Cassady, H. K. Schnoes, A. L. Burlingame, *J. Org. Chem.* **34**, 3867 (1969).
6. S. M. Kupchan, J. E. Kelsey, M. Maruyama, J. M. Cassady, *Tetrahedron Lett.* **1968**, 3517 (1968); S. M. Kupchan, J. E. Kelsey, M. Maruyama, J. M. Cassady, J. C. Hemingway, J. R. Knox, *J. Org. Chem.* **34**, 3876 (1969).
7. S. M. Kupchan, R. J. Hemingway, D. Werner, A. Karim, A. J. McPhail, G. A. Sim, *J. Amer. Chem. Soc.* **90**, 3596 (1968); S. M. Kupchan, R. J. Hemingway, D. Werner, A. Karim, *J. Org. Chem.* **34**, 3903 (1969).
8. D. R. Grassetti and J. F. Murray, Jr., *Arch. Biochem. Biophys.* **119**, 41 (1967).
9. Calculated from data of E. S. G. Barron, *Advan. Enzymol.* **11**, 213 (1951).
10. Calculated from data of G. Gorin, P. A. Martic, G. Doughty, *Arch. Biochem. Biophys.* **115**, 593 (1966).
11. S. M. Kupchan, T. J. Giacobbe, I. S. Krull, in preparation.
12. Cytotoxicity against KB cell culture was assayed under the auspices of the Cancer Chemotherapy National Service Center by procedures described in *Cancer Chemother. Rep.* **25**, 1 (1962).
13. Supported by grant CA-04500 from NIH, grant T-275 from the American Cancer Society, and NIH postdoctoral fellowships to D.C.F. and T.J.G.
* Present address: Department of Chemistry, University of Virginia, Charlottesville 22901.

21 January 1970

Inhibition of Phosphofructokinase by Quinone Methide and α-Methylene Lactone Tumor Inhibitors

Abstract. *The plant-derived tumor inhibitors taxodone, taxodione, vernolepin, eupacunin, and euparotin acetate each inhibit the sulfhydryl enzyme, phosphofructokinase. The substrates, fructose-6-phosphate and adenosine triphosphate, protect the enzyme from this inhibition as does the addition of dithiothreitol to the inhibitors. Incubation of taxodione with phosphofructokinase is associated with the loss of about one sulfhydryl group per inhibitor molecule, and the substrates protect six sulfhydryl groups per protomer of 93,000 daltons.*

The conjugated α-methylene lactones and quinone methides taxodone, taxodione, vernolepin, euparotin acetate, and eupacunin have been isolated from plants; they inhibit growth in vivo of the Walker intramuscular carcinosarcoma 256 in rats and inhibit growth in vitro of cells derived from human carcinoma of the nasopharynx (KB) (*1*).

The results of a study of the reactions of conjugated α-methylene lactones with model biological nucleophiles lent support to the view that Michael-type addition of sulfhydryl-bearing compounds may play a significant role in the mechanisms by which the lactones exert their biological activities (*2*). Furthermore, the well-known sensitivity of quinone methides to nucleophilic addition (*3*) and the observation of the rapid addition of the sulfhydryl group of glutathione to taxodone and taxodione (*4*) suggested that these compounds may also act via alkylation of biologically important sulfhydryl groups.

To test the possibility that these inhibitors may be reacting with sulfhydryl enzymes, we have studied their effects on phosphofructokinase, which contains 16 to 18 sulfhydryl groups per protomer of 93,200 daltons (*5, 6*). In their study of the reactivity of these groups with Ellman's reagent at *p*H 7, Kemp and Forest (*5*) found that four of the sulfhydryls titrate more rapidly in the native than in the denatured state and that two sulfhydryls essential for enzymatic activity can be protected by fructose-6-phosphate or adenine nucleotides. Five more sulfhydryl groups become available as the *p*H is increased from 7 to 9. Similarly, Younathan, Paetkau, and Lardy (*6*) found that, at *p*H 8, six sulfhydryl groups react immediately with Ellman's reagent and that two of these can be protected by the addition of fructose-6-phosphate. Younathan *et al.* also found that 60 percent of the enzyme's activity is lost by alkylation of six sulfhydryl groups with iodoacetamide, and that fructose-6-phosphate and magnesium adenosine triphosphate (ATP) protect against this loss of activity.

We now show that the tumor inhibitors inhibit phosphofrucktokinase (ATP: D-fructose-6-phosphate 1-phosphotransferase, E.C. 2.7.1.11) and present evidence that the inhibition results from their reaction with the sulfhydryl groups of this enzyme.

