Kasahara, M., & Hinkle, P. C. (1977) *J. Biol. Chem. 252*, 7384–7390.
Korenstein, R., & Hess, B. (1977) *FEBS Lett. 82*, 7–11.
Lindsey, H., Petersen, N. O., & Chan, S. I. (1979) *Biochim. Biophys. Acta 555*, 147–167.
Lozier, R. H., Niederberger, W., Ottolenghi, M., Sivorinovsky, G., & Stoeckenius, W. (1978) in *Energetics and Structure of Halophilic Microorganisms* (Caplan, S. F., & Ginzburg, M., Eds.) pp 123–141, Elsevier/North-Holland, Amsterdam.
Mowery, P. C., Lozier, R. H., Chae, Q., Tseng, Y.-W., Taylor, M., & Stoeckenius, W. (1979) *Biochemistry 18*, 4100–4107.
Nagle, J. F., & Wilkinson, A. D. (1978) *Biophys. J. 23*, 159–175.
Oesterhelt, D., & Stoeckenius, W. (1971) *Nature (London), New Biol. 233*, 149–152.
Oesterhelt, D., & Stoeckenius, W. (1974) *Methods Enzymol., 31*, 667–678.
Oesterhelt, D., Meentzen, M., & Schuhmann, L. (1973) *Eur. J. Biochem. 40*, 453–463.
Ohno, K., Takeuchi, Y., & Yoshida, M. (1977) *Biochim. Biophys. Acta 462*, 575–582.
Pettei, M., Yudd, A. P., Nakanishi, K., Henselman, R., & Stoeckenius, W. (1977) *Biochemistry 16*, 1955–1959.
Reynolds, J. A., & Stoeckenius, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2803–2804.
Sperling, W., Carl, P., Rafferty, Ch. N., & Dencher, N. A. (1977) *Biophys. Struct. Mech. 3*, 79–94.
Stoeckenius, W., Lozier, R. H., & Bogomolni, R. A. (1979) *Biochim. Biophys. Acta 505*, 215–278.
Sumper, M., Reitmeier, H., & Oesterhelt, D. (1976) *Angew. Chem., Int. Ed. Engl. 15*, 187–194.

In Vitro Inhibition of Vitamin K Dependent Carboxylation by Tetrachloropyridinol and the Imidazopyridines†

Paul A. Friedman* and Anne E. Griep

ABSTRACT: The compounds 2,3,5,6-tetrachloro-4-pyridinol (TCP) and the structurally related imidazopyridines (IP) cause hemorrhage and lower the plasma prothrombin level in animals. In vitro, TCP and the IP are more potent inhibitors of both the vitamin K dependent carboxylase which catalyzes the posttranslational γ-carboxylation of specific glutamyl residues in proteins and the related vitamin K epoxidase activity than they are either of vitamin K epoxide reductase or of NAD(P)H-K oxidoreductase. TCP and IP, as is the case with the coumarin and indandione anticoagulants, are competitive inhibitors of NAD(P)H-K oxidoreductase with respect to NADH. The epoxide reductase from coumarin-resistant rats is quite resistant to inhibition not only by warfarin but also by the IP, and to a lesser extent by TCP. When interpreted in light of published in vivo experiments, the data suggest that the principal site of anticoagulant action of the IP, but not TCP, is the epoxide reductase. The anticoagulant effect of TCP may be inhibition of the carboxylase itself. TCP is a significantly more potent inhibitor of the carboxylase and epoxidase than the IP; it inhibits both the enzymatic activities to the same degree with 50% inhibition observed at about 10^{-5} M. Inhibition of the carboxylase by TCP is not competitive with respect to the pentapeptide substrate phenylalanylleucylglutamylglutamylleucine nor with respect to the following components of the in vitro carboxylase assay: imidazole, pyridoxal 5′-phosphate, dithiothreitol, KCl, sodium bicarbonate, oxygen, and vitamin K. The order of addition of components of the assay relative to the addition of inhibitor did not affect the degree of inhibition. Inhibition is readily reversed in experiments designed to dissociate an enzyme–inhibitor complex. Analysis of double-inhibitor experiments suggests that TCP and IP have the same binding site on the carboxylase.

