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INHIBITION OF DEHYDROGENASE ENZYMES BY HEXACHLOROPHENE

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Abstract—Low concentrations of the antibacterial agent hexachlorophene (HCP) inhibited a number of pyridine nucleotide-linked dehydrogenase enzymes, including bovine liver glutamate dehydrogenase (GDH), beef heart malate dehydrogenase (MDH), torula yeast glucose 6-phosphate dehydrogenase (G-6-P-D), horse liver alcohol dehydrogenase (ADH), pig heart isocitrate dehydrogenase (ICD), and beef heart lactate dehydrogenase (LDH). Initial velocity studies at appropriate enzyme concentrations gave I_{50} values for the dehydrogenases which ranged between 1.6 μM for GDH and 105 μM for ICD and LDH. More detailed kinetic analyses of G-6-P-D, ICD and GDH showed that inhibition by HCP in most cases was of the noncompetitive type. Calculations made from the kinetic data gave apparent K_i values with G-6-P-D, of 59 μM for $NADP^+$ and of 38 μM for glucose 6-phosphate; with ICD, of 1.0 μM for $NADP^+$ and of 25 μM for isocitrate; and, with GDH, of 2.0 μM for NADH, of 7.4 μM for α-ketoglutarate, and of 2.3 μM for ammonium acetate.

The halogenated antibacterial compound, hexachloropohene (HCP), was used extensively in soaps and cosmetics until the recent finding of mammalian toxicity [1–3], particularly in the central nervous system [1, 2]. Relatively little is known, however, about the biochemical mechanism for this toxicity. HCP has been shown to be a potent uncoupler of oxidative phosphorylation [4–6] and to inhibit several enzymes [7–9]. HCP also inhibits lactate, formate, glucose, and butanol dehydrogenases of *Bacillus subtilis* and *Escherichia coli*; malate and NADH dehydrogenases from *Bacillus megaterium* [10]; and lactate dehydrogenase of animal origin [11, 12].

In the present study, we report the inhibition by HCP of a number of pyridine nucleotide-dependent dehydrogenases, including alcohol dehydrogenase (ADH), glucose 6-phosphate dehydrogenase (G-6-P-D), glutamate dehydrogenase (GDH), isocitrate dehydrogenase (ICD), lactate dehydrogenase (LDH), and malate dehydrogenase (MDH). The kinetic parameters for the inhibition of G-6-P-D, GDH and ICD, by HCP, are presented also. A preliminary report of these investigations appeared earlier [13].

MATERIALS AND METHODS

Enzymes. Horse liver ADH type IX (alcohol: NAD^+-oxidoreductase, EC 1.1.1.1), 2.2 units/mg of protein; G-6-P-D from *Candida* [*Torulopsis*] *utilis* type XII (D-glucose 6-phosphate: $NADP^+$-oxidoreductase, EC 1.1.1.49), 405 units/mg of protein; bovine liver GDH type I (L-glutamate: NAD^+-oxidoreductase, EC 1.4.1.2), 2.7 units/mg of protein; pig heart ICD type IV (threo-D_s-isocitrate:$NADP^+$-oxidoreductase, EC 1.1.1.42), 7.3 units/mg of protein; beef heart LDH type III (L-lactate:NAD^+-oxidoreductase, EC 1.1.1.27), 560 units/mg of protein; and beef heart MDH (L-malate:NAD^+-oxidoreductase, EC 1.1.1.37), 2181 units/mg of protein, were obtained from the Sigma Chemical Co., St. Louis, MO.

Chemicals. NAD^+, NADH, $NADP^+$, and Tris were obtained from the Sigma Chemical Co. HCP (U.S.P. grade) was kindly donated by the Givaudan Corp., Clifton, NJ, and was twice recrystallized from isopropanol–water to yield a chromatographically pure product, m.p. 165.0–165.5°. All other chemicals used were from the Mallinckrodt Chemical Co., St. Louis, MO.

Determination of dehydrogenase activities. All dehydrogenase activities were measured spectrophotometrically at 30° by following the initial velocity of pyridine nucleotide oxidation or reduction as an increase or decrease of absorbance with respect to time at 340 nm. All enzyme assays were carried out in triplicate (except where indicated otherwise) in 1-ml quartz cuvettes in a Gilford model 2000 spectrophotometer equipped with a thermostated cell compartment maintained at 30°. A tight fitting aluminum holder held at the same temperature was utilized to prewarm the cuvettes and their contents. Reactions were initiated by the addition of enzymes, which had been stored at 0°, into the prewarmed reaction mixture. The amount of enzyme added was adjusted so that the initial rate of reduction or oxidation of pyridine nucleotide was linear, and proportional to the enzyme concentration.

