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Coprecipitation of Carbonic Anhydrase by 1,1-Bis(*p*-chlorophenyl)-2,2,2-trichloroethane, 1,1-Bis(*p*-chlorophenyl)-2,2-dichloroethylene, and Dieldrin*

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ABSTRACT: The effect of 1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethane, (DDT), 1,1-bis(*p*-chorophenyl)-2,2-dichloroethylene (DDE), and dieldrin on the enzyme carbonic anhydrase from bovine erythrocytes and from spinach is carefully examined. Contrary to beliefs expressed in the literature, we found that these compounds are not true inhibitors of enzyme action. The esterase activity of bovine carbonic anhydrase (BCA) and the hydrase activity of spinach carbonic anhydrase (SCA) are found to be totally unaffected by any amount of DDT, DDE, or dieldrin present. However, the hydrase activity of BCA is reduced by DDT, DDE, and dieldrin; but only when these compounds are added in excess of their respective solubility limits. An amount of precipitate merely visible as a slight turbidity is sufficient to occlude from solution small amounts of enzyme (up to 7 μg/ml). It is shown that these amounts of occluded BCA represent a significant percentage of the total enzyme present in studies of hydrase activity, and furthermore that the reduction in enzymatic activity is *entirely* due to the removal of BCA from solution. We find no true inhibition whatsoever by DDT, DDE, or dieldrin.

The fact that SCA is not similarly coprecipitated is attributed to the fact that while BCA is a monomer with a molecular weight of ~30,000, SCA is probably a hexamer with a molecular weight around 180,000 and apparently does not present the proper surfaces for facile adsorption.

The growing world-wide concern for the environment has given rise to considerable interest in the effects of DDT [1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethane],[1] of DDE [1,1-bis-(*p*-chlorophenyl)-2,2-dichloroethylene, a stable metabolite of DDT], and dieldrin [1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-*endo,exo*-1,4:5,8-dimethononaphthalene]. One of the effects of large concentrations of DDT, DDE, and dieldrin in birds has been shown to be a decrease in egg shell thickness (Peakall, 1970). Since it is felt by some that carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1) plays an important role in egg shell production,[2] one is led to look for inhibition of carbonic anhydrase activity by these compounds (Bitman *et al.*, 1969).

It was reported that small amounts of DDT can be measured very accurately with carbonic anhydrase by the inhibitory effect on the enzyme-catalyzed dehydration of HCO_3^- (Keller, 1952). Peakall found that injection of DDE into a ringdove shortly before egg laying severely depressed carbonic anhydrase activity and brought a marked decrease in the thickness of the egg shell (Peakall, 1970). Similar findings were reported (Bitman *et al.*, 1970) with Japanese quail using DDT. They observed carbonic anhydrase activity depression of 16–19% in the shell-forming gland and 22–24% in blood. Such evidence is often taken to imply that carbonic anhydrase is inhibited by DDT, DDE, and dieldrin. However, no correlation was found between shell deformability and carbonic anhydrase activity for three strains of hens (Heald *et al.*, 1968). This would tend to indicate that the effect of these compounds, in birds at least, may be more complex than a mere inhibitory action on carbonic anhydrase. Since we have been performing extensive studies on competitive and noncompetitive inhibitors of bovine carbonic anhydrase (BCA) (Pocker and Meany, 1965, 1967; Pocker and Dickerson, 1965; Pocker and Stone, 1968a,b), it was natural to investigate the inhibitory effect of

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[1] Abbreviations used are: DDT, 1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethane; DDE, 1,1-bis(*p*-chlorophenyl)-2,2-dichloroethylene; dieldrin, 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-*endo,exo*-1,4:5,8-dimethylnonaphthalene; BCA, bovine carbonic anhydrase.

[2] The main evidence in support of the intimate involvement of carbonic anhydrase in eggshell formation is that certain sulfonamides of the type $ArSO_2NH_2$ inhibit the calcification of eggs. These sulfonamides are known to be powerful and specific inhibitors of carbonic anhydrase.

DDT, DDE, and dieldrin in an effort to further elucidate the role of these pesticides. Our investigation of both esterase and hydrase activity of various carbonic anhydrases has shown that DDT, DDE, and dieldrin are in fact not true inhibitors of these enzymes. We report here primarily on the enzyme from bovine erythrocytes (BCA) which we have studied in some depth.

The esterase activity of BCA has been thoroughly examined in recent years (Pocker and Stone, 1965, 1967, 1968a,b; Pocker and Storm, 1968; *cf.* also Tashian *et al.*, 1964; Malmström *et al.*, 1964; Armstrong *et al.*, 1966; Duff and Coleman, 1966). Observation of the enzyme-catalyzed hydrolysis of *p*-nitrophenyl acetate coupled with its complete inhibition by acetazolamide has been established as a standard assay for BCA activity. Employing this procedure we have been unable to detect any inhibition of the esterase activity of carbonic anhydrase by either DDT, DDE, or dieldrin.

