSEPH LARNER, A. M. THOMAS AND S. MORRIS KUPCHAN
Departments of Pharmacology and Chemistry, University of Virginia, Charlottesville, Va. 22903 (U.S.A.)

(Received February 28th, 1972)

SUMMARY

Vernolepin, a tumor inhibitor extracted from *Vernonia hymenolepis*, is known to react with thiol compounds to form thioethers. It has now been found to inactivate the enzyme glycogen synthase (UDPG:glycogen α-4-glucosyltransferase, EC 2.4.1.11), purified from rabbit muscle.

To measure the stoichiometry of the reaction with the enzyme protein, a procedure for tritiation of vernolepin was developed. Reaction with 3 moles of radioactive reagent per 90 000-dalton subunit caused virtually complete loss of activity. The concurrent disappearance of 3 titratable thiol groups (out of 6) indicates that thioether formation is the major mode of binding to the protein. The persistence of 1 to 2 residual thiol groups in the enzyme subunit, even after incubation with vernolepin in the presence of sodium dodecyl sulfate, points up the selectivity in the reaction with vernolepin.

Inactivation is kinetically pseudo-first-order and biphasic, suggesting intermediate formation of partially active enzyme species. In the initial portion of the reaction, a comparison of the rates of incorporation and inactivation suggests a phase transition of previously modified protein. In the final portion of the reaction, the correspondence of the two rates indicates a direct relationship between the two processes. Based on these studies, a model of the reaction course has been formulated.

INTRODUCTION

Vernolepin (Fig. 1) and other naturally occurring α-methylene γ-lactones have been shown to inhibit tumor proliferation *in vitro* and *in vivo*[1]. Vernolepin has also been shown to be a reversible inhibitor of plant growth (ref. 2 and L. Sequeira and S.

* Present address: Department of Pathology, Washington University School of Medicine, St. Louis, Missouri 63110.

M. Kupchan, unpublished observations). Comparison of the relative cytotoxicities of sesquiterpene lactones and their derivatives has demonstrated that the α-methylene lactone function is essential to activity[3].

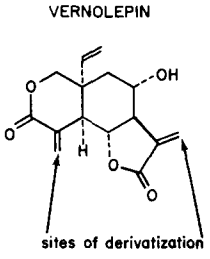

Fig. 1. Vernolepin molecule. The two arrows indicate the sites of derivatization.

Several lines of evidence indicate that the reaction of α,β-unsaturated lactones with thiols may underlie the growth inhibitory properties of these compounds. The inhibition of plant growth by protoanemonin[4], heliangine[5,6], and vernolepin (ref. 2 and L. Sequeira and S. M. Kupchan, unpublished observations) is prevented by 2,3-dimercaptopropanol and other sulfhydryl compounds. Cysteine antagonizes the selective growth-inhibitory action of Δ-hexanolactone on certain animal tissues[7]. A direct and reversible reaction between the lactone and the thiol grouping was demonstrated[8–10]. A study of the reactions of tumor inhibitory α-methylene lactones with model biological nucleophiles revealed that thiols were the most reactive of the nucleophiles investigated, and that biological activity decreased, markedly, with successive addition of cysteine to bis-unsaturated lactones[11]. Recently, the tumor-inhibitory α-methylene lactones were shown to inhibit phosphofructokinase with concurrent loss of enzyme sulfhydryl groups[12]. All of the foregoing evidence thus indicates that the addition of the biologically important thiols to the α,β-unsaturated carbonyl function is the major mechanism by which sesquiterpene lactones exert their effects on cell growth.

It has been shown that the degree of cytoxicity of α-methylene lactones varies with their hydrophobic character[3]. This relationship may result either from an effect upon the transport of the tumor inhibitor into the cell, or from the chemical environment of the specific intracellular thiol to be alkylated. Considerations of this nature led to the speculation that sesquiterpene lactones may have a dual selectivity; generally for thiols over other nucleophiles, and specifically for particular sulfhydryl groups within the protein molecule. To explore this possibility, as well as the potential usefulness of these compounds in following the kinetics of enzyme inactivation in a system of inherent interest, we have studied the interaction of vernolepin with muscle glycogen synthase (UDPG:glycogen α-4-glucosyltransferase, EC 2.4.1.11). This enzyme exists in two forms differing in their state of phosphorylation[13,14]. It has been shown recently to be composed of 90 000-dalton subunits[15,16]. Each of these subunits contains six sulfhydryl groups and six phosphorylatable serine sites. With this information on the protein structure, it was of interest to study the inactivation of the enzyme with vernolepin. A procedure for tritiation of vernolepin was therefore developed. This labelled reagent was used to determine the stoichiometry of complete en-

zyme inactivation and to compare the rates of enzyme inactivation and vernolepin incorporation.