The posttranslational γ-carboxylation of specific glutamyl residues in proteins (Stenflo et al., 1974; Nelsestuen et al., 1974) is catalyzed by a vitamin K dependent carboxylase (Esmon et al., 1975) which is located in the endoplasmic reticulum (Helgelund, 1977). The basic requirements of the carboxylase, which has not yet been purified extensively, include oxygen, CO_2 or bicarbonate, and the hydroquinone form of the vitamin (Esmon et al., 1975; Friedman & Shia, 1976; Jones et al., 1976; Girardot et al., 1976). In addition to the carboxylase, several other enzymes are required for the normal in vivo function of vitamin K. In particular, NAD(P)H-K oxidoreductase ("DT-diaphorase", EC 1.6.99.2) or a very similar enzyme is required for the reduction of vitamin K quinone to the active hydroquinone (Wallin et al., 1978). In vivo and during in vitro incubations with microsomes prepared from a variety of tissues, vitamin K is converted enzymatically to a 2,3-epoxide (Matschiner et al., 1970; Willingham & Matschiner, 1974). Although epoxidation appears to be associated with the vitamin K dependent carboxylation event (Willingham & Matschiner, 1974; Willingham et al., 1976; Friedman & Smith, 1977, 1979), coupling of the two reactions has not been demonstrated. The epoxide itself is inactive in supporting carboxylation, yet another enzyme, vitamin K epoxide reductase, also located in the endoplasmic membrane, will reduce the epoxide to the quinone form of the vitamin (Matschiner et al., 1974; Zimmerman & Matschiner, 1974; Willingham & Matschiner, 1974; Whitlon et al., 1978).

The anticoagulants, the coumarins and indandiones, are potent inhibitors of both the DT-diaphorase (Ernster et al., 1962) and the epoxide reductase activities (Bell & Caldwell,

† From the Center for Blood Research, Boston, Massachusetts 02115, and the Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115. *Received October 11, 1979.* This study was supported by U.S. Public Health Service Grant HL 25066. P.A.F. is a recipient of U.S. Public Health Service Research Career Development Award HD 00023.

0006-2960/80/0419-3381$01.00/0

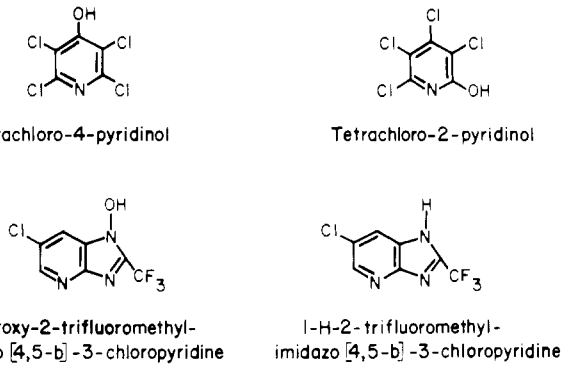

FIGURE 1: Structures of the compounds studied.

1973; Matschiner et al., 1974; Willingham & Matschiner, 1974; Whitlon et al., 1978) but not of the vitamin K dependent carboxylase or the vitamin K epoxidase activity (Whitlon et al., 1978). Recent data would suggest that their anticoagulant action results primarily from inhibition of the epoxide reductase (Whitlon et al., 1978). The compounds 2,3,5,6-tetrachloro-4-pyridinol (TCP)[1] (Marshall, 1972a; Ren et at., 1974) and the structurally related imidazopyridines (IP) (Bang et al., 1975) (Figure 1) cause hemorrhage and lower the plasma prothrombin level in animals. TCP inhibits both carboxylation (Friedman et al., 1979) and epoxidation (Willingham & Matschiner, 1974) in microsomal preparations derived from rat liver. In this paper, we report a detailed study of the effects in vitro of TCP and IP on vitamin K dependent carboxylase, as well as their effects on the NAD(P)H-K oxidoreductase, the vitamin K epoxidase, and the vitamin K epoxide reductase.

Materials and Methods

Animals. Either male CD or Holtzman rats (250-g body weight) were fed on a vitamin K deficient diet for at least 10 days in wire-bottomed, coprophagy-minimizing cages. Male coumarin-resistant rats, kindly provided by J. W. Suttie, Madison, WI, were treated in an identical way, but for only 7 days.

Enzyme Preparations. Rats were starved overnight and then decapitated and exsanguinated. The liver was removed and homogenized (2 mL/g wet weight) in 0.025 M imidazole hydrochloride, pH 7.2, containing 0.25 M sucrose (buffer A). All operations were carried out in the cold (0–4 °C). The homogenate was centrifuged at 10000*g* for 10 min, and the resulting supernatant was then centrifuged for 1 h at 100000*g*. The supernatant from this latter centrifugation was aliquoted and frozen at −80 °C until used in the assay for NAD(P)H-K oxidoreductase. Microsomal pellets, used to assay for carboxylation, epoxidation, or epoxide reductase activity, were either resuspended in buffer A, solubilized in buffer A containing 1% Triton X-100, or extracted with acetone. In the last instance, the extracted protein was dried in vacuo and stored as a dried powder at 4 °C until used for enzyme assays.