G-6-P-D was assayed by a modification of the method described in the *Worthington Enzyme Manual* [14]. The assay mixture contained 0.05 M Tris–HCl buffer (pH 7.5), 0.48 mM $NADP^+$, 0.85 mM

glucose 6-phosphate, 0.02 M $MgCl_2$, and 0.2 to 0.3 μg enzyme/ml (or as indicated in the text). GDH was analyzed [15] using 4 μg enzyme/ml. ICD activity was measured [16] using 4 μg enzyme/ml, but the final buffer was 0.05 M. MDH was measured [17] at a concentration of 0.9 μg enzyme/ml. This enzyme was quite unstable and, therefore, the final values were corrected for decay. ADH was analyzed [18] using 2.2 μg enzyme/ml. LDH activity was followed [19] using 0.1 μg enzyme/ml; glycylglycine buffer, pH 7.5, however, was used in place of phosphate buffer, pH 7.0, to give higher dissolved HCP concentrations.

HCP was added to the assay mixtures dissolved either in 0.02 N NaOH or 95% ethanol. In most instances, the chlorinated bisphenol was initially dissolved in 0.4 N NaOH, diluted 20-fold with distilled water and then a 0.02-ml aliquot of this solution was added to the assay mixture. In order to prepare higher concentrations of HCP for the I_{50} determinations, however, HCP was dissolved in 95% ethanol, and then a 0.02-ml aliquot of this solution was added to the assay mixture. Controls contained comparable amounts of dilute NaOH or ethanol.

Presentation of data and calculation of results. Mean reaction rates from two to five assays were used in an interactive computer program [20] to prepare Lineweaver–Burk plots [21] and to calculate Hill or interaction coefficients [22]. Computer outputs gave values of K_m (Michaelis–Menten constant), V_{max} (maximum reaction rate), slope of Lineweaver–Burk plot, Y_{int} (intersection of the line of the Lineweaver–Burk plot on Y axis), and n or n' (Hill or interaction coefficient) of substrate binding sites or inhibitor binding sites respectively. The experimental inhibition constants, $K_{i_{slope}}$ (values varied with inhibitor concentration), were calculated using the computer output kinetic values to fit Webb's method [23, 24]. Apparent inhibition constants (K_i values) were estimated according to Segel [25] by replotting the reciprocal of the $K_{i_{slope}}$ values vs the corresponding HCP concentration. The I_{50} values (inhibitor concentrations which caused 50 per cent inhibition under the experimental conditions) were determined visually from semilogarithmic plots of percentage inhibition vs inhibitor concentration.

RESULTS

Effect of HCP on various dehydrogenases. HCP, in the micromolar range, inhibited all of the pyridine nucleotide dehydrogenases tested, yielding I_{50} values ranging between 1.6 μM for GDH and 105 μH for ICD and LDH (Fig. 1, Table 1). The sigmoid shape of the inhibition curves (Fig. 1A) suggested the possibility of cooperative interactions between inhibitory binding sites on the enzyme molecule [26]. These data were plotted, therefore, according to the following empirical inhibition Hill equation [22, 25]:

$$\log \frac{v_i}{v - v_i} = \log K - n' \log [I]$$

where v_i is the reaction velocity in the presence of inhibitor, v is the reaction velocity in the absence of inhibitor, K is the apparent product of x dissociation constants for the EI_x complex in an assumed equilibrium $E + I_x \rightleftharpoons (E \cdot I_x)$, $[I]$ is the inhibitor concentration, and n' is the interaction coefficient between inhibitor binding sites on the enzyme molecule. The slopes of the resulting plots were not constant (Fig. 1B), however, and the n' values for the various enzymes increased with HCP concentration, ultimately reaching values that ranged between 1.62 and 3.33 (the n' values for each linear portion of the Hill plots are given in Table 1).