METHODS

Preparation of enzyme

Glycogen synthase, as the fully phosphorylated dependent or D form was prepared as described by Brown and Larner[17]. For study of inactivation with non-radioactive vernolepin, the enzyme preparation was carried to the stage of the glycogen–enzyme complex obtained by precipitation with 15% ethanol. For incubation with radioactive vernolepin, amylase digestion and gel filtration with Sepharose 4B were employed to render the enzyme virtually homogeneous[16]. Amylase (EC 3.2.1.1) digestion was conducted at room temperature and gel filtration at 4 °C to preserve maximum activity. After elution from the column, glycogen was added to a concentration of 0.5 mg/ml and the enzyme was concentrated with Ficoll as described previously[17]. Therefore, all of the experiments reported have been done with enzyme in the presence of glycogen.

Enzyme inactivation

Vernolepin (isolated from *Vernonia hymenolepis*[18]) was dissolved in methanol to give a stock solution of 10–20 mg/ml. For measurement of enzyme inactivation and radioactivity incorporation, glycogen synthase was diluted with 50 mM Tris, 5 mM EDTA and 0.1% glycogen to give protein concentrations of 0.05–0.3 mg/ml. Vernolepin was then added and the reaction mixture incubated at 30 °C. When the protein concentration exceeded 0.15 mg/ml, a wrist action shaker was used during incubation to minimize any tendency of the enzyme to precipitate. For enzyme assay, aliquots of the reaction mixture were removed and added to a solution of the same composition as that used for dilution of the stock enzyme, but containing mercaptoethanol at a final concentration of 50 mM. The diluted aliquots were incubated for 30 min at 30 °C, to render inert the excess vernolepin, and then cooled in ice. All samples from a 3-h vernolepin incubation were then assayed together.

Measurement of radioactive vernolepin incorporation into protein

To minimize variation between samples, a modification of the protein kinase[19] (EC 2.7.1.37) and glycogen synthase[20] assays was employed for measurement of radioactivity incorporation. Aliquots of the reaction mixture were removed and pipetted onto rectangular pieces of 31 ET filter paper, 0.5 cm × 1 cm. The papers were dropped into a beaker of 15% trichloroacetic acid containing 50 mM mercaptoethanol maintained at 4 °C. The solution was mixed with a magnetic stirrer shielded by a stainless steel screen. The protein precipitate was retained within the fibers of the paper. Papers from a given assay were treated in this solution for 20 min after the final sample was taken, and then washed 4 times with methanol, each for 0.5 h, to remove remaining non-protein bound radioactivity.

Papers still wet with methanol were placed in individual counting vials and 1 ml of 90% aqueous NCS Solubilizer (Nuclear-Chicago) was added. The vials were incubated for 4 h at 45 °C to solubilize the radioactivity; 10 ml of a solution containing 0.5% PPO and 0.03% POPOP in toluene was then added, and the ^3H counted in a

Model 3375 Packard Tri-Carb, or a Beckman Model LS 233 liquid scintillation counter. Quenching from the liquid medium was evaluated using an external standard. In addition, blanks, consisting of vernolepin added to the glycogen containing solution used for enzyme dilution, were pipetted onto papers and carried through the entire washing and solubilization procedure.

This procedure gave radioactivity yields equal to those obtained by collecting and washing the protein precipitates on glass fiber filters. To assess the reproducibility and completeness of solubilization, multiple samples were taken after 30 min inactivation and the time course of extraction studied. Extract radioactivity counts reached a maximum after 3 h incubation at 45 °C and did not further increase with up to 24 h extraction. Variation was less than 5% between four samples washed and solubilized together. Blank values (subtracted from the sample counts) were 150 and 400 cpm, approximately 1/7 to 1/2 the sample counts.