Enzyme Assays. The assay for NAD(P)H-K oxidoreductase was essentially as described by Dallner (1963). The enzyme was incubated at 25 °C in a final volume of 1.3 mL containing 0.05 M potassium phosphate, pH 7.5, 2.7 mg/mL Tween-20, 6.4×10^{-5} M dichloroindophenol (DCPIP), and NADH at 0.1 mM or less as indicated, as well as inhibitor when included at the concentrations indicated. Reduction of DCPIP was measured spectrophotometrically as a decrease in absorbance at 600 nm. Assay of the vitamin K dependent carboxylase was done as we have reported previously, with the synthetic substrate phenylalanylleucylglutamylglutamylleucine (FLEEL) (Friedman & Shia, 1977; Friedman et al., 1979). Vitamin K epoxidase was assayed by using tritiated vitamin K_1 with conversion to vitamin K_1 epoxide determined by reversed-phase thin-layer chromatography (Willingham & Matschiner, 1974; Friedman & Smith, 1977). To study the epoxide reductase, we converted tritiated vitamin K_1 to tritiated vitamin K_1 epoxide by using the method of Fieser et al. (1941); after incubation of the epoxide with microsomes, conversion of epoxide to the quinone form of the vitamin was measured by reversed-phase thin-layer chromatography (Zimmerman & Matschiner, 1974).

Chemicals. Vitamin K_1, pyridoxal 5′-phosphate, NADH, Triton X-100, imidazole, warfarin, and DCPIP were purchased from Sigma Chemical Co., St. Louis, MO. $NaH^{14}CO_3$ (sp act. 50 mCi/mmol) was purchased from New England Nuclear, Boston, MA. The pentapeptide FLEEL was purchased from Vega Biochemicals, Tucson, AZ. Tritiated K_1 was a generous gift from Drs. U. Gloor and F. Weber, F. Hoffmann-La Roche and Co., Basel, Switzerland; menaquinone-3 (MK-3) was provided by Ed Hensen. TCP was provided by Dr. F. Marshall, Dow Chemicals, Midland, MI, and the IP by Eli Lilly and Co., Indianapolis, IN. All other chemicals were of reagent grade or better.

Table I: Inhibition of Carboxylation by TCP, 1-H-IP, and 1-OH-IP[a]

	vitamin			
	MK-3		K_1H_2	
inhibitor (10^6 M)	cpm	inhibn (%)	cpm	inhibn (%)
	4128		2236	
TCP (5)	2876	30	1705	27
(10)	1738	58	1097	53
(20)	878	79	638	73
(40)	655	84	524	78
(100)	161	96	212	91
(200)	81	98	191	92
(400)	39	99	76	97
TC2P (400)	3381	20		
1-H-IP (400)	2855	31		
1-OH-IP (400)	2731	34		

[a] Incubations were for 30 min at 28 °C in a total volume of 136 μL. The reaction mixture contained 100 μL of acetone powder resuspended to 25 mg/mL in buffer A containing 0.05 M KCl and 1 mM DTT with (K_1H_2 assay) or without (MK-3 assay) 1% Triton X-100, 2 mM pyridoxal 5-phosphate, 2 mM FLEEL, 2 mM NADH (only for assays with MK-3), inhibitors at the concentrations indicated [either 5 μL of MK-3 (1 mg/mL in ethanol) or vitamin K_1H_2 (2 mg/mL in ethanol)], and 0.7 μCi of $NaH^{14}CO_3$. Reactions were terminated by addition of 250 μL of 10% perchloric acid; after centrifugation to remove precipitated protein, 350 μL of the supernatant was assayed for ^{14}C. The results are the average of duplicate samples from which a background of 150 cpm obtained from incubations without vitamin has been subtracted.

Results

When the source of carboxylase is an acetone-powder preparation, TCP inhibits both MK-3- and phylloquinol-driven carboxylation of the synthetic substrate FLEEL (Table I). Fifty percent inhibition is seen at approximately 10^{-5} M TCP.

[1] Abbreviations used: TCP, 2,3,5,6-tetrachloro-4-pyridinol; TC2P, 3,4,5,6-tetrachloro-2-pyridinol; IP, imidazopyridines; 1-H-IP, 6-chloro-2-(trifluoromethyl)-1*H*-imidazo[4,5-*b*]pyridine; 1-OH-IP, 6-chloro-1-hydroxy-2-(trifluoromethyl)imidazo[4,5-*b*]pyridine; DCPIP, dichloroindophenol; FLEEL, the pentapeptide phenylalanylleucylglutamylglutamylleucine; vitamin K_1, phylloquinone or 2-methyl-3-phytyl-1,4-naphthoquinone; vitamin MK-3, menaquinone-3 or 2-methyl-3-farnesyl-1,4-naphthoquinone; vitamin K_1H_2, phylloquinol or 2-methyl-3-farnesyl-1,4-naphthoquinol; vitamin K_1O, phylloquinone 2,3-epoxide.

Table II: Comparison of Inhibition of Carboxylation and Epoxidation

inhibitor (10^6 M)	carboxylation[a] cpm	carboxylation[a] inhibn (%)	epoxidation[b] $K_1 \rightarrow K_1O$ (%)	epoxidation[b] inhibn (%)
	2110		55	
TCP (5)	1524	28	39	28
TCP (10)	923	56	28	49
1-OH-IP (240)	1492	29	36	34
1-OH-IP (960)	575	73	15	73

[a] Conditions for the carboxylation assay were as described under Table I except that 5 μg of vitamin K_1 was used. [b] Incubations for epoxidation were run separately and contained [^{3}H]K_1 (1 μg; 150 000 cpm). The results (expressed as percent conversion of $K_1 \rightarrow K_1O$) are the average of duplicate determinations from which a control (either no incubation or an incubated boiled enzyme), having 3% tritium in the K_1O region of the chromatogram, has been subtracted.