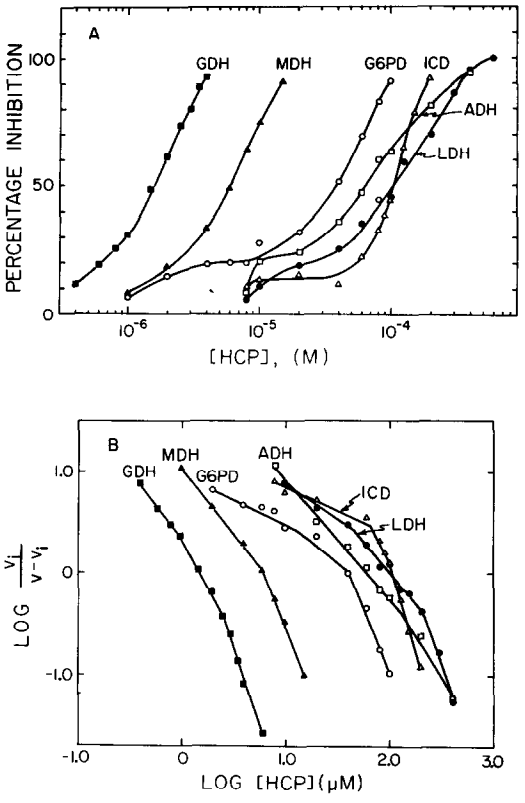

Fig. 1. Inhibition of various dehydrogenases by HCP. Panel A: effect of HCP concentration on the initial reaction rate of bovine liver GDH, beef heart MDH, torula yeast G-6-P-D, pig heart ICD, horse liver ADH, and beef heart LDH. Panel B: determination of interaction coefficient (n') values from the data presented in Panel A. The n' values are summarized in Table 1.

Table 1. Interaction coefficient (n') and I_{50} values for the inhibition of various dehydrogenases by HCP

Enzyme	n' Values*	I_{50} (μM)
Glutamate dehydrogenase	1.32, 1.95, 3.09	1.6
Malate dehydrogenase	1.29, 2.59	6.0
Glucose 6-phosphate dehydrogenase	0.49, 0.79, 2.52	39
Alcohol dehydrogenase	1.18, 1.62	65
Isocitrate dehydrogenase	0.45, 2.07, 3.33	105
Lactate dehydrogenase	0.70, 1.21, 2.86	105

*Interaction coefficient (n') values for linear portions of the Hill plots shown in Fig. 1B.

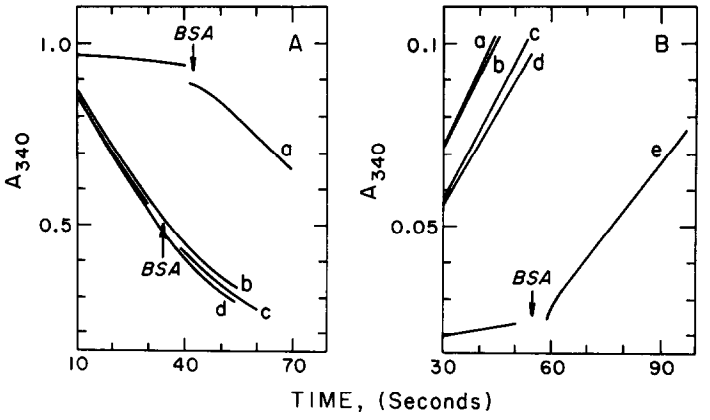

Fig. 2. Effect of BSA on the inhibition of bovine liver GDH and pig heart ICD by HCP. Panel A: reversal of HCP inhibition of GDH by BSA. Various components of the standard assay mixture were added in the sequence indicated and, after enzyme had been in contact with HCP for the time period indicated, the reaction was initiated by addition of α-ketoglutarate. The HCP concentration was 3 μM and BSA was added to give a final concentration of 0.5 mg/ml as described or indicated. Curve a: preincubation components added in the order enzyme, HCP (enzyme and HCP were in contact for 90 sec prior to initiation of the reaction), NADH and ammonium acetate. BSA were added after iniation of the reaction at the indicated time. Curve b: preincubation components added in the order enzyme, HCP (enzyme and HCP were in contact for 90 sec to initiation of the reaction), BSA, NADH and ammonium acetate. Curve c: preincubation components added in the order enzyme, NADH and ammonium acetate. BSA was added after initiation of reaction, NADH and ammonium acetate BSA were added after initiation of the reaction at the indicated (enzyme and HCP were in contact for 60 sec prior to initiation of the reaction), NADH and ammonium acetate. Panel B: reversal of HCP inhibition of ICD by BSA. Various components of the standard assay mixture were added in the sequence indicated and, after the enzyme had been in contact with the enzyme for 30 sec, the reaction was initiated by the addition of isocitrate. The HCP concentrtaion was 15 mM and BSA was added to give a final concentration of 0.5 mg/ml as described or indicated. Curve a: preincubation components added in the order enzyme, BSA, HCP and $NADP^+$. Curve b: preincubation components added in the order enzyme, BSA and $NADP^+$. Curve c: preincubation components added in the order enzyme and $NADP^+$. Curve d: preincubation components added in the order enzyme, HCP, BSA and $NADP^+$. Curve e: preincubation components added in the order enzyme, HCP and $NADP^+$. BSA was added after initiation of the reaction at the time indicated.