Determination of [^{3}H]vernolepin specific activity

The concentration of a sample of labeled vernolepin in methanol was determined accurately, spectroscopically. A standard solution of vernolepin was prepared and the extinction coefficient determined at 210 nm with a Zeiss PMQ-II spectrophotometer. The absorbance of the sample was then determined immediately with the same instrument and its concentration calculated. Aliquots of this same sample were then counted in the scintillation mixture described above.

Analytical procedures

Glycogen synthase activity, protein, and protein sulfhydryl content were all measured as previously described[16]. The incubation mixture for assay of glycogen synthase contained 10 mM glucose 6-phosphate and 10 mM Na_2SO_4.

Melting points were determined on a Mettler FP_2 hotstage and are corrected. Mass spectra of the deuterated vernolepin samples were measured using a Hitachi Perkin–Elmer RMU-6E Mass Spectrometer.

For measurement of sulfhydryl groups lost from the enzyme during vernolepin incubation, mercaptoethanol was added to the reaction mixture to give a final concentration of 50 mM. After 30 min of additional incubation at 30 °C, 0.1 vol. of 10% sodium dodecylsulfate was added and the solution (0.3–0.8 ml) was dialyzed 24–36 h against frequent changes of a solution of 50 mM Tris, 5 mM EDTA, 0.2% sodium dodecylsulfate. Protein and protein sulfhydryl content were then measured. Sodium dodecylsulfate was added to the blank and standard tubes of the Folin determination to give a concentration equal to that added with the synthase mixture.

RESULTS

Enzyme inactivation

Initial experiments showed that incubation of glycogen synthase with a methanolic solution of vernolepin at 30 °C resulted in progressive loss of activity. With vernolepin concentrations of 0.3 to 1.5 mg/ml, inactivation was found to be complete (95 to 99%) within 3 h. In control studies, incubation with the same volume of methanol for 3 h caused no change in activity.

Glycogen synthase had been demonstrated to contain 6 sulfhydryl groups in

each 90 000-dalton subunit. Vernolepin (Fig. 1) has 2 functional groups, each capable of reacting with a cysteine sulfhydryl. To understand the mechanism of its reaction with the enzyme, it was necessary to determine the stoichiometric relationship between thiol groups reacted and vernolepin incorporated into protein at complete inactivation. This was feasible only if a method for determination of incorporated vernolepin could be developed. The preparation of ^{3}H-labeled vernolepin was therefore undertaken.

Preparation of tritiated vernolepin

Conditions for the isotopic labeling of vernolepin were determined using ^2H_2O in phosphoric acid as a model for tritiation. Three separate samples of vernolepin (20 mg) were dissolved in 0.2 ml of phosphoric acid containing 0.1 ml ^2H_2O and 5% palladium on charcoal catalyst. These slurries were heated, with stirring, at 50 °C for 2, 4, and 24 h. Each reaction mixture was worked up by the following method. The catalyst was removed by filtration and washed with 10% aqueous acetone (11 ml). The combined filtrates were concentrated under reduced pressure, the residue was dissolved in 10% aqueous acetone (11 ml), and the solution distilled under reduced pressure (twice). The residual liquid was neutralized with 5% $NaHCO_3$, and the resultant solution extracted 6 times with 10 ml portions of chloroform. The chloroform extracts were evaporated to a gummy solid. The gum was further purified by preparative thin-layer chromatography on 0.5-mm silica gel G plates (E. Merck), eluted with 15% acetone in chloroform, and the vernolepin crystallized from chloroform–hexane.

Both the 2- and 4-h reactions yielded 5–6 mg of vernolepin, m.p. 178–179 °C (lit.[18] m.p. 179–180 °C), containing about 0.8 deuterium atoms per molecule (calculated from the ratio of the *m/e* 276 and 277 peaks in the mass spectrum). The 24-h reaction gave no isolatable vernolepin; thin-layer chromatography showed a high R_F decomposition product.