Similarly, the carbonic anhydrase catalyzed hydration of carbon dioxide has been extensively studied with respect to the inhibitory effects of both anions (Keilin and Mann, 1940; Roughton and Booth, 1946; DeVoe and Kistiakowski, 1961; Kernohan, 1965) and sulfonamides (Mann and Keilin, 1940; Davis, 1959; Krebs, 1948; Maren *et al.*, 1954; Liebman *et al.*, 1967). We have now extended these kinetic measurements to include a detailed examination of the effects of DDT, DDE, and dieldrin on the BCA-catalyzed hydration of carbon dioxide. These pesticides are extremely insollube in water (<1 ppm) and are introduced with the help of an organic cosolvent. No inhibition can be detected in clear test solutions. However, when DDT, DDE, or dieldrin are added in excess of their respective solubility limits, a slowly forming precipitate or suspension is produced which occludes minute amounts of enzyme resulting in an apparent decrease in the hydrase activity of carbonic anhydrase. We are certain that it is this coprecipitation phenomenon that is actually responsible for the reported (Keller, 1952) inhibition of carbonic anhydrase activity by DDT. Indeed, the reduction in enzymatic activity can only be documented in opalescent test solutions in which the insecticides are present in excess of their solubility limit.

Experimental Section

Materials. Bovine carbonic anhydrase, BCA (carbonate hydrolyase, EC 4.2.1.1), was a product of Mann Research Laboratories prepared and purified from bovine erythrocytes by the method of Keilin and Mann (1940). For control purposes, small amounts of enzyme were also purified by the method of Lindskog (1960). Standardization of BCA solutions was accomplished by zinc analysis employing atomic absorption spectrophotometry. Concentrations based on zinc content, assuming a molecular weight of 30,000, were in accord with a molar extinction coefficient, ϵ_M, at 280 mμ of 54,000 M^{-1} cm^{-1}. Further confirmation of the standardization results was achieved by examining the effect of the specific inhibitor acetazolamide on both the hydrase (carbon dioxide hydration) and the esterase (*p*-nitrophenyl acetate hydrolysis) activity of the enzyme. The BCA was shown to contain a mixture of A and B isomers in the ratio of *ca.* 1:2. This was ascertained by the electrophoretic pattern obtained in 7% polyacrylamide gels on a Canalco Model 12 EPH apparatus and by chromatography on DEAE-cellulose. The two resolved isozymes have essentially identical catalytic properties (Nyman and Lindskog, 1964; Pocker and Stone, 1967; Pocker and Dickerson, 1968) and amino acid composition but differ in electrophoretic mobility. Both isozymes are equally well occluded to within the margin of error associated with the Wilbur–Anderson (1948) assay as applied by Rickli *et al.* (1964). Although the relatively concentrated stock solutions (10^{-4} to 10^{-6} M) of the enzyme were stable, some activity was lost on dilution (10^{-8} to 10^{-9} M). In some instances (indicated by specific footnotes to the tables) we resorted to the device used by others (DeVoe and Kistiakowsky, 1961; Gibbons and Edsall, 1964) and stabilized our very dilute solutions by the addition of bovine serum albumin (in place of peptone) and/or EDTA. Plant carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1) was prepared in our laboratories from spinach leaves and was purified by the procedures of Kondo *et al.* (1952) and Tobin (1970). The enzyme comprises approximately 1% of the total protein extract of spinach leaves and is a powerful catalyst for the reversible hydration of carbon dioxide. It also reversibly catalyzes the hydration of acetaldehyde. Zinc analysis indicates the presence of one zinc atom per 30,000 molecular weight subunits, and the simplest interpretation of the molecular weight data (Tobin, 1970; Y. Pocker and J. Ng, unpublished observations) is that the plant enzyme has a molecular weight of approximately 180,000 and is composed of 6 subunits. The purification and properties of *p*-nitrophenol and *p*-nitrophenyl acetate were previously described (Pocker and Stone, 1967). Tris was obtained from Aldrich and was purified by sublimation. Triethylamine was obtained from Eastman Kodak Co. and was purified by fractional distillation, bp 89–90° (760 mm). EDTA came from Eastman Kodak as the tetrasodium salt. Diethylmalonic acid diethyl ester was obtained from Aldrich. Diethylmalonic acid was prepared by hydrolysis of the diethyl ester and recrystallization of the acid from ether–benzene, mp 128–129°. Reagent Grade monobasic sodium phosphate, dibasic sodium phosphate, and sodium chloride were obtained from Allied Chemical and used without further purification. Sodium diethylbarbiturate (Veronal) was supplied by Merck. Water-soluble bromothymol blue, *p,p'*-DDT (99+%, Gold Label), and DDE were obtained from Aldrich. Reagent Grade dimethylformamide and acetonitrile were obtained from the J. T. Baker Chemical Co. Primary standard dieldrin (recrystallized, 99+% purity) came from Shell Chemicals. The bovine serum albumin was 100% electrophoretically pure material from Behringwerke AG.