Reversibility of HCP inhibition. Bovine serum albumin (BSA) binds HCP strongly;* hence, this protein was used to remove HCP from the reaction mixture containing the dehydrogenase enzyme and HCP.

Reversibility of HCP inhibition to three enzymes was tested. Addition of BSA (0.5 mg/ml) to bovine liver GDH and pig heart ICD in the presence of HCP resulted in almost complete restoration of the enzyme activity (Fig. 2A and B). The addition of BSA (0.2 mg/ml) to the inhibited torula yeast G-6-P-D reaction mixture, on the other hand, showed only a partial (20–30 per cent) reversal of the inhibition by BSA (data not shown).

Kinetic studies of the interaction of HCP with G-6-P-D, ICD and GDH. Three of the dehydrogenases were selected for more detailed kinetic studies. Lineweaver–Burk plots in the presence of various concentrations of HCP are shown for G-6-P-D (Fig. 3A and B), ICD (Fig. 4A and B) and GDH (Fig. 5). In most cases, noncompetitive inhibition of the three dehydrogenases occurred with HCP. Kinetic constants for the various dehydrogenases were calculated [23–25] and are presented in Table 2. HCP exhibited no effect on substrate binding as evidence by Hill coefficient (n) values [27] that varied between 0.97 and 1.09, whether HCP was present or not.

The secondary plot or replot [25, 28] of the slopes of the Lineweaver–Burk plot for HCP inhibition of G-6-P-D was almost a linear function of HCP concentration (Fig. 3C), but showed a slight curvature for the replot of the intercepts (Fig. 3D). While the slope and intercept replots for HCP inhibition of ICD were curved strongly in the case of isocitrate, similar replots for this enzyme with $NADP^+$ as the variable substrate were nearly linear (Fig. 4C and D). Replots of slopes and intercepts for HCP inhibition of GDH (Fig. 6) yielded parabolic curves for both ammonium acetate and α-ketoglutarate (Fig. 6A and B). However, for NADH the slope replot was linear (Fig. 6C) and the intercept replot curved (Fig. 6D).

*T. L. Miller and D. R. Buhler, manuscript in preparation.

DISCUSSION

The results of the present study and those of previous investigators [10–12] show that HCP is an effective inhibitor of dehydrogenase enzymes of both bacterial and animal origin. The sigmoid shape of the inhibition