In accordance with these results, vernolepin (11 mg) was heated at 50 °C, with stirring, for 4 h, in 0.2 ml phosphoric acid containing 10 Ci of ^3H_2O and 15 mg of 5% palladium on charcoal catalyst. After removal of the catalyst, the labile ^{3}H was removed by dilution and evaporation, under vacuum, with 10% aqueous acetone (two 10-ml portions)*. The residue was purified as described above, and diluted with 10 mg of vernolepin to afford product with m.p. 178–180 °C. This material had a specific activity of $10.95 \cdot 10^6$ dpm/μmole.

The low recovery (0.5 mg) of vernolepin from the reaction was explained by the presence of decomposition products, seen on preparative thin-layer chromatography of the crude reaction product. This extensive decomposition probably took place during the interval (2 weeks) between the time that the exchange was carried out and the final product isolated (see Discussion).

Determination of stoichiometry

To determine the stoichiometry of loss of sulfhydryl groups corresponding to complete enzyme inactivation, one portion of the virtually homogeneous enzyme ob-

* The tritium exchange and removal of labile tritium was carried out by New England Nuclear Corp., Boston, Mass.

tained by amylase digestion and gel filtration was incubated 3 h with non-radioactive vernolepin under the conditions previously shown to result in complete loss of activity. A second portion of enzyme was incubated with vernolepin in the presence of 1% sodium dodecyl sulfate, to determine the number of sulfhydryl groups which react with vernolepin under denaturing conditions. A control portion was incubated with methanol under the same concentration but without vernolepin. All enzyme samples contained added glycogen as detailed in Methods. Mercaptoethanol was then added and the excess vernolepin and mercaptoethanol removed by dialysis. Protein and sulfhydryl content were determined.

The results are shown in Table I. In agreement with earlier results[16], 6 thiol groups per 90 000-dalton subunit were found in the control unreacted enzyme. Incubation with vernolepin alone under conditions leading to complete enzyme inactivation resulted in the reaction of 3 sulfhydryl groups. Thus, direct analysis of the enzyme demonstrated that reaction of 3 thiol groups per subunit resulted in virtually complete loss of activity. Incubation with vernolepin in the presence of sodium dodecylsulfate led to reaction of between 4 and 5 sulfhydryl groups, leaving 1 to 2 residual sulfhydryl unreacted even under these denaturing conditions.

TABLE I

CHANGE IN SULFHYDRYL CONTENT WITH VERNOLEPIN INCUBATION

Glycogen synthase was incubated 3 h at 38 °C with methanol (control), vernolepin and methanol, or vernolepin, methanol and 1% sodium dodecylsulfate. Residual thiol content was measured with Ellman's reagent. Reacting thiol groups were calculated by subtraction of the residual thiol content from that of the control. Results are expressed as SH equivalents/90 000 g protein. Values are mean ± S.E., of 3 determinations.

	Residual thiol content (equivalents/subunit)	*Reacting thiol groups (equivalents/subunit)*
Control	6.35 ± 0.04	—
Vernolepin	3.33 ± 0.29	3.02
Vernolepin and sodium dodecylsulfate	1.77 ± 0.12	4.58

Since vernolepin is a bifunctional reagent, it was necessary to determine the number of moles reacting with the enzyme protein at complete enzyme inactivation. For this purpose, the essentially homogeneous enzyme preparation was incubated 3 h with tritiated vernolepin. Activity decreased to approximately 10% of its original value and 2.7 moles of vernolepin were incorporated into each 90 000-dalton subunit (mean of 3 determinations). In conjunction with the earlier finding of 3 thiols derivatized, this result indicated that each mole of vernolepin reacts with one thiol and inactivation thus occurs by monoadduct formation.

Kinetics of inactivation and sulfhydryl reaction

With a knowledge of the overall stoichiometry, the intermediate course of the reaction could next be studied. Initial investigation was conducted with non-radioactive vernolepin using the glycogen-enzyme complex obtained by ethanol precipitation. The time course of inactivation was determined at three vernolepin concentrations and the results are plotted in Fig. 2. Assuming a protein sulfhydryl content of

one per 15 000 molecular weight[16], the vernolepin concentrations used are at least several 100-fold in excess. Therefore, one would expect pseudo-first-order kinetics and accordingly fractional remaining activity is plotted on a logarithmic scale against time. With each of the three concentrations of vernolepin used, a biphasic character of the plot is apparent. Furthermore, the slopes of each portion of each curve vary in direct proportion to the vernolepin concentrations.