Kinetics. The reactions run in an attempt to find inhibition of esterase activity of carbonic anhydrase by DDT or DDE were followed on a Beckman DU-2 spectrophotometer with a water-jacketed cell compartment held at 25.0°. Appearance of *p*-nitrophenol or *p*-nitrophenolate produced by *p*-nitrophenyl acetate hydrolysis was monitored at 348 mμ (Pocker and Stone, 1967). Rates were measured in buffered 1 and 10% acetonitrile–water solutions, and in 1, 10, and 34% dimethylformamide–water solutions. The buffers used were phosphate, diethylmalonate, Tris, and triethylamine acetate prepared at 0.05 M concentration and held at a constant ionic strength of μ = 0.55 by addition of sodium chloride. The pH range of 7.5–10 was examined as determined on a Beckman 101900 research pH meter. Bovine carbonic anhydrase concentration in the buffers was 5×10^{-6} M. At each point examined, one set of three runs was made with no DDE or DDT present and then a second set of three runs was made with DDE or DDT present. The per cent inhibition could then be calculated by the following formula: % inhibition = $100\% - (v_2/v_1) \times 100\%$, where v_1 = average rate of reaction with no DDT or DDE present at a given pH, v_2 = average rate of reaction with DDT or DDE present at the same pH. For the ester-inhibition runs, the enzyme solution was saturated with DDT or DDE

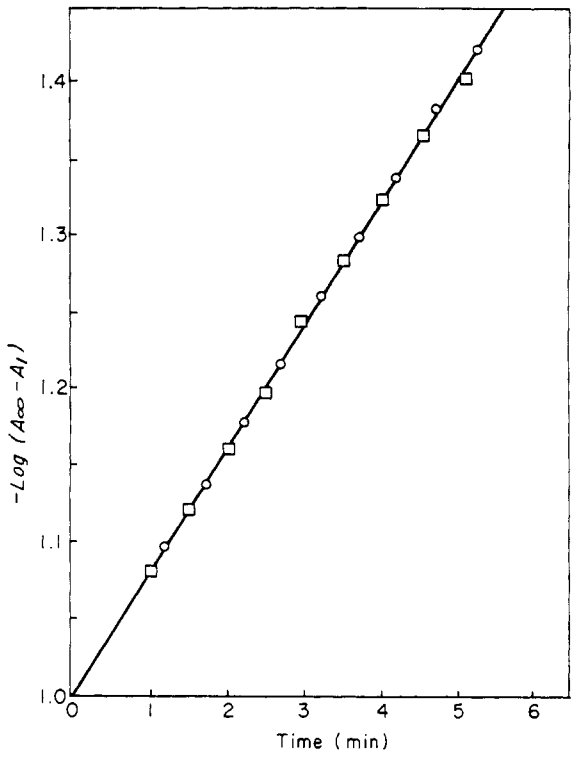

FIGURE 1: Typical first-order rate plot for BCA-catalyzed hydrolysis of *p*-nitrophenyl acetate (1.6×10^{-4} M) in Tris buffer (0.05 M, μ = 0.55, pH 9.12) with 10% (v/v) dimethylformamide and $\sim 3 \times 10^{-6}$ M BCA (corresponding to one zinc atom per enzyme molecule); ○, no DDT present; □, 1×10^{-4} M DDT added.

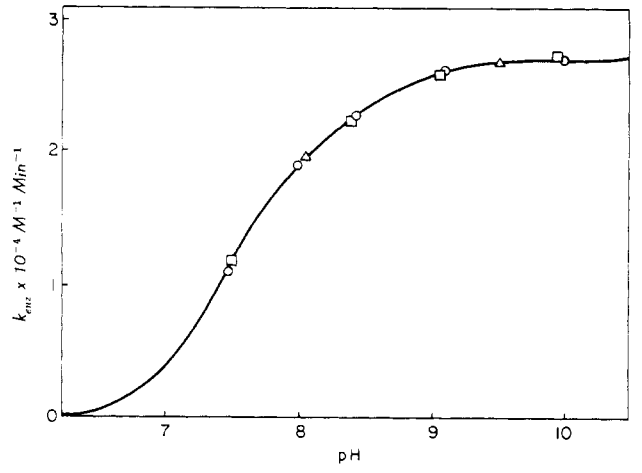

FIGURE 2: The BCA-catalyzed hydrolysis of *p*-nitrophenyl acetate (1.6×10^{-4} M) as a function of pH at 25.0°; $\sim 3 \times 10^{-6}$ M BCA (corrected for actual per cent zinc present); 10% (v/v) dimethylformamide; μ = 0.55; ○, no added DDT or DDE; □, 1×10^{-4} M DDT added; ◇, 1×10^{-3} M DDT added; △, 1×10^{-3} M DDE added.