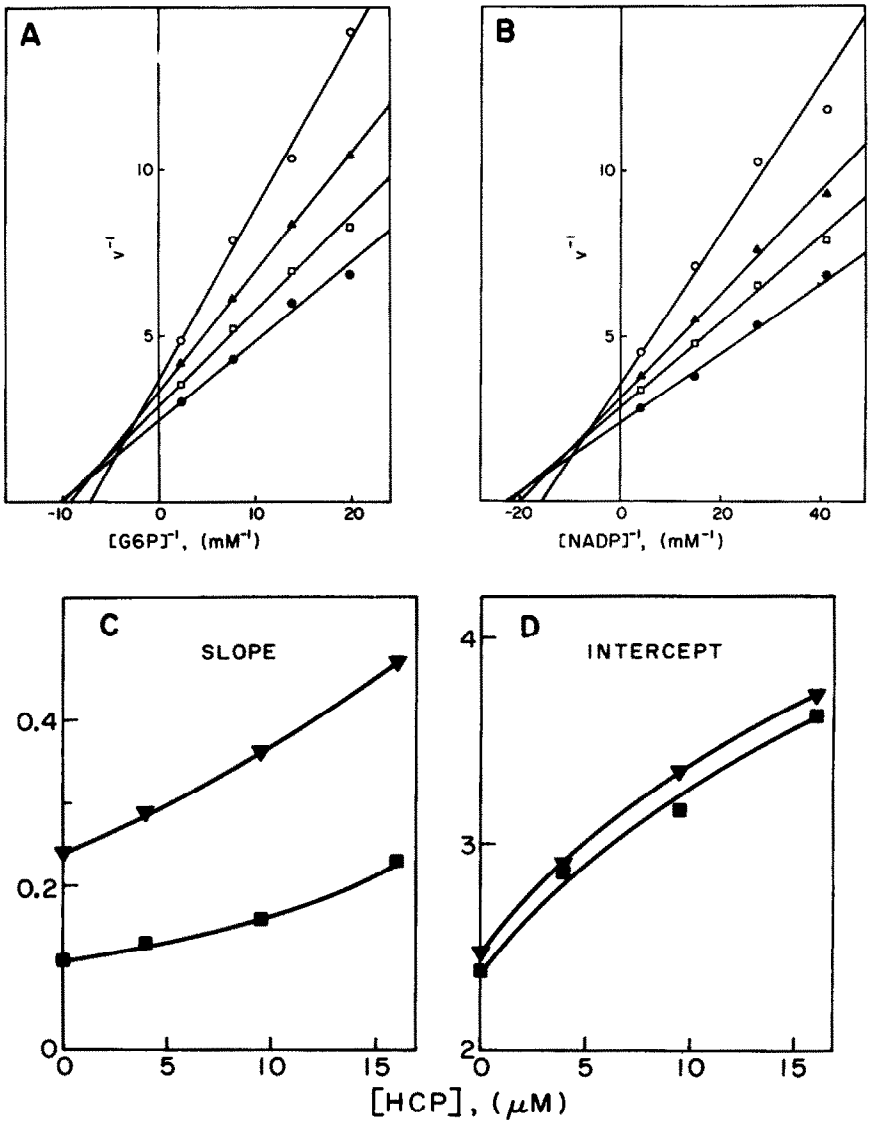

Fig. 3. Lineweaver–Burk plots and replots for the inhibition of torula yeast G-6-P-D by HCP at various concentrations of substrates. Panel A: [$NADP^+$] fixed at 0.48 mM and [G-6-P] varied. Panel B: [G-6-P] fixed at 0.85 mM and [$NADP^+$] varied. Key: without HCP (●——●); 4 μM HCP (□——□): 9.4 μM HCP (▲——▲); and 16 μM HCP (○——○). Panel C: replot of slopes. Panel D: replot of intercepts. Key: [G-6-P] varied (▼——▼); and [$NADP^+$] varied (■——■).

curves (Fig. 1A) suggested the possibility of cooperative interactions between inhibition binding sites on the enzyme [26]. The relationship between velocity and inhibitor concentration was examined further by the use of the Hill equation [22, 25]. The slopes (n' values) of the resulting Hill plots (Fig. 1B) were generally greater than 1.0, providing further evidence that HCP interacted with the enzymes cooperatively. Interaction coefficients (n' value) for HCP with GDH, MDH, G-6-P-D, ADH, ICD or LDH ranged between 1.62 and 3.33 (Table 1), suggesting that the minimum number of inhibitory binding sites per enzyme molecule may be two or three [22, 25, 29, 30], assuming that HCP did not irreversibly inhibit or denature the enzymes tested.

The slopes of the Hill plots for the inhibitor were not constant but increased with rather sharp transitions as the concentration of HCP increased. Such results might be explained by the presence of multiple inhibitory sites with different affinities for the bisphenol inhibitor on the enzyme [25] or by the enzyme changing from a state of low affinity to states of higher affinity for the inhibitor [31]. Slopes of Hill plots that vary with inhibitor concentration have also been observed for purine ribonucleotide inhibitors of glutamine phosphoribosylpyrophosphate amidotransferase [32], for prephenate inhibition of 3-deoxy-D-arabino-heptulosonate 7-phosphate [22], and for the inhibition of pyruvate kinase by triethyltin [29].