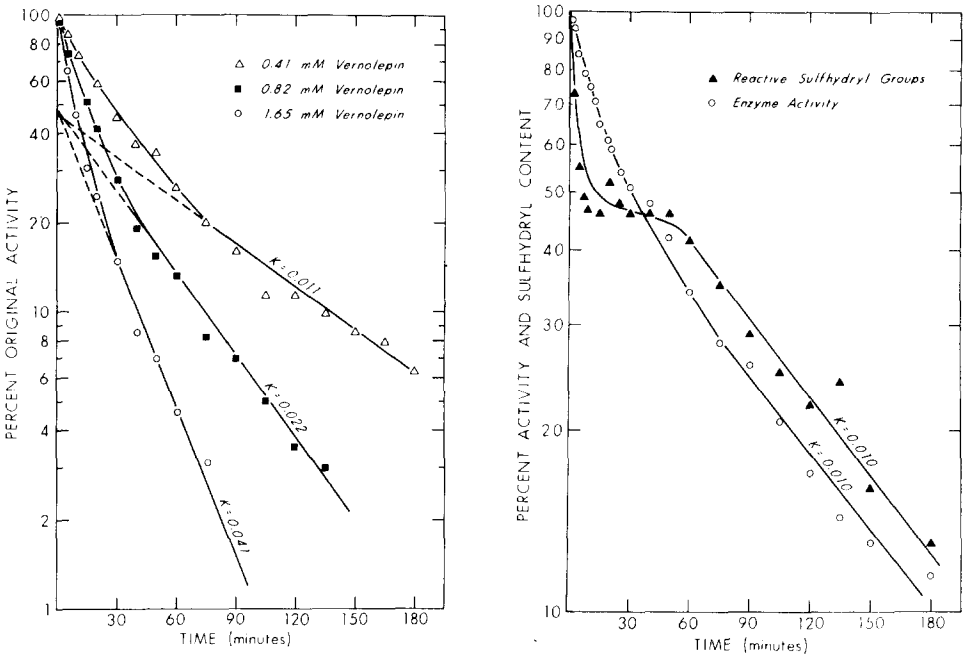

Fig. 2. Kinetics of inactivation of glycogen synthase by vernolepin. The glycogen–enzyme complex was incubated in three test tubes at separate vernolepin concentrations. As described in the text, the final portion has been extrapolated back to the vertical intercept to give F_1, the fractional activity of the rapidly formed intermediate species.

Fig. 3. Kinetics of inactivation and vernolepin incorporation. One portion of the virtually homogeneous preparation was incubated with tritiated vernolepin and aliquots were removed for measurement of enzyme activity and radioactivity incorporation. The incorporation curve shows the percent of the total of three reactive sulfhydryl groups per subunit remaining at each time point.

Ray and Koshland[21] have provided an analysis of biphasic curves of this type. This analysis strongly suggests that in the present inactivation, more than one class of thiol group is involved, and that there is intermediate formation of a partially active enzyme species. It is considered that one group reacts rapidly to yield a partly active molecule, which then reacts with additional inhibitor molecules to give a virtually inactive form. Inactivation must thus involve at least 2 steps with differing rate constants. As described by Ray and Koshland[21], the negative slope of the final portion of the inactivation curve gives the rate constant of the reaction of the slowest reacting sulfhydryl class. The extrapolated zero time intercept of the final portion of the curve

gives the fractional activity of the rapidly formed intermediate species*. In the experiment shown in the figure, this is 0.48. Repeated studies gave similar biphasic curves with fractional values of 0.45 to 0.60.

The simultaneous time courses of enzyme inactivation and radioactive vernolepin incorporation were next studied and are shown in Fig. 3. There is an initial rapid incorporation of vernolepin corresponding to the reaction of between 1 and 2 sulfhydryl groups per 90 000-dalton subunit, which occurs somewhat faster than the accompanying loss of enzyme activity. Following this phase, the enzyme activity decreases steadily while the vernolepin incorporation is considerably slower. In the final portion of the reaction, the rate of enzyme inactivation is identical to that of vernolepin incorporation.