either immediately before the run or 48 hr before the run with no noticeable difference in observed rate. DDT and DDE were added as much as 100-fold in excess of solubility. Any precipitate was removed by centrifuging immediately prior to a kinetic run which was initiated by injection of *p*-nitrophenyl acetate in acetonitrile stock solution into the buffered enzyme solution.

For the investigation of the enzyme-catalyzed hydration of carbon dioxide, a modification of the colorimetric method of

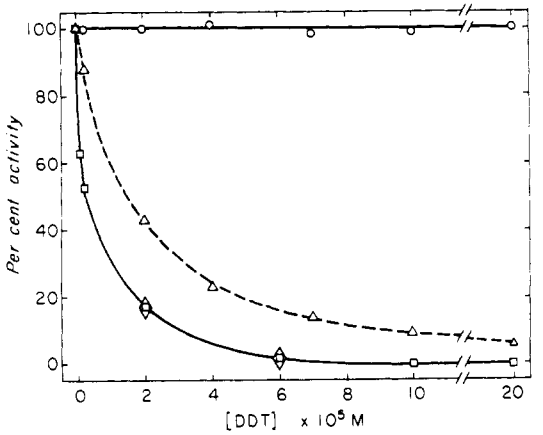

FIGURE 3: The coprecipitation of BCA and BCA protected with bovine serum albumin by DDT. The per cent activity (in phosphate buffers) is the per cent enzyme remaining in solution; ○, 34% (v/v) dimethylformamide and 6.3×10^{-9} M BCA; △, 1% (v/v) dimethylformamide and 8.8×10^{-9} M BCA with 20 μg of bovine serum albumin added per μg of BCA: □, 1% (v/v) dimethylformamide and 8.8×10^{-9} M BCA; ◇, 1% (v/v) dimethylformamide and 8.8×10^{-9} M BCA with 2 μg of bovine serum albumin added per μg of BCA.

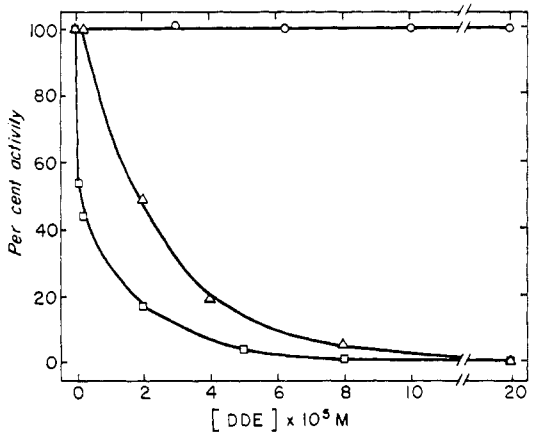

FIGURE 4: The coprecipitation of BCA by DDE in phosphate buffers. The per cent BCA remaining in solution (given as per cent activity) is shown as a function of added DDE; ○, 34% (v/v) dimethylformamide and 6.3×10^{-9} M BCA; △, 10% (v/v) dimethylformamide and 7.0×10^{-9} M BCA; □, 1% (v/v) dimethylformamide and 8.8×10^{-9} M BCA.

Wilbur and Anderson (1948) was used.[3] All work employed deionized, distilled water. A solution of 0.0167 M phosphate buffer containing 0.005% bromothymol blue and $\sim 10^{-8}$ M carbonic anhydrase at pH 8.2 was equilibrated in an ice–water bath. To this was added either pure dimethylformamide or dimethylformamide containing DDT, DDE, or dieldrin and the mixture was allowed to stand for 1 hr. The run was initiated by adding a saturated solution of CO_2 in deionized, distilled water at 0°. The time for the pH to change from the initial pH of 8.2 to pH 6.35 was recorded. The end point was determined by comparison to a standard tube containing

[3] It would have been desirable to perform the study on our Model 13000 Durrum-Gibson stopped-flow apparatus, but the turbidity produced when DDT or DDE was present in excess of solubility precluded this possibility. Employing the Wilbur and Anderson method and repeating each point at least five times, we were able to obtain reproducible (±10%) results.

TABLE I: The Effect of DDT on the Carbonic Anhydrase Catalyzed Hydration of Carbon Dioxide.