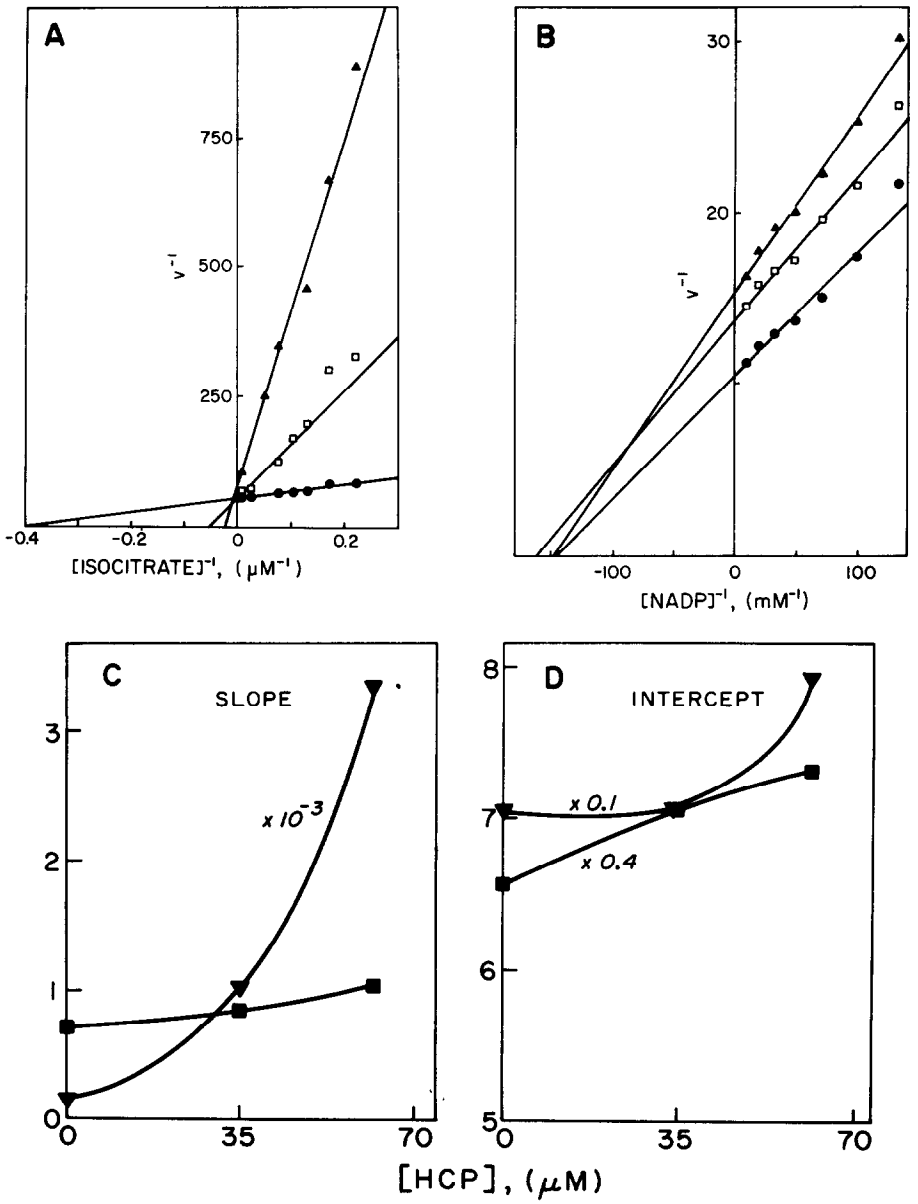

Fig. 4. Lineweaver–Burk plots and replots for the inhibition of pig heart ICD by HCP at various concentrations of substrates. Panel A: [$NADP^+$] fixed at 0.1 mM and [isocitrate] varied. Panel B: [isocitrate] fixed at 1.3 mM and [$NADP^+$] varied. Key: without HCP (●——●); 35.1 μM HCP (□——□); and 61.5 μM HCP (▲——▲). Panel C: replot of slopes. Panel D: replot of intercepts. Key: [isocitrate] varied (▼——▼); and [$NADP^+$] varied (■——■).