These relationships are better seen in Fig. 4, which is a stoichiometric plot of enzyme activity (as per cent of original) *vs* vernolepin incorporation. The initial

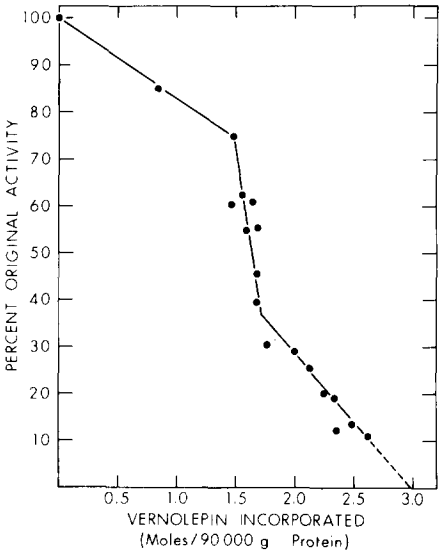

Fig. 4. Stoichiometry of vernolepin incorporation and glycogen synthase inactivation. Data are based on the same experiments as the rate curves shown in Fig. 3.

incorporation (1.5 moles/90 000 g protein) corresponds to the loss of about 25% of enzyme activity. Then the enzyme activity decreases to about 40% of original, with only little additional incorporation of vernolepin as the plot becomes nearly vertical.

* Such mechanism has been represented in the literature as follows:[21,22]

$$\begin{array}{ccc} & \overset{k_1}{\longrightarrow} & \\ E_0 & \underset{}{\longleftarrow} & E_1 \\ k_2 \downarrow & & \downarrow k_2 \\ E_2 & \underset{k_1}{\longleftarrow} & E_{1,2} \end{array}$$

E_1, E_2 and $E_{1,2}$ represent enzyme molecules in which sulfhydryls of class 1, 2 or both have reacted with reagent, and k_1 and k_2 are rate constants for reaction of two sulfhydryl classes. E_0 is fully active enzyme, and $E_{1,2}$ totally inactive. E_1 and E_2 have fractional activities F_1 and F_2. As described in the text, the inactivation plot permits determination of F_1 and k_2.

In the final portion, the curve slopes less steeply and can be extrapolated to 3 bound sulfhydryl groups, at the horizontal intercept.

DISCUSSION

This study has confirmed the reactivity of α-methylene lactones toward sulfhydryl groups and their ability to inactive enzymes. The results agree with those reported for phosphofructokinase (EC 2.7.1.11)[12] and extend the findings to demonstrate their usefulness in kinetic studies of inactivation. In this regard, the increased water solubility of vernolepin relative to the other lactones offers a significant advantage.

The activity of the radioactive vernolepin preparation was lower than predicted by comparisons with the deuteration experiments. This could arise from a poor exchange rate, or from increased decomposition of vernolepin. If isotope effects are ignored, it is possible to calculate a theoretical activity, based on the relative concentration of deuterium and tritium in the two experiments. Each μmole of vernolepin surviving the exchange should have an activity of 72 μCi. The vernolepin actually isolated, after dilution with unlabeled vernolepin, had an activity of 4.95 μCi/μmole. Since 95% of this material was added carrier, the material actually surviving the exchange had an activity of 99 μCi/μmole, in good agreement with theory. This together with the fact that only about 25% of the expected 2.3 mg was recovered, strongly suggested that the low recovery resulted from extensive decomposition from acid exposure during shipment*.

The correspondence between incorporation of vernolepin into the protein and loss of titratable sulfhydryl groups indicates that vernolepin reacts entirely, or almost entirely, with thiol groups of the enzyme and furthermore behaves as a monofunctional reagent. After denaturation with sodium dodecylsulfate, only 4 to 5 thiol groups of the enzyme subunit reacted with vernolepin during a 3-h incubation. Vernolepin thus appears more selective for particular thiol groups than Ellman's reagent, which at approximately the same concentration reacts with 6 sulfhydryl groups within 15–20 min[16]. A similar difference in reactivity between the two reagents was reported in studies with phosphofructokinase[12].