[BCA][a] M × 10^9	[DDT] M × 10^5	DMF (%)	Activity Units[b] ($U \times 10^{-8}$)	% BCA in Solution
	A. Bovine Carbonic Anhydrase			
1.75	0.0	1	110[c]	100
1.75	0.2	1	87[c]	79
1.75	2.0	1	22[c]	20
1.75	6	1	8.2[c]	7.5
1.75	10	1	4.4[c]	4
1.75	20	1	1.0[c]	1
8.8	0	1	29[d]	100
8.8	0.04	1	19[d]	66
8.8	0.2	1	14[d]	48
8.8	2	1	4.9[d]	17
8.8	6	1	1.4[d]	5
8.8	10	1	0.3[d]	1
8.8	20	1	0.0[d]	0
8.8	100	1	0.0[d]	0
8.8	0.2[e]	1	29[d]	100
8.8	2.0[e]	1	29[d]	100
8.8	20[e]	1	29[d]	100
8.8	0	1	50[f]	100
8.8	0.2	1	43[f]	86
8.8	2	1	20[f]	40
8.8	4	1	11.5[f]	23
8.8	7	1	7[f]	14
8.8	10	1	4.5[f]	9
8.8	20	1	2.5[f]	5
8.8	0	1	29[g]	100
8.8	2.0	1	4.9[g]	17
8.8	6.0	1	1.4[g]	5
6.3	0.2	34	11.7[d]	100
6.3	2	34	11.7[d]	100
6.3	4	34	11.6[d]	100
6.3	7	34	11.7[d]	100
6.3	10	34	11.7[d]	100
6.3	20	34	11.7[d]	100

[SCA][h] M × 10^9	[DDT] M × 10^5	DMF (%)	Activity Units[b] ($U \times 10^{-3}$)	Activity (%)
	B. Spinach Carbonic Anhydrase			
8	0.0	0.1[i]	6.7[d]	100
8	10.0	0.1[i]	6.6[d]	99
8	0.0	1.0[i]	5.1[d]	100
8	100.0	1.0[i]	5.1[d]	100
8	1.0	0.1	6.7[d]	100
8	10.0	0.1	6.5[d]	97
8	50.0	0.1	6.3[d]	95

[a] Bovine carbonic anhydrase concentration is calculated assuming a molecular weight of 30,000 and correcting for the per cent zinc actually present in the enzyme preparation, as determined by atomic absorption spectroscopy. [b] The activity units (U) are calculated according to the formula (Rickli *et al.*, 1964), $U = 10\,[(T_b/T_e) - 1]$/mg of protein, where T_b is the time for the uncatalyzed reaction and T_e is the time for the enzyme-catalyzed reaction. Dilute solutions of the enzyme (10^{-7} to 10^{-9} M) in the buffer–indicator mixtures are very sensitive to trace heavy-metal cations, to anions, and to added organic solvents. Therefore, for each set of conditions employed, we calculated a reference activity (U) with no added insecticide. [c] Performed using Veronal buffer (the standard Wilbur–Anderson test conditions) containing 10^{-4} M EDTA added to complex any trace heavy-metal ions present. [d] Phosphate was used in place of Veronal buffer and no EDTA was added. Enzymic activity appears lower for two reasons: (i) in the pH region of the Wilbur–Anderson test phosphate has a higher buffer capacity than veronal and (ii) at these high dilutions the enzyme shows a diminished activity unless protected by bovine serum albumin and/or EDTA. Nevertheless this modified procedure has the advantage of allowing a wider range of enzyme concentrations to be studied, coupled with an improved reproducibility in the magnitude of the coprecipitation phenomenon. Also, higher enzyme concentrations assayed by this method more closely approximate the enzyme concentrations encountered in nature (Maren, 1967). [e] Reversed order of addition. The BCA was added after precipitation of DDT was complete instead of prior to injection of the DDT solution. [f] Phosphate buffer was used with 20 μg of added bovine serum albumin per μg of BCA. [g] Phosphate buffer was used with 2 μg of bovine serum albumin added per μg of BCA. [h] Spinach carbonic anhydrase concentration is calculated assuming a molecular weight of 180,000. [i] Acetonitrile was used as the solvent for the DDT in place of DMF.

bromothymol blue at pH 6.35. Both bovine carbonic anhydrase and spinach carbonic anhydrase were tested. DDT, DDE, and dieldrin concentrations were varied over the range 10^{-6} to 10^{-3} M in 1, 10, and 34% dimethylformamide.

Two modifications of the above procedure were also used. In one modification, varying amounts of bovine serum albumin were added to the phosphate buffer to help stabilize the enzyme. In a second modification, the phosphate buffer was replaced with a 0.0167 M Veronal buffer containing 0.001% bromothymol blue and 10^{-4} M EDTA (to remove inhibition by trace heavy-metal ions in solution).