Inhibition of G-6-P-D, ICD and GDH by HCP was generally noncompetitive, as evidenced by the Lineweaver–Burk plots (Figs. 3A-B, 4A-B and 5A-B). While HCP inhibition of membrane-associated MDH from *B. megaterium* was also noncompetitive [10], the bisphenol inhibited soluble MDH from the same source by a partially competitive mechanism.

More detailed characterization of the type of inhibition seen in the present studies may be obtained from replots of the slopes and intercepts of the reciprocal plots [25, 28, 33]. Such secondary plots for G-6-P-D (Fig. 3C and D) are almost linear for the slope, and probably hyperbolic for the intercept with respect to both substrates. Following a more complete designation [28, 33]. inhibition of G-6-P-D by HCP may, therefore, be classified as slope-linear, intercept-hyperbolic, noncompetitive inhibition. Nonlinear secondary plots are indicative of complex inhibition involving at least two mechanisms for inhibition of the enzyme [25,34].

Inhibition of ICD by HCP appeared to be noncompetitive with respect to $NADP^+$ but competitive for

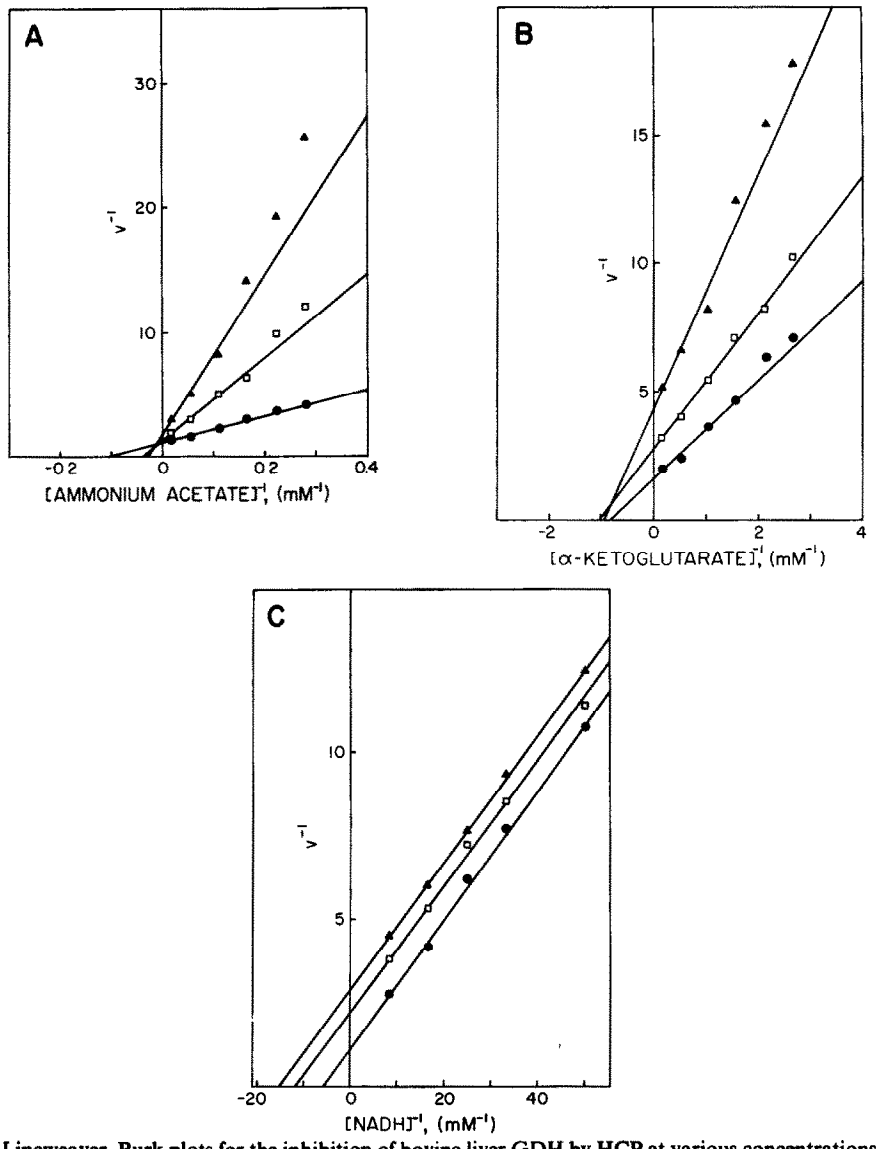

Fig. 5. Lineweaver–Burk plots for the inhibition of bovine liver GDH by HCP at various concentrations of substrates. Fixed substrate concentrations were: [ammonium acetate] 60 mM; [α-ketoglutarate] 6.6 mM; and [NADH] 0.11 mM. Panel A: [ammonium acetate] varied. Panel B: [α-ketoglutarate] varied. Panel C: [NADH] varied. Key without HCP (●——●); 0.67 μM HCP (□——□); and 0.86 μM HCP (▲——▲).