Crystalline phosphofructokinase from rabbit skeletal muscle was prepared by the method of Ling *et al.* (*7*). The crystalline enzyme suspension was dialyzed against a 27 m*M* borate (sodium)–0.03 m*M* EDTA buffer system at *p*H 8. Protein concentrations were determined assuming an optical density of 1.09 mg^{-1} ml^{-1} cm^{-1} in 0.1*N* NaOH (*8*).

The *p*H stat assay for phosphofructokinase was used (*9*). The basic system contained, in a volume of 4.00 ml at *p*H 7 and 30°C: 80 m*M* KCl, 2 m*M* sodium fructose-6-phosphate, 1.2 m*M* *o*-phenanthroline, 5 m*M* sodium ATP, and 5 m*M* $MgSO_4$. Ordinarily, dithiothreitol is used in the assay to protect the sulfhydryls of the enzyme, but *o*-phenanthroline can be substituted for dithiothreitol (*10*). The dialyzed enzyme was diluted about tenfold into 0.1*M* potassium phosphate, 1.2 m*M* *o*-phenanthroline, *p*H 8; and 0.01 ml of this solution was added to the assay system. The NaOH was standardized with potassium biphthalate.

Table 1. The assay system is described in the text. Incubations were at 30°C, *p*H 7.2 to 7.5. In each assay 10.2 μg of phosphofructokinase were used. When there were no additions to system, KCl, *o*-phenanthroline, and inhibitor were incubated for 5 minutes, then phosphofructokinase was added. After 5 minutes, the enzymatic reaction was initiated by addition of ATP, fructose-6-phosphate, and $MgSO_4$. When dithiothreitol was added before phosphofructokinase, KCl, *o*-phenanthroline, inhibitor, and dithiothreitol (4 or 20 μmole in the case of iodoacetamide) were incubated for 5 minutes (or for 10 minutes in the case of iodoacetamide); then phosphofructokinase was added. After 5 minutes the enzymatic reaction was initiated by addition of ATP, fructose-6-phosphate, and $MgSO_4$. When dithiothreitol was added after phosphofructokinase, KCl, *o*-phenanthroline, inhibitor, and phosphofructokinase were incubated for 5 minutes; then dithiothreitol (4 or 20 μmole in the case of iodoacetamide) was added. After 5 minutes (or 10 minutes in the case of iodoacetamide) the enzymatic reaction was initiated by addition of ATP, fructose-6-phosphate, and $MgSO_4$. (The large amount of iodoacetamide used required a larger amount of dithiothreitol to be added and required incubation for a longer time.)

System		Reaction rate with:		
Inhibitor	Amount (μg)	No addition	Dithiothreitol	
			Before PFK	After PFK
None		0.795	0.968	
Taxodone	3.76	0.168	1.15	0.308
Taxodione	0.5	0.78	1.08	0.214
Vernolepin	200	0.165	0.968	0.250
Euparotin acetate	200	0.239	1.09	0.469
Eupacunin	200	0.242	1.00	0.466
None		0.760	1.00	
N-Ethylmaleimide	2	0.101	1.04	0.054
Iodoacetamide	3×10^3	0.187	0.676	0.104
Ellman's reagent	1	0.137	0.987	1.030

For determination of the number of sulfhydryl groups per enzyme molecule, solutions of phosphofructokinase were passed through a Bio-Gel P-10 column and assayed for sulfhydryl content by Ellman's procedure (*11*) and for protein by ultraviolet absorption in 0.1*N* NaOH as described by Younathan *et al.* (*6*).

Taxodione (*12*) was the most potent of the tumor inhibitors in diminishing phosphofructokinase activity. Addition of only 1.6 moles of taxodione per protomer of enzyme reduced activity by 50 percent (Fig. 1). The other quinone methide, taxodone (*12*), inhibited 50 percent at a relative concentration of 32, and the α,β-unsaturated lactones, euparotin acetate (*13*), eupacunin (*14*), and vernolepin (*15*) inhibit 50 percent at relative concentrations of 1000 to 2000. In comparison with some standard sulfhydryl reagents, taxodione is about as effective an inhibitor as *N*-ethylmaleimide and Ellman's reagent, whereas the α,β-unsaturated lactones are about ten times as effective as iodoacetamide. The substrates, fructose-6-phosphate and ATP, protect the enzyme from each of the inhibitors. Concentrations of the quinone methides and standard sulfhydryl reagents required for 50 percent inhibition are 10 to 100 times higher when the substrates are present than when they are absent.