The selective reaction of vernolepin with sulfhydryl groups of proteins is in keeping with the previously noted extreme rapidity of its reaction with model thiols relative to amines[11]. With cysteine, however, bisadducts were formed, whereas with glycogen synthase only monoadduct formation occurred, presumably because of the size and structure of the enzyme. Also, not unexpectedly, the rate of reaction with cysteine as observed previously[11] is very much faster than the reaction with protein sulfhydryl groups observed in this study.

The stoichiometry of the reaction with glycogen synthase indicates that when 3 sulfhydryls of the total of 6 on each 90 000 molecular weight subunit react, the enzyme is virtually inactivated. The biphasic time course of inactivation strongly suggests the intermediate formation of a partially active enzyme species, as depicted earlier. The complete inactivation of glycogen synthase with vernolepin therefore occurs by a pro-

* Immediate removal of acid before shipment should markedly increase recovery.

cess different from the all-or-none mechanism demonstrated so elegantly by Hellerman and coworkers[23] for D-amino acid oxidase (EC 1.4.3.3).

Initially, incorporation of vernolepin into glycogen synthase proceeds considerably faster than activity loss. Inactivation then accelerates as the rate of vernolepin incorporation slows markedly. One possibility that might explain this observation is a change in state of the enzyme; for example, a change in conformation or in degree of aggregation after initial reaction. In the final linear portion of the time course, the rate of inactivation corresponds closely to the rate of incorporation. In this phase, activity loss appears to be directly dependent upon alkylation of the remaining reactive sulfhydryl groups.

Based on these studies, a tentative model for the reaction can be formulated.

$$E + V \xrightarrow{\text{rapid}} \underset{\text{(active)}}{EV_1} \xrightarrow[k_1]{V} \underset{\text{(partially active)}}{EV_2} \xrightarrow[k_2]{V} \underset{\text{(inactive)}}{EV_3} \qquad (1)$$

The native enzyme (E) reacts rapidly with vernolepin (V) to form EV_1 with the loss of 1.5 sulfhydryl groups and a small loss in activity. EV_1 is then converted to EV_2 (rate constant k_1), which is the partially inactivated form with an additional small incorporation of vernolepin. EV_2 in turn reacts with vernolepin (rate constant k_2) to form the completely inactive form, EV_3, with loss of a total of 3 sulfhydryl groups per subunit. The slowest reaction rate constant is k_2. A possibility which might explain the initial course is the participation of two different types of 90 000-dalton subunits which react at different rates.

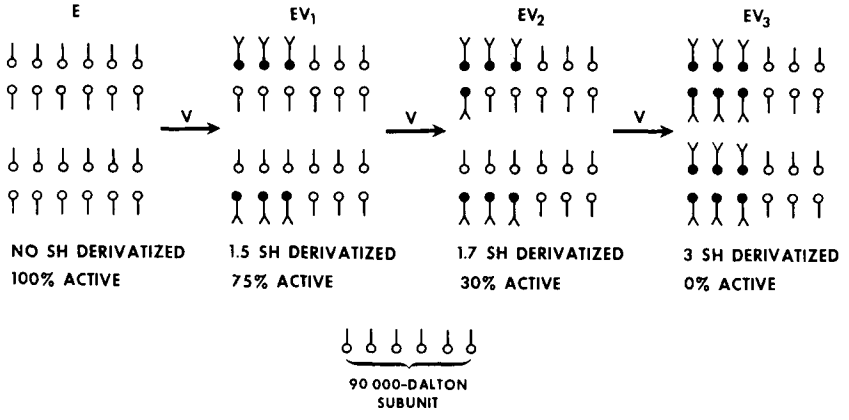

Fig. 5. Physical model of glycogen synthase inactivation. | represents a free sulfhydryl group and Y a thiol derivatized with vernolepin. The enzyme is shown as a tetramer of four 90 000-dalton subunits, each of which contains 6 thiol groups. Formation of the three derivitized forms is discussed in the text.

A physical model to illustrate this mechanism is shown in Fig. 5. The active enzyme is pictured as a tetramer composed of four 90 000-dalton subunits each containing 6 sulfhydryl groups*. The 3 derivatized forms are as shown and labelled according to Eqn 1. Note the large loss in activity accompanying the conversion of EV_1

* Evidence for tetramerer configuration for the enzyme has developed from electron microscopic studies[24].

to EV_2 associated with the derivatization of the third 90 000-dalton subunit, and the complete loss in activity when all four 90 000-dalton subunits are derivatized (EV_3). The fractional activity of an intermediate form determined from the inactivation studies (45 to 60%) would probably represent an average of EV_1 and EV_2.