The per cent inhibition produced was calculated in the following manner, % activity = $U_i/U_o \times 100\%$, % inhibition = 100% − % activity, where $U_o = 10\,[(T_b/T_e) - 1]$/mg of protein, $U_i = 10\,[(T_b/T_{ei}) - 1]$/mg of protein, and T_b is the time for the buffer pH to change from 8.2 to 6.35 with no enzyme present, T_e, the time of the enzyme-catalyzed reaction, and T_{ei}, the time of the enzyme-catalyzed reaction with inhibitor present (Rickli *et al.*, 1964).

Results

The bovine carbonic anhydrase catalyzed rate of hydrolysis of *p*-nitrophenyl acetate was not affected by addition of DDT, DDE, or dieldrin. Even attempted addition of 10^{-3} M quantities to 10% dimethylformamide–water solution with subsequent formation of copious precipitate produced no decrease in rate. The solubility of these pesticides is less than

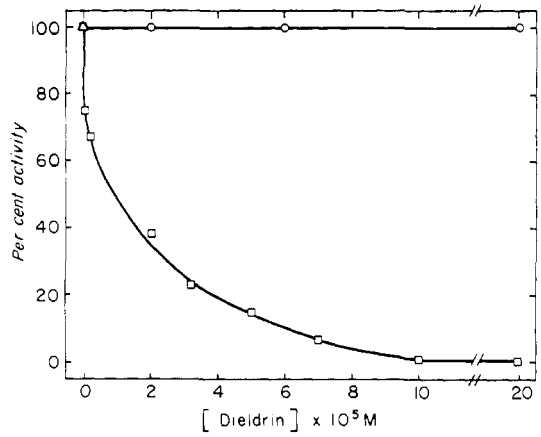

FIGURE 5: The coprecipitation of BCA by dieldrin in phosphate buffers. The per cent BCA remaining in solution (given as per cent activity) is shown as a function of added dieldrin; ○, 34% (v/v) dimethylformamide and 6.3×10^{-9} M BCA; □, 1% (v/v) dimethylformamide and 8.8×10^{-9} M BCA.

10^{-4} M in 10% acetonitrile–water or 10% dimethylformamide–water solution. In 34% dimethylformamide–water solutions, the solubility of DDT, DDE, and dieldrin increased to around 10^{-3} M. However, we were still unable to observe inhibition of the esterase activity of carbonic anhydrase. The BCA-catalyzed hydration of *p*-nitrophenyl acetate was first order, and runs with and without DDT, DDE, or dieldrin were superimposable (Figures 1 and 2).

In contrast, when we examined the hydrase activity of the enzyme, a phenomenon to be referred to as "apparent inhibition" of bovine carbonic anhydrase (BCA) catalyzed hydration of CO_2 was observed if DDT, DDE, or dieldrin was added in excess of its solubility limit. The apparent losses of activity (see Figures 3–5) looked qualitatively like inhibition curves for well-established inhibitors. The percentage inhibition increased in a less than linear fashion as the concentration of inhibitor increased from 10^{-5} to 10^{-3} M. These results were obtained in 1% and 10% dimethylformamide–water solution. By increasing the amount of dimethylformamide to 34% we were able to keep $\sim 10^{-3}$ M DDT, DDE, or dieldrin in solution. In the 34% dimethylformamide–water solution, where no precipitate of DDT, DDE, or dieldrin formed, apparent inhibition could no longer be observed. The results are given in Tables I–III.[4]

Unambiguous proof that we were observing only a precipitation of the enzyme was obtained by isolating the precipitate formed after addition of DDT, DDE, or dieldrin, redissolving the precipitate, and analyzing for BCA activity.[5] The amount of activity found in the redissolved precipitate accounted for the activity decrease that had been produced when the precipitate formed originally. Also, addition of BCA to a solution of DDT or DDE after the precipitate had been formed produced no apparent inhibition of the enzyme, indicating that the coprecipitation occurs during crystal growth and is not a postprecipitation adsorption phenomenon. Finally, since DDT undergoes a general base-catalyzed E2 elimination to form DDE (England and McLennan, 1966), we carried out control experiments which indicate that even under extreme conditions (1×10^{-5} M BCA at pH 10.0 for 1000 half-lives of

TABLE II: The Effect of DDE on the Bovine Carbonic Anhydrase Catalyzed Hydration of Carbon Dioxide.