Neither the form of the vitamin nor the presence of Triton X-100 affects the degree of inhibition. Identical results are obtained if the same experiment is performed with microsomes suspended in buffer A plus 1% Triton X-100 as the carboxylase source (data not shown). Inhibition by TCP is of the carboxylase itself and not of either NAD(P)H-K oxidoreductase or vitamin K epoxide reductase, since in the K_1H_2-driven reaction NADH is omitted and inhibition is the same regardless of the presence or absence of sulfhydryl compounds which are required for epoxide reductase activity. 3,4,5,6-Tetrachloro-2-pyridinol (TC2P), a very weak anticoagulant in vivo, is much less potent than TCP at inhibiting carboxylation (Table I). The IP are also less potent than TCP; at concentrations of 5×10^{-4} M, they inhibit the carboxylase only 30% (Table I).

The effects of these compounds on epoxidase, epoxide reductase, and NAD(P)H-K oxidoreductase activities were studied. When microsomes are suspended in buffer A containing 1% Triton X-100 and a comparison is made between inhibition of epoxidase and carboxylase activities, the amount of inhibition of each activity is the same at the several concentrations of inhibitors tested (Table II). These results are not unexpected since it has been demonstrated previously that conditions which stimulate or inhibit the carboxylase also stimulate or inhibit the epoxidase (Willingham & Matschiner, 1974; Willingham et al., 1976; Friedman & Smith, 1977, 1979); indeed, conversion of the vitamin to the epoxide may occur during the oxygen-dependent carboxylation event. TCP and the IP inhibit NAD(P)H-K oxidoreductase but are only one-tenth as potent inhibitors of this enzyme as they are of the carboxylase. Both the coumarin and indandione anticoagulants are competitive inhibitors of NAD(P)H-K oxidoreductase with respect to NADH (Ernster et al., 1962; Rase et al., 1975). As shown in Figure 2, the IP as well as TCP also are competitive with respect to NADH. Finally, epoxide reductase was assayed with tritiated vitamin K_1 epoxide (K_1O). Although this enzyme also is inhibited by both TCP and the IP, in vitro it is the least sensitive of the enzymes studied (Table III).

While the data demonstrate that, in vitro, TCP and the IP are more potent inhibitors of the carboxylase (and epoxidase) than of either NAD(P)H-K oxidoreductase or epoxide reductase, a discrepancy exists with in vivo experiments. Although TCP is reported to be as effective an anticoagulant in coumarin-resistance rats as in coumarin-sensitive ones (Ren et al., 1974), the IP are ineffective anticoagulants in coumarin-resistant rats (Bang et al., 1975). The inhibition by TCP

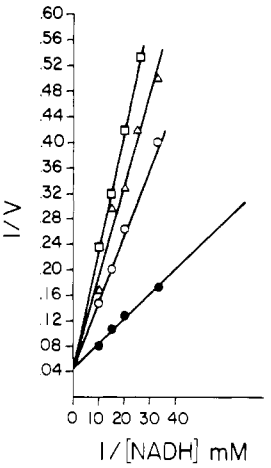

FIGURE 2: Competitive inhibition by 1-OH-IP, warfarin, and TCP of NADH-stimulated NAD(P)H-K oxidoreductase activity. Initial rates of reduction of DCPIP were measured as described under Materials and Methods at each of several NADH concentrations in the presence of no inhibitor (●), 2.5×10^{-5} M warfarin (○), 3.5×10^{-4} M TCP (△), or 2.1×10^{-3} M 1-OH-IP (□). A double-reciprocal plot of the data is presented.

Table III: Inhibition of Epoxide Reductase[a]

inhibitor (10^4 M)	$K_1O \rightarrow K_1$ (%)	inhibn (%)
	22.3	
TCP (4)	13.5	39
TCP (8)	10	55
TCP (16)	6.5	71
1-OH-IP (8)	13.5	39
1-OH-IP (16)	11	51
1-OH-IP (32)	9.5	57

[a] Incubations were for 30 min at 28 °C in a volume of 220 μL and contained 100 μL of microsomes resuspended in buffer A containing 0.05 M KCl to the volume of the homogenate from which they were derived, 2.5 mM dithiothreitol, [^{3}H]K_1O (1 μg; 156 000 cpm), and inhibitors at the concentrations indicated. The results (expressed as percent conversion of K_1O to K_1) are the average of duplicate determinations from which a control (either no incubation or an incubated boiled enzyme), having 2% tritium in the K_1 region of the chromatogram, has been subtracted.