If these compounds are inhibiting phosphofructokinase because of their affinity for sulfhydryl groups, then

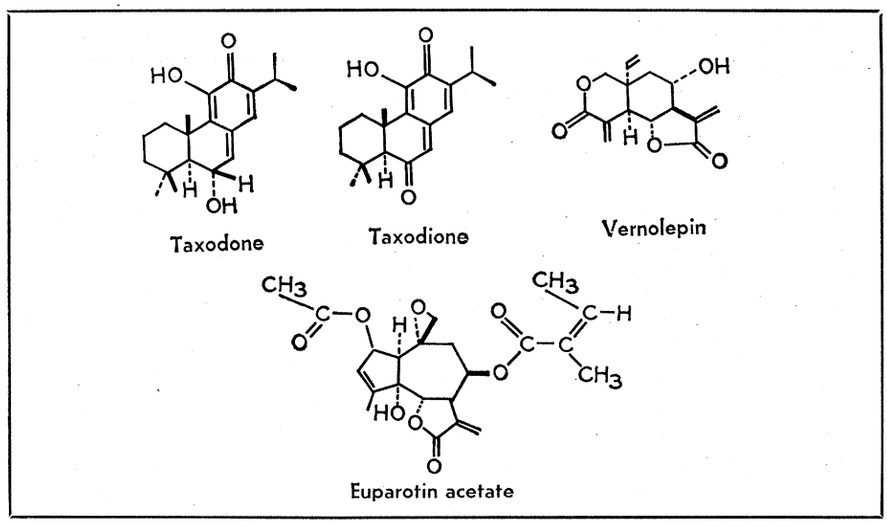

Table 2. Loss of sulfhydryls from phosphofructokinase by reaction with taxodione. Each of four tubes contained 2.36×10^{-2} μmole of phosphofructokinase in 0.5 ml of 16 m*M* borate (sodium), 0.02 m*M* EDTA, *p*H 8. Additions to each tube are indicated in the table. The taxodione concentration in a stock solution was determined spectrophotometrically; the molar extinction ϵ was 25,000 and 26,000 at 320 nm and 332 nm, respectively, in methanol (*12*). The tubes were incubated at room temperature, and 0.1 ml of 100 m*M* dithiothreitol was added to stop each reaction. Dithiothreitol was added to tubes 1 and 2 at 30 minutes and to tubes 3 and 4 at 5 minutes. At 45 minutes, 0.025 ml of 110 m*M* sodium dodecyl sulfate was added to tubes 2, 3, and 4, and the sulfhydryl groups per enzyme molecule were then determined for each tube.

Tube No.	Additions	SH groups per enzyme molecule			
		Experiment 1		Experiment 2	
		Present	Lost	Present	Lost
1	None	18.6	0	18.5	0
2	Taxodione (0.236 μmole), 5.5 m*M* sodium dodecyl sulfate	6.1	12.5	6.6	11.9
3	Taxodione (0.236 μmole)	10.1	8.5	9.0	9.5
4	Taxodione (0.236 μmole), 5 m*M* ATP 2 m*M* Fructose-6-phosphate	16.3	2.3	15.9	2.6