[BCA][a] M × 10^9	[DDE] M × 10^5	DMF (%)	Activity Units[b] ($U \times 10^{-3}$)	% BCA in Solution
1.75	0.0	1	110[c]	100
1.75	0.2	1	108[c]	98
1.75	2	1	16.5[c]	15
1.75	5	1	6.3[c]	5.7
1.75	8	1	3.3[c]	3
1.75	20	1	1.1[c]	1
1.75	100	1	0.0[c]	0
8.8	0.0	1	29[d]	100
8.8	0.4	1	16[d]	55
8.8	0.2	1	12[d]	41
8.8	2.0	1	4.7[d]	16
8.8	5.0	1	1.3[d]	4.5
8.8	8.0	1	0.4[d]	1.4
8.8	20	1	0.0[d]	0
8.8[e]	0.2	1	29[d]	100
8.8[e]	2.0	1	29[d]	100
8.8[e]	20	1	29[d]	100
7.0	0.0	10	17[d]	100
7.0	0.04	10	17[d]	100
7.0	0.2	10	17[d]	100
7.0	2.0	10	8.3[d]	49
7.0	4.0	10	3.4[d]	20
7.0	8.0	10	0.9[d]	5
7.0	20	10	0.0[d]	0
6.3	0	34	11.7[d]	100
6.3	3.0	34	11.8[d]	100
6.3	6.2	34	11.7[d]	100
6.3	10	34	11.6[d]	100
6.3	20	34	11.7[d]	100

[a] BCA concentration is calculated as in footnote *a*, Table I. [b] Activity units (*U*) are calculated as in footnote *b*, Table I. [c] Refer to footnote *c*, Table I. [d] Refer to footnote *d*, Table I. [e] Refer to footnote *e*, Table I.

[4] The results reported in these tables represent maximum effects. The insecticide solution is forcefully syringed into a very well-stirred buffered solution containing 10^{-8} to 10^{-9} M enzyme. Addition with inadequate mixing leads to smaller losses of activity because enzyme adsorption takes place predominantly during precipitate formation. It was also noted that the more impure enzyme preparations are somewhat less susceptible to coprecipitation but even these could be completely removed from solution by increasing the amount of precipitating insecticide. We have no evidence for preferential coprecipitation of either isozyme A or B.

[5] This procedure requires great care and is performed as follows. The precipitate is separated by centrifugation, washed rapidly with a small amount of cold, deionized water and brought back into solution by the addition of a buffered solution of phosphate or veronal containing 10% dimethylformamide. In cases where this amount of dimethylformamide is insufficient to completely solubilize the precipitate the concentration of dimethylformamide was increased up to 34%, if necessary. The BCA content is then deduced by a Wilbur–Anderson assay of the resultant solution. The highly pure enzyme preparations (formally 10^{-7} to 10^{-9} M) generally tended to lose some activity either upon or after coprecipitation. However, when bovine serum albumin was added prior to coprecipitation (20-fold excess over BCA) or when less pure enzyme preparations were employed, the enzyme could be recovered from the precipitate with little or no denaturation or other loss in activity.

the carbon dioxide hydration reaction), there is no detectable elimination.

With carbonic anhydrase isolated from spinach, no apparent inhibition of enzyme-catalyzed CO_2 hydration could be found. Even DDT or DDE 100-fold in excess of solubility caused no rate decrease in the hydration of CO_2 by the plant enzyme.

Discussion and Conclusions

We have now shown that DDT, DDE, and dieldrin are not true inhibitors of carbonic anhydrase esterase or hydrase activity. They simply have the ability to coprecipitate minute amounts of enzyme from solution. A graphic plot of these data shows that when the respective solubility limits of DDT, DDE, and dieldrin are exceeded, the enzyme carbonic anhydrase from bovine erythrocytes is carried down with or in the precipitates (Figures 3–5). We believe that in these cases carbonic anhydrase is not incorporated in the crystal lattice, but that it is adsorbed during the growth of these crystals. Control experiments show that we are not dealing here with mixed crystal formation or surface adsorption by the precipitate after it is formed. It seems to us that the views expressed in the literature that DDT is an inhibitor of carbonic anhydrase activity must be revised in favor of a physical phenomenon of coprecipitation attributed to and governed by adsorption phenomena during the growth of the crystals. A purely chemical interpretation based on the formation of chemical complexes between carbonic anhydrase and DDT, DDE, or dieldrin must be rejected. The observation that plant carbonic anhydrase is not similarly subject to coprecipitation can probably be attributed to the fact that while carbonic anhydrases so far isolated from animal sources are monomers (Lindskog, 1960; Maren, 1967), the plant enzyme is a hexamer (Tobin, 1970; Y. Pocker and J. Ng, unpublished observations). The hexameric unit is either too large to be carried down or does not have the proper surfaces exposed for adsorption to take place.[6]

It was found that 10^{-5} M precipitating DDT, DDE, or dieldrin is capable of coprecipitating about 10^{-8} M of bovine enzyme, but increasing the formal amount of DDT, DDE, or dieldrin to 10^{-3} M still only caused coprecipitation of about 10^{-7} to 10^{-8} M BCA. Removal of enzyme from solutions of these concentrations is significant in CO_2 hydration studies since CO_2 work is performed on 10^{-8} M concentrations. However, in esterase studies, where one must work with enzyme concentrations varying between 10^{-6} M and 10^{-4} M, the removal of amounts of enzyme on the order of 10^{-8} M would go undetected. We also believe, however, that at these higher concentrations the enzyme solutions are more stable and less susceptible to coprecipitation. Thus, even repeated injections of insecticide produced no notable decrease in the esterase activity of BCA.