and the IP of each of the enzymes derived from both coumarin-resistant and control rats was examined to evaluate this conflict (Table IV). As has been shown by other investigators (Bell & Caldwell, 1973; Matschiner et al., 1974; Whitlon et al., 1978), the epoxide reductase from the coumarin-resistant rats is inhibited significantly less by warfarin than is the enzyme from coumarin-sensitive rats. By contrast, the NAD(P)H-K oxidoreductase from the coumarin-resistant rats is slightly more sensitive to warfarin than is the enzyme from the control rats. Carboxylase and epoxidase are inhibited significantly only at very high concentrations of warfarin (Whitlon et al., 1978), and the inhibition by warfarin of these enzymatic activities from each strain was not evaluated. When the effects of TCP and 1-OH-IP were studied, each was as inhibitory to the carboxylase, epoxidase, and NAD(P)H-K oxidoreductase from the coumarin-resistant rats as they were to these enzymes from the coumarin-sensitive rats. However, the epoxide reductase from coumarin-resistant rats is inhibited far less by 1-OH-IP and somewhat less by TCP than the enzyme from coumarin-sensitive rats. Thus, these data, when interpreted in light of the aforementioned in vivo experiments, suggest that, despite the fact that, in vitro, the IP inhibit the carboxylase (epoxidase) at lower concentrations than they inhibit the epoxide reductase, the major site of anticoagulant

Table IV: Inhibition of Carboxylase, Epoxidase, Epoxide Reductase, and NAD(P)H-K Oxidoreductase in Warfarin-Sensitive and Warfarin-Resistant Rats

		warfarin-sensitive rats		warfarin-resistant rats	
enzymatic activity assayed	inhibitor (M)		inhibn (%)		inhibn (%)
		cpm		cpm	
carboxylase[a]		1932		3486	
	TCP (5×10^{-6})	1577	18	3026	13
	TCP (2×10^{-5})	962	50	1320	62
	1-OH-IP (4×10^{-4})	1437	26	2200	37
	1-OH-IP (9×10^{-4})	845	56	1389	60
		$K_1 \rightarrow K_1O$ (%)		$K_1 \rightarrow K_1O$ (%)	
epoxidase[b]		40		46	
	TCP (5×10^{-6})	33	17	39	15
	TCP (2×10^{-5})	21	47	29	37
	1-OH-IP (4×10^{-4})	24	40	28	39
	1-OH-IP (9×10^{-4})	18	55	22	52
		$K_1O \rightarrow K_1$ (%)		$K_1O \rightarrow K_1$ (%)	
epoxide reductase[c]		29		11	
	TCP (4×10^{-4})	6	79	9	18
	TCP (2×10^{-3})	4	86	6	45
	1-OH-IP (1×10^{-3})	11	62	11	0
	1-OH-IP (4×10^{-3})	7	76	10	9
	warfarin (1×10^{-6})	17	41	11	0
	warfarin (1×10^{-5})	10	66	9	18
	warfarin (1×10^{-4})	4	86	4	64
		DCPIP redn (mol/min $\times 10^9$)		DCPIP redn (mol/min $\times 10^9$)	
NAD(P)H-K oxidoreductase[d]		9.8		9.7	
	TCP (7×10^{-5})	7.8	20	5.7	42
	TCP (7×10^{-4})	3.8	61	2.4	75
	1-OH-IP (7×10^{-4})	6.7	32	5.0	49
	1-OH-IP (1×10^{-3})	5.3	46	3.6	63
	warfarin (8×10^{-6})	8.1	17	6.8	30
	warfarin (8×10^{-5})	4.6	53	3.3	66
	warfarin (8×10^{-4})	2.3	76	1.2	88

[a] Conditions were as described under Table I with buffer A and 1% Triton X-100; the vitamin was K_1 (5 μg). [b] Conditions were as described under Table II. [c] Conditions were as described under Table III. [d] Conditions were as described under Figure 2.

action is probably the epoxide reductase. Since TCP is as effective an anticoagulant in coumarin-resistant as in coumarin-sensitive rats and since vitamin K_1O can reverse the anticoagulant effect of TCP, but not that of warfarin or phenylindandione (Ren et al., 1974), its major site of anticoagulant action must be other than the epoxide reductase.

Additional experiments were performed to define further the nature of the inhibition of the carboxylase by TCP. When the concentration of the pentapeptide substrate FLEEL is varied over 6-fold, the percent inhibition by a fixed amount of TCP is unchanged (Table V). Thus, TCP is not a competitive inhibitor with respect to the peptide substrate. The following components of the assay also were examined for their ability to reverse the inhibitory effect of TCP: imidazole (over a concentration range from 0.025 to 0.5 M), pyridoxal 5′-phosphate (2–20 mM), dithiothreitol (2–20 mM), KCl (0.05–0.25 M), sodium bicarbonate (0.2–5 mM), oxygen (20–100%), and vitamin K_1H_2 (4–40 μg/mL). None are effective over the concentration ranges tested.