Table 2. Inhibition of glucose 6-phosphate dehydrogenase, isocitrate dehydrogenase and glutamate dehydrogenase by HCP*

Enzyme	Variable substrate	K_m† (μM)	Apparent K_i (μM)
G-6-P-D	Glucose 6-PO_3	97.2	38
	$NADP^+$	44.1	59
ICD	Isocitrate	2.5	25
	$NADP^+$	6.8	1000
GDH	Ammonium acetate	9600	2.3
	α-Ketoglutarate	1250	7.4
	NADH	170	2.0

*Enzymes were assayed under standard conditions.
†Michaelis–Menten constant for the uninhibited reaction.

isocitrate (Fig. 4A and B). The inhibition kinetics for GDH were more complex, apparently being competitive for the ammonium ion (Fig. 5A), noncompetitive with respect to α-ketoglutarate (Fig. 5B) but uncompetitive for NADH (Fig. 5C). Secondary plots of the slopes and intercepts from the reciprocal plots for HCP inhibition of these enzymes were generally parabolic in nature, although linear curves were found with some substrates (Figs. 4C and D, and 6).

The source of curvature in the slope and intercept replots is most likely due to the presence of at least two inhibitory sites on the enzyme molecules [25, 34—36]. As an example of such a mechanism, two phenol molecules were shown recently [36] to bind to two separate inhibitory sites on porcine heart MDH. One molecule was bound as phenol, the other as a charge transfer complex of phenol with NAD^+

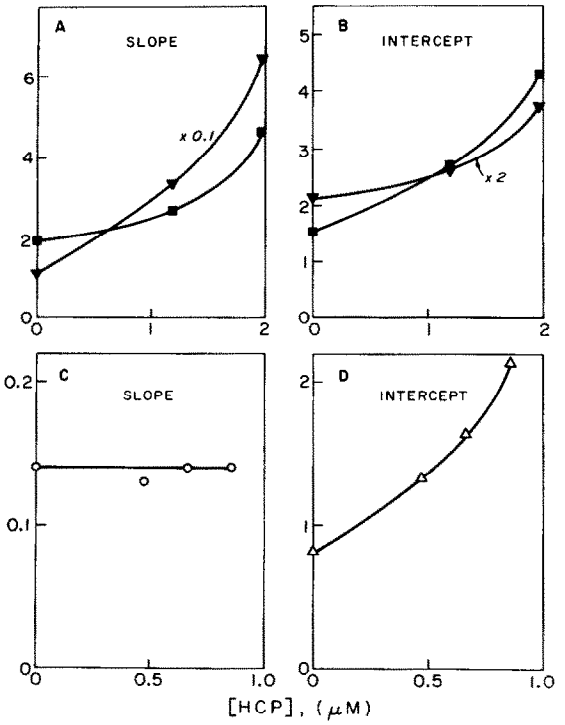

Fig. 6. Replots of slopes and intercepts of Lineweaver–Burk plots of HCP inhibition of GDH. Panels A and C: replots of slopes: [ammonium acetate] varied (▼——▼); [α-ketoglutarate] varied (■——■); and [NADH] varied (○——○). Panels B and D: replots of intercepts: [ammonium acetate] varied (▼——▼); [α-ketoglutarate] varied (■——■); and [NADH] varied (△——△).

Thus, there could be two or more HCP inhibition sites on each molecule of G-6-P-D, ICD or GDH. Alternatively, as in the case of MDH and phenol [36], there may be separate inhibitory sites that bind HCP and a HCP–substrate complex respectively.

Since pyridine-nucleotide-dependent dehydrogenases catalyze oxidation–reduction reactions essential in controlling cellular metabolism, their inhibition by HCP could have serious adverse effects on biological systems. Whether such inhibition occurs *in vivo* and plays a role in the toxicity of HCP [1–4], however, remains to be established.

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