This investigation has demonstrated that vernolepin reacts with the thiol groups of glycogen synthase by monoadduct formation. Complete inactivation results from reaction of 3 out of 6 thiols per 90 000-dalton subunit. Kinetic studies of inactivation and vernolepin incorporation suggest a model involving intermediate forms with partial activity and partial derivatization of thiols of different subunits. The mechanism of inactivation of this enzyme with this sulfhydryl reagent is therefore clearly different from the all or none one observed with D-amino acid oxidase.

ACKNOWLEDGEMENTS

We thank Dr James Ogilvie of the Department of Biochemistry for his help in analysis of the kinetic data.

This work was supported by grants from the National Institutes of Health, U.S.P.H.S. AM 14334-02 and CA 12059-02. C.H.S. was supported by Grant 5R01, HD 02182, Research Career Development Award, National Institutes of Health. A.M.T. is a Postdoctoral Fellow of the National Instituses of Health (1969–1971).

REFERENCES

1 S. M. Kupchan, *Trans. N.Y. Acad. Sci.*, 32 (1970) 85.
2 L. Sequeira, R. J. Hemingway and S. M. Kupchan, *Science* 161 (1968) 789.
3 S. M. Kupchan, M. A. Eakin and A. M. Thomas, *J. Med. Chem.*, 14 (1971) 1147.
4 K. V. Thimann and W. D. Bonner, Jr, *Proc. Natl. Acad. Sci. U.S.*, 35 (1945) 272.
5 H. Shibacka, *Plant Cell Physiol.*, 2 (1961) 175.
6 H. Shibacka, M. Shimokoriyama, S. Iriuchijima and S. Tamura, *Plant Cell Physiol.*, 8 (1967) 297.
7 T. S. Hauschka, G. Toennies and A. P. Swain, *Science*, 101 (1945) 383.
8 W. B. Geiger and J. E. Conn, *J. Am. Chem. Soc.*, 67 (1945) 112.
9 C. J. Cavallito and T. H. Haskell, *J. Am. Chem. Soc.*, 67 (1945) 1991.
10 L. J. Haynes, *Q. Rev. London*, 2 (1948) 46.
11 S. M. Kupchan, D. C. Fessler, M. A. Eakin and T. J. Giacobbe, *Science*, 168 (1970) 376.
12 R. L. Hanson, H. A. Lardy and S. M. Kupchan, *Science*, 168 (1970) 378.
13 M. Rosell-Perez, C. Villar-Palasi and J. Larner, *Biochemistry*, 1 (1962) 763.
14 D. L. Friedman and J. Larner, *Biochemistry*, 2 (1963) 669.
15 T. R. Soderling, J. P. Hickenbottom, E. M. Reimann, F. L. Hunkler, D. H. Walsh and E. G. Krebs, *J. Biol. Chem.*, 245 (1970) 6317.
16 C. H. Smith, N. E. Brown and J. Larner, *Biochim. Biophys. Acta*, 242 (1971) 81.
17 N. E. Brown and J. Larner, *Biochim. Biophys. Acta*, 242 (1971) 69.
18 S. M. Kupchan, R. J. Hemingway, D. Werner and A. Karim, *J. Org. Chem.*, 34 (1969) 3903.
19 E. M. Reimann, D. A. Walsh and E. G. Krebs, *J. Biol. Chem.*, 246 (1971) 1986.
20 J. A. Thomas, K. K. Schlender and J. Larner, *Anal. Biochem.*, 25 (1968) 486.
21 W. J. Ray and D. E. Koshland, Jr, *J. Biol. Chem.*, 236 (1961) 1973.
22 W. Donhan, Jr and E. E. Snell, *J. Biol. Chem.*, 245 (1970) 4618.
23 L. Hellerman, D. S. Coffey and A. H. Neims, *J. Biol. Chem.*, 240 (1965) 290.
24 L. Rebhum, C. H. Smith and J. Larner, *Abstr. Int. Congr. of Pharmacology*, (1972) in the press.