The effect of time of addition of TCP to the reaction was evaluated. Carboxylation was initiated with addition of vitamin in the presence of unlabeled $NaHCO_3$, and after 2, 5, or 10 min $NaH^{14}CO_3$ with or without TCP was added and a further incubation ensued; the percent inhibition by TCP relative to the appropriate time controls lacking TCP is the same and equal to the percent inhibition seen when TCP is present from zero time. Thus, initiation of enzymatic activity does not protect the carboxylase from inhibition. The order of addition of components of the assay relative to the addition of TCP also does not affect the degree of inhibition. However,

Table V: Inhibition of Carboxylation by TCP vs. FLEEL Concentrations[a]

FLEEL (μg/mL)	cpm −TCP	cpm +TCP	inhibn (%)
634	1887	400	79
1269	2930	619	79
2537	3671	713	81
3807	3562	621	83

[a] Conditions were as described under Table I with the following exceptions: incubations were for 15 min at 28 °C, TCP when present was at 2×10^{-5} M, and FLEEL (substrate) was at the concentrations indicated.

if TCP is added to resuspended acetone powder prior to the other reactants and the carboxylase activity is pelleted by centrifugation and resuspended in fresh buffer lacking TCP, carboxylation is equal to that found in control incubations utilizing enzyme which had been pelleted and resuspended in the absence of TCP. Thus, TCP inhibition is readily reversible, presumably as a result of dissociation of inhibitor from the enzyme.

An analysis of double-inhibitor experiments was done as described by Yonetani & Theorell (1964) to determine if TCP and the IP interact at the same or different sites on the carboxylase. For this, carboxylase incubations were done in which the concentrations of all reactants were held constant while the concentration of one of the inhibitors was varied at different fixed levels of the other inhibitor. Initial velocities were determined and plotted as $1/V$ vs. the varying inhibitor concentration. Such plots are either parallel (if no complex

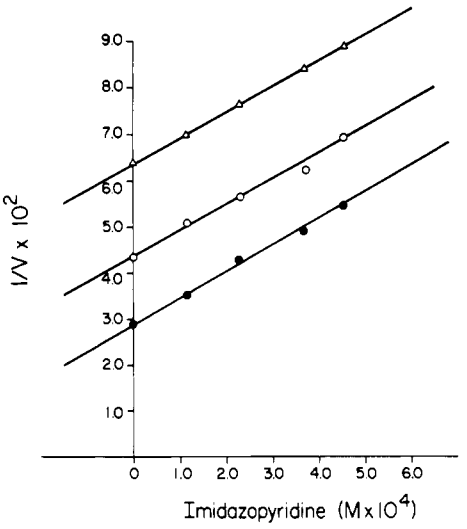

FIGURE 3: Interaction between 1-OH-IP and TCP with respect to inhibition of carboxylation. Incubation was for 15 min with the acetone-powder preparation as described under Table I with MK-3 (5 μg) as vitamin. 1-OH-IP alone (●), 1-OH-IP plus 4.3×10^{-6} M TCP (○), and 1-OH-IP plus 8.6×10^{-6} M TCP (△).

composed of both the inhibitors and the enzyme can form) or intersect to the left of the vertical axis (if such a complex can form). The position of the intersection relative to the horizontal axis is dependent upon the relative affinities of the two inhibitors for the enzyme. As shown in Figure 3, when inhibition of the carboxylase by TCP and the IP is analyzed in this way, parallel lines are obtained. On the basis of these data and the structural similarities between the inhibitors (Figure 1), it is likely that they have the same binding site on the carboxylase to which TCP either binds more readily and/or is a more effective inhibitor once bound.

Discussion

TCP and the IP inhibit the vitamin K dependent carboxylase and the related epoxidase activities as well as the NAD(P)H-K oxidoreductase and the vitamin K epoxide reductase activities. TCP is a more potent inhibitor than the IP, and each appears to have a greater effect on carboxylase and epoxidase than on the other two enzymes in vitro. Nonetheless, our results comparing the effects of these inhibitors on the enzymes from coumarin-resistant and coumarin-sensitive rats, when interpreted in light of in vivo experiments (Bang et al., 1975), support the conclusion that the site of the anticoagulant effect of the IP is the epoxide reductase. Whether the IP interact with the epoxide reductase at the same site(s) as either the coumarins or the indandiones remains to be determined. Why the IP are more potent inhibitors of the carboxylase (and epoxidase) than of the epoxide reductase in vitro is unclear. A possible explanation is that in vivo distribution of the IP favors delivery to the epoxide reductase. TCP, however, does not exert its anticoagulant effect by inhibiting the epoxide reductase (Ren et al., 1974); our data suggest the possibility that the carboxylase is its major site of anticoagulant action.