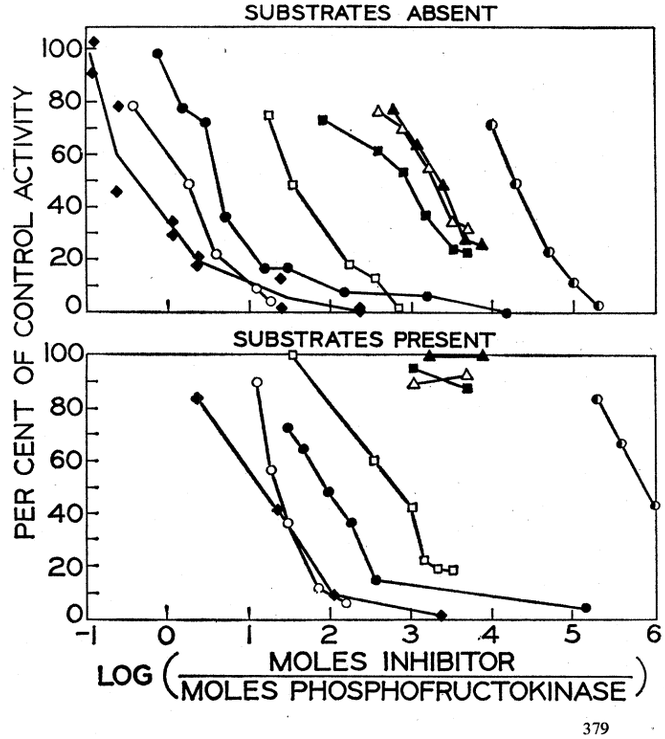

Fig. 1. Inhibition of phosphofructokinase by tumor inhibitors and by some sulfhydryl reagents. The assay system is described in the text. In these experiments 10.2 to 13.9 μg of phosphofructokinase were used. Substrates absent: KCl, *o*-phenanthroline, phosphofructokinase, and inhibitor were incubated for 5 minutes at 30°C, *p*H 7.2 to 7.5. The substrates, fructose-6-phosphate and adenosine triphosphate (ATP), were then added, and 2 minutes later the reaction was initiated by addition of $MgSO_4$. (The tumor inhibitors and *N*-ethylmaleimide were dissolved in 0.06 ml or less of methanol. This amount of methanol was shown not to diminish phosphofructokinase activity. Ellman's reagent and iodoacetamide were dissolved in water.) Substrates present: KCl, *o*-phenanthroline, fructose-6-phosphate, ATP, phosphofructokinase, and inhibitor were incubated for 5 minutes at *p*H 7.2 to 7.5 and at 30°C; then the reaction was initiated by addition of $MgSO_4$. ♦, Ellman's reagent [5,5′-dithiobis-(2-nitrobenzoic acid)]; ○, taxodione; ●, *N*-ethylmaleimide; □, taxodone; ■, euparotin acetate; △, eupacunin; ▲, vernolepin; ◐, iodoacetamide.

treatment of the inhibitors with an excess of dithiothreitol should render them ineffective. The results in Table 1 show that neither the tumor inhibitors nor the standard sulfhydryl reagents inhibit phosphofructokinase if they have first been allowed to react with dithiothreitol (except for iodoacetamide which inhibited 32 percent when treated with dithiothreitol compared with 75 percent when not treated). When dithiothreitol was added after the tumor inhibitor was incubated with enzyme, activity was increased only slightly over that with inhibitor alone. As was expected, the inhibition of phosphofructokinase by *N*-ethylmaleimide or iodoacetamide, each of which forms a carbon-sulfur bond with the enzyme, was not reversed by dithiothreitol; but inhibition by Ellman's reagent, which forms a disulfide bond with the enzyme, was reversible.

Direct evidence for a reaction of taxodione with the sulfhydryls of phosphofructokinase is shown in Table 2. When phosphofructokinase was denatured with sodium dodecyl sulfate, ten molecules of taxodione per enzyme protomer resulted in the loss of about 12 sulfhydryl groups, indicating about a 1:1 reaction. When taxodione (10 moles per mole of enzyme) was incubated with the enzyme for 5 minutes, 5 m*M* ATP plus 2 m*M* fructose-6-phosphate protected six to seven of the sulfhydryl groups of the protomer. This incubation time and the substrate concentration are the same as those used in the kinetics experiment of Fig. 1; thus the prevention of inhibition by substrates is correlated with the protection of sulfhydryl groups. In this experiment, a concentration of phosphofructokinase about 800 times that used in the kinetics experiments was required in order to have sufficient protein for accurate determination of the enzyme's sulfhydryl content. Because of the larger relative concentrations of the other tumor inhibitors required for inhibition and their limited water solubility, they could not be tested in this system (*16*).

RONALD L. HANSON
HENRY A. LARDY
Institute for Enzyme Research, University of Wisconsin, Madison 53706
S. MORRIS KUPCHAN*
Department of Pharmaceutical Chemistry, University of Wisconsin