[6] It could be argued that since spinach carbonic anhydrase (SCA) is more difficult to purify than the enzyme from bovine erythrocytes, the former might actually contain enough inactive protein to preclude SCA from being coprecipitated by the DDT, DDE, or dieldrin. We believe that this cannot be so for two reasons. Firstly, SCA prepared as described above, appeared as a single component in analytical polyacrylamide gel electrophoresis. Secondly, the addition of bovine serum albumin does not stop BCA from being coprecipitated by DDT, DDE, or dieldrin. Thus, the addition of 2 μg of bovine serum albumin per μg of BCA produced no measurable effect. Even the addition of 20 μg of bovine serum albumin per μg of BCA provided relatively little protection from coprecipitation by the insecticides (see Figure 3).

TABLE III: The Effect of Dieldrin on the Bovine Carbonic Anhydrase Catalyzed Hydration of Carbon Dioxide.

[BCA][a] M × 10^6	[Dieldrin] M × 10^5	DMF (%)	Activity Units[b] ($U \times 10^{-3}$)	% BCA in Solution
1.75	0.0	1	110[c]	100
1.75	0.2	1	108[c]	98
1.75	2	1	75[c]	68
1.75	5	1	27[c]	30
1.75	7	1	22[c]	20
1.75	10	1	10.9[c]	9.9
1.75	20	1	4.4[c]	4
1.75	100	1	2.2[c]	2
8.8	0	1	29[d]	100
8.8	0.04	1	22[d]	75
8.8	0.2	1	19[d]	65
8.8	2.0	1	11[d]	38
8.8	3.2	1	6.6[d]	23
8.8	5.0	1	4.3[d]	15
8.8	7.0	1	2.0[d]	7
8.8	10	1	0.3[d]	1
8.8	20	1	0.15[d]	0.5
8.8	100	1	0.0[d]	0
6.3	0	34	11.7[d]	100
6.3	2	34	11.7[d]	100
6.3	6	34	11.7[d]	100
6.3	20	34	11.7[d]	100

[a] See footnote *a*, Table I. [b] See footnote *b*, Table I. [c] See footnote *c*, Table I. [d] See footnote *d*, Table I.

It should be noted that the exact effects of DDT, DDE, and dieldrin on eggshell formation remain in question. A purely chemical inhibition of carbonic anhydrase can definitely be ruled out. However, it is difficult to predict what the effect of these insecticides would be *in vivo* since the DDT, DDE, and dieldrin generally concentrate in the fatty tissues and are not easily available for interaction with carbonic anhydrase. Were insecticide precipitation to occur, the potential for coprecipitating carbonic anhydrase would exist since the enzyme is known to be present in various tissues in concentrations varying between ~10^{-8} and 10^{-6} M (Maren, 1967). It is known that DDT affects many other enzymes (Kagan *et al.*, 1969) and conclusions about specific environmental effects on DDT and DDE should await further investigation. We would caution that other studies also include DDE as some people are tempted to conclude that DDE is a harmless decomposition product of DDT. This study, for example, shows that with respect to occlusion the effect of DDE on carbonic anhydrase is virtually identical with the effect of DDT and dieldrin.

We have shown that the effect of DDT, DDE, and dieldrin on carbonic anhydrase is a purely physical phenomenon. We would suggest that in investigating the effect of compounds such as DDT, DDE, and dieldrin where a very low solubility in water is encountered, one must be alert to a number of biochemical and physiological consequences which arise from a purely physical action of precipitation and occlusion. Furthermore, the search for a chemical explanation of the inhibitory capacity of such relatively inert compounds should be widened to include parallel investigations of their power to occlude enzymes from solution. It has not escaped our notice that this

phenomenon could be used in the exploration of the relative topography of a number of enzymes, especially the multiple molecular forms of erythrocyte carbonic anhydrase.

It is important to note that close to the lower limit of solubility, the amount of precipitate formed is so small as to be barely visible. Since the material is held in suspension, merely forming a slightly opaque solution, one may tend not to realize immediately that is is a precipitation phenomena under observation. This would lead to the incorrect conclusion that a true inhibition was taking place.

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