The nature of the binding site on the carboxylase for TCP and the IP is undefined; detailed studies of this sort will require a more highly purified enzyme. However, we have demonstrated that the inhibition is readily reversed and that the inhibitors are not competitive with respect to any of the required constituents of the carboxylase system. In particular, vitamin K did not reverse the inhibition by TCP. Since, in vivo, vitamin K_1 can reverse the anticoagulant effect of TCP (Marshall, 1972b; Ren et al., 1974), a conclusion that the anticoagulant effect of TCP results directly from the inhibition of the carboxylase will require resolution of this discrepancy.

Recently, we reported that cleavage of the γ-carbon–hydrogen bond of peptide-bound glutamic acid residues is a vitamin K dependent step which precedes carboxylation (Friedman et al., 1979). TCP (Friedman et al., 1979) and IP (P. A. Friedman, unpublished experiments) inhibit the cleavage of the γ-carbon–hydrogen bonds to the same degree that they inhibit carboxylation. Thus, since fixation of $^{14}CO_2$ measures the overall carboxylation reaction, these compounds exert their effect on carboxylation by blocking the initial γ-carbon–hydrogen bond cleavage.

The fact that the inhibition of NAD(P)H-K oxidoreductase by TCP and IP is competitive with respect to reduced pyridine nucleotide is interesting. Such a competition with NADH is noted with the coumarin and indandione anticoagulants. In view of the fact that these agents are structurally dissimilar to TCP and IP, it seems somewhat unlikely that NADH competes for a single binding site with all of these inhibitors.

References

Bang, N. U., O'Doherty, G. O. P., & Barton, R. D. (1975) *Clin. Res. 23*, 521A.

Bell, R. G., & Caldwell, P. T. (1973) *Biochemistry 12*, 1759.

Dallner, G. A. (1963) *Acta Pathol. Microbiol. Scand., Suppl. 166*, 24.

Ernster, L., Danielson, L., & Ljungren, M. (1962) *Biochim. Biophys. Acta 58*, 171.

Esmon, C., Sadowski, J., & Suttie, J. (1975) *J. Biol. Chem. 250*, 4744.

Fieser, L. F., Tishler, M., & Sampson, W. L. (1941) *J. Biol. Chem. 137*, 659.

Friedman, P. A., & Shia, M. A. (1976) *Biochem. Biophys. Res. Commun. 70*, 647.

Friedman, P. A., & Shia, M. A. (1977) *Biochem. J. 163*, 39.

Friedman, P. A., & Smith, M. W. (1977) *Biochem. Pharmacol. 26*, 804.

Friedman, P. A., & Smith, M. W. (1979) *Biochem. Pharmacol. 28*, 937.

Friedman, P. A., Shia, M. A., Gallop, P. A., & Griep, A. E. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3126.

Girardot, J. M., Mack, D. O., Floyd, R. A., & Connor-Johnson, B. (1976) *Biochem. Biophys. Res. Commun. 70*, 655.

Helgelund, L. (1977) *Biochim. Biophys. Acta 499*, 181.

Jones, J. P., Fausto, A., Houser, R. M., Gardner, E. J., & Olsen, R. E. (1976) *Biochem. Biophys. Res. Commun. 72*, 589.

Marshall, F. N. (1972a) *Proc. Soc. Exp. Biol. Med. 139*, 223.

Marshall, F. N. (1972b) *Proc. Soc. Exp. Biol. Med. 139*, 806.

Matschiner, J. T., Bell, R. G., Amellotti, J. M., & Knauer, T. E. (1970) *Biochim. Biophys. Acta 201*, 309.

Matschiner, J. T., Zimmerman, A., & Bell, R. G. (1974) *Thromb. Diath. Haemorrh., Suppl. 57*, 45.

Nelsestuen, G., Zytkovicz, T., & Howard, J. (1974) *J. Biol. Chem. 249*, 6347.

Rase, B., Bartfei, T., & Ernster, L. (1975) *Arch. Biochem. Biophys. 172*, 380.

Ren, P., Laliberte, R. E., & Bell, R. G. (1974) *Mol. Pharmacol. 10*, 373.

Stenflo, J., Fernlund, P., Egan, W., & Roepstorff, P. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2730.

Wallin, R., Gebhardt, O., & Prydz, H. (1978) *Biochem. J. 169*, 95.

Whitlon, D. S., Sadowski, J. A., & Suttie, J. W. (1978) *Biochemistry 17*, 1371.

Willingham, A. K., & Matschiner, J. T. (1974) *Biochem. J. 140*, 435.
Willingham, A. K., Laliberte, R. E., & Bell, R. G. (1976) *Biochem. Pharmacol. 25*, 1063.
Yonetani, T., & Thoerell, H. (1964) *Arch. Biochem. Biophys. 106*, 243.
Zimmerman, A., & Matschiner, J. T. (1974) *Biochem. Pharmacol. 23*, 1033.