References and Notes

1. S. M. Kupchan, *Trans. N.Y. Acad. Sci.* **32**, 85 (1970).
2. ———, D. C. Fessler, M. A. Eakin, T. J. Giacobbe, *Science*, this issue.
3. A. B. Turner, *Progr. Chem. Org. Natur. Prod.* **24**, 288 (1966).
4. S. M. Kupchan and M. A. Eakin, unpublished observations.
5. R. G. Kemp and P. B. Forest, *Biochemistry* **7**, 2596 (1968).
6. E. S. Younathan, V. Paetkau, H. A. Lardy, *J. Biol. Chem.* **243**, 1603 (1968).
7. K. H. Ling, V. Paetkau, F. Marcus, H. A. Lardy, *Meth. Enzymol.* **9**, 425 (1966).
8. V. H. Paetkau, E. S. Younathan, H. A. Lardy, *J. Mol. Biol.* **33**, 721 (1968).
9. J. E. Dyson and E. A. Noltmann, *Anal. Biochem.* **11**, 362 (1965).
10. V. Paetkau and H. A. Lardy, *J. Biol. Chem.* **242**, 2035 (1967).
11. G. L. Ellman, *Arch. Biochem. Biophys.* **82**, 70 (1959).
12. S. M. Kupchan, A. Karim, C. Marcks, *J. Amer. Chem. Soc.* **90**, 5923 (1968).
13. S. M. Kupchan, J. C. Hemingway, J. M. Cassady, J. R. Knox, A. T. McPhail, G. A. Sim, *ibid.* **89**, 465 (1967).
14. S. M. Kupchan, M. Maruyama, R. J. Hemingway, S. Shibuya, in preparation.
15. S. M. Kupchan, R. J. Hemingway, D. Werner, A. Karim, A. T. McPhail, G. A. Sim, *J. Amer. Chem. Soc.* **90**, 3596 (1968).
16. Dr. H. I. Hadler (personal communication) has found that vernolepin acts as a thiol reagent in a mitochondrial system.
17. This research was supported in part by NIH grant AM-10334 and by NSF grant GB-6676X. We thank Miss M. Wagner for technical assistance.
* Present address: Department of Chemistry, University of Virginia, Charlottesville 22901.

19 January 1970

Identity of Very Low Density Lipoprotein Apoproteins of Plasma and Liver Golgi Apparatus

Abstract. *In the rat, very low density lipoproteins isolated from hepatocyte Golgi apparatus, liver perfusates, and whole plasma appear identical in many respects. With specific immunochemical techniques and polyacrylamide-gel electrophoresis it can be demonstrated that the very low density lipoproteins from all three sources contain the same major lipoprotein apoproteins.*

The liver is the site of biosynthesis of at least one of the families of plasma lipoproteins, namely the triglyceride-rich, very low density lipoproteins (VLDL) (*1*, *2*). An additional site of origin for VLDL is the intestine (see *2*).

Evidence has been presented from electron microscopy suggesting that the 300- to 1000-Å particles within the hepatocyte Golgi apparatus are the precursors of plasma VLDL (*3*). A cell fraction rich in Golgi apparatus, isolated from rat liver, contains lipoprotein particles similar to plasma VLDL in morphology, immunochemical reactivity, and chemical composition (*4*). Delipidated plasma VLDL in man contains a number of apoproteins whose functional importance and site of origin remain unclear (*5*). It is not known whether all these apoproteins are assembled into VLDL in the liver, reflect VLDL from several tissues, or are partially adsorbed onto the VLDL after its release into the circulation. The first of these alternatives seems likely in that the major apoproteins of plasma VLDL have now been found in the VLDL recovered from liver Golgi apparatus and in the VLDL released from liver during perfusion of the isolated organ.

The Golgi apparatus were isolated from rat livers with the use of a barbital-buffered homogenizing medium and an unbuffered sucrose gradient (*4*). Lipoprotein particles were obtained by sonication of the Golgi apparatus cell fraction in barbital buffer (37.5 m*M* sodium diethyl barbiturate; 7.3 m*M* diethyl barbituric acid at *p*H 8.4) for 20 to 25 seconds (at tap No. 2 with a Branson Sonifier, model S110, equipped with a 13 by 0.3 cm microtip and operated at 3 amperes). The Golgi VLDL were floated free from the fragmented Golgi membranes at a density of 1.006 by ultracentrifugation at 100,000*g* for 16 hours (No. 50 Spinco rotor). They were removed in a small volume, mixed with 0.15*M* sodium chloride and recentrifuged in a similar manner. The low density lipoproteins (LDL) were isolated at densities of 1.006 to 1.035 (*6*).

Livers of 290-g rats were perfused in situ for 6 hours at 37°C at a flow rate of 20 ml/min with a plasma-free medium (*6*). The perfusate consisted of Krebs-Ringer bicarbonate buffer plus 5 percent (weight to volume) crystalline bovine serum albumin, 0.1 percent glucose, a sufficient number of washed rat erythrocytes to give a hematocrit of 25 percent, and a mixture of 11 amino acids (*7*). Perfusate VLDL were isolated and washed twice in 0.15*M* sodium chloride by ultracentrifugation at 100,000*g* for 16 hours. The VLDL isolated from the Golgi apparatus, perfusate, and plasma were dialyzed against 0.01 percent ethylenediaminetetraacetate in distilled water (*p*H 7) for 24 to 36 hours. These native lipo-