Comparison of Glycopeptides from Control and Virus-Transformed Baby Hamster Kidney Fibroblasts†

Diana L. Blithe,‡ Clayton A. Buck, and Leonard Warren*

ABSTRACT: Glucosamine-labeled glycopeptides from control and virus-transformed BHK fibroblasts were characterized by size, lectin affinity, charge, and composition. As already demonstrated, on the basis of elution position on a column of Sephadex G-50, transformed cells contained a greater proportion of large glycopeptides than did control cells. Transformed cells also contained a larger proportion of glycopeptides which do not bind to Con A–Sepharose. By sequential chromatography on Sephadex G-50, Con A–Sepharose, and DEAE-Sephadex, ~40 individual peaks were partially or completely resolved. If sialic acid was removed from the glycopeptides prior to analysis by ion-exchange chromatography, 95% of the glycopeptides from control cells and 85% of the glycopeptides from transformed cells were no longer bound by DEAE-Sephadex. It was concluded that the DEAE-Sephadex elution properties of the glycopeptides are determined almost entirely by the sialic acid content of the molecules. A comparison of the profiles of control and transformed cell glycopeptides simultaneously eluting from columns of DEAE-Sephadex revealed that the differences between the two cells were largely quantitative; however, the possibility of the existence of qualitative differences as well cannot be excluded. In particular, there was one component present on the surface of transformed cells that was virtually absent in control cells. It was degraded by nitrous acid hydrolysis and heparinase and appeared to be heparan sulfate like material. After fractionation, each isolated glycopeptide population was analyzed for carbohydrate and, in some cases, amino acid content. The apparently larger glycopeptides, group A, the dominant population in transformed cells, were found to contain 3 to 4 mannose residues/glycopeptide when the sugars were normalized to sialic acid content. On the basis of the same criteria, group B glycopeptides contained 4–6 mannose residues/glycopeptide. The carbohydrate and amino acid compositions of the glycopeptides from transformed cells were, with a few exceptions, similar to those from control cells. Some isolated glycopeptides appeared to contain both O-glycosidic and N-glycosidic linkages on the same oligopeptide.

The role of bound carbohydrate in growth control and malignancy has been a subject of great interest in recent years. It has been found that malignant cells in culture are enriched in a population of glycopeptides (group A) (Buck et al., 1970, 1971a,b; Van Beek et al., 1975; Smets et al., 1976). This enrichment has been observed by analysis of limit digest products of Pronase-treated glycoproteins on columns of Sephadex G-50. The degree of increase in the high molecular weight population has been correlated with tumorigenicity (Glick et al., 1973; Smets et al., 1976) and with an active growth state (Buck et al., 1971a,b; Muramatsu et al., 1973). The larger glycopeptides, group A, have an approximate apparent molecular weight range of 4200–5500. The bulk of the glycopeptides from control cells elutes from Sephadex G-50 in regions called groups B and C, which correspond to approximate apparent molecular weight ranges of 3000–4200 and 1500–3000, respectively (Warren et al., 1974).

An increase in group A glycopeptides following transformation has been observed in several species and appears to be independent of the transforming agent (e.g., DNA- or RNA-containing virus, chemical agents, or spontaneous transformation) (Buck et al., 1971a,b; Smets et al., 1976). The increase in the population of group A glycopeptides has been demonstrated in material released from the cell surface by trypsin, as well as in cell pellets and in membranes from each major organelle isolated by cell fractionation (Buck et al., 1974; Keshgegian & Glick, 1973).

A recent study (Glick, 1979) has shown that the cell surface glycopeptides can be separated into seven or more groups by chromatography on DEAE-cellulose.[1] Comparison of the glycopeptides from control and transformed cells by this procedure indicated that the differences between the two cell types were quantitative with the transformed cell type containing an increase in larger glycopeptides. A partial structure for such glycopeptides was proposed (Santer & Glick, 1979; Ogata et al., 1976). In the work presented here, we extended these findings on a more detailed level. We cofractionated cell surface glycopeptides from control and virus-transformed

† From The Wistar Institute, Philadelphia, Pennsylvania 19104. *Received November 15, 1979.* This work was supported by Grants CA-19130, CA-19144, and CA-10815 and Training Grant CA-09171 from the U.S. Public Health Service.

‡ Presented to the Graduate Faculty of the University of Pennsylvania in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[1] Abbreviations used: Con A, concanavalin A; Con A(−), glycopeptides which do not bind to a column of Con A–Sepharose; Con A(+), glycopeptides which bind to a column of Con A–Sepharose; C13, BHK_{21}/C_{13}; B4, C_{13}/B_4; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetate; L-fucose, 6-deoxy-L-galactose; sialic acid, *N*-acetylneuraminic acid; Man and mannose, D-mannose; galactose, D-galactose; glucose, D-glucose; GlcNAc and *N*-acetylglucosamine, 2-acetamido-2-deoxy-D-glucose; *N*-acetylgalactosamine, 2-acetamido-2-deoxy-D-galactose; Asn, asparagine.

0006-2960/80/0419-3386$01.00/0