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STUDIES ON THE ANIMAL METABOLISM OF PHENOXYACETIC HERBICIDES*)

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In conjunction with previously reported work on the distribution and elimination of chlorinated phenoxyacetic acids in animals (*Erne* 1966 b), some aspects of the metabolism of such compounds in the animal organism were studied. The experiments to be described below include studies of 1) the plasma protein binding of 2,4-dichlorophenoxyacetic acid (2,4-D) in pigs using the gel filtration technique, 2) the urinary conjugation of 2,4-D in pigs using hydrolytic methods and thin-layer chromatography and 3) the biological splitting of a 2,4-D ester in pigs and rats.

MATERIALS AND METHODS

Determination of phenoxyacetic acids

Unless otherwise stated, 2,4-D was determined in sample extracts according to the previously described method (*Erne* 1966 a), involving a buffer-extraction step followed by thin-layer chromatography and photometric determination. The method will be referred to as the standard procedure.

Gel filtration

Sephadex G-25, fine, (Pharmacia, Uppsala, Sweden) was hydrated in buffer (M/15 phosphate buffer pH 7.4—physiological saline (1:4)), allowed to settle and the "fines" decanted. Taking care to obtain a uniform packing, a column, 2.5×40 cm, was prepared with the hydrated gel, the details following *Flodin* (1962). If another eluant

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than buffer was to be used, the column was equilibrated with the eluant overnight before analysis. A 5-ml sample (2,4-D in plasma diluted with an equal volume of buffer) was introduced and elution performed with buffer, unless otherwise stated, at a flow rate of 8 ml/hr. and 8-ml fractions collected. The separation was followed by scanning at 280 mμ. Free 2,4-D (in the absence of protein) was measured by ultraviolet spectrophotometry. For determination of protein-bound 2,4-D the standard procedure, modified to include a deproteination step, was used. Twenty-five % (w/w) phosphotungstic acid (0.5 vol.) and 5 M sulphuric acid (0.1 vol.) were added and the mixture filtered after 5—10 minutes. The filter was washed with 50 % ethanol (2×5 ml), the combined filtrates extracted with chloroform (3×15 ml) and the chloroform extracts analyzed by the quantitative thin-layer chromatographic procedure.

The partition ratio, K_D, between the two aqueous phases of the gel system was calculated according to *Gelotte* (1960).

Hydrolysis

1. *Acid-hydrolyzable conjugates.* Urine samples after dilution with 2 volumes of water were adjusted to 1 N in respect of sulphuric acid and refluxed for 1 hour. The hydrolyzates were extracted with chloroform and the extracts analyzed for 2,4-D according to the standard procedure. Unhydrolyzed samples were run in parallel, and the per cent conjugation was calculated from the difference in results between hydrolyzed and unhydrolyzed samples.

2. *Alkali-hydrolyzable conjugates.* The procedure was analogous to that under 1, except that 0.2 N sodium hydroxide was used for the hydrolysis and the hydrolyzate was acidified before chloroform extraction.

3. *2,4-D esters.* Esters were determined, together with free 2,4-D, by an extended standard procedure. The preliminary hydrolysis steps of the standard procedure (acid reflux of urine samples and alkali treatment at evaporation of solvent extracts) were omitted. In addition, the organic phase remaining after extraction of free phenoxy acids into buffer and containing any phenoxy esters present was retained. After addition of 5 M sodium hydroxide (5 ml) and water (50 ml) the solvent was evaporated on a water bath, the mixture was cooled, acidified and extracted with chloroform, and the extract analyzed for 2,4-D by the quantitative thin-layer chromatographic procedure.

Thin-layer chromatography

Unless otherwise stated, thin-layer chromatography was performed as described in the standard procedure. For chromatography of urine or crude sample extracts silica gel-coated plates with a layer thickness of 0.50 mm were used and the sample applied as a band. 2,4-D standard was also added to each plate. After development with

ethyl acetate, n-hexane, formic acid (35:65:0.3, by vol.), the zones were located in ultraviolet light or by means of the methylumbelliferone and silver reagents and eluted with ammoniacal methanol for further characterization.

RESULTS

A. *Plasma protein binding*

Five hundred µg of 2,4-D, equilibrated for 24 hours with 5 ml of 50 % horse plasma in buffer pH 7.4, were applied to the Sephadex column, and elution performed with the buffer. 2,4-D was eluted in two peaks, the higher (K_D 2.2), containing the free acid, and the other (K_D 0), containing about 2 % of the total 2,4-D associated with the plasma protein.

The experiment was repeated after equilibrating the column with 50 % plasma and using 50 % plasma as the eluant. This time 2,4-D was eluted in a single peak and at a considerably reduced K_D value (0.95).

B. *Conjugation*

Pigs were given single and repeated oral doses of 2,4-D amine and urine samples analyzed for acid-hydrolyzable conjugates. The results are given in Table 1.

Table 1. Conjugation of 2,4-D in urine of pigs given 2,4-D amine, as determined by differential acid hydrolysis. Values given are the means of duplicate determinations.

Animal no.	2,4-D found, µg/ml		Conjugation %
	Before hydrolysis	After hydrolysis	
4 A[a])	200	214	7
4 A[b])	490	550	11
16 E[c])	293	297	1
18 A[c])	100	105	5
18 B[c])	81	99	18
18 E[c])	384	376	0

[a]) 3 doses of 50 mg/kg.
[b]) 23 „ „ 50 „
[c]) 500 p.p.m. in feed for 5 months.

Considering the variability of the analytical results (the standard deviation of the method is 3—5 %) the differences found are probably not significant except for the values for

samples no. 4 A (at 23 doses) and 18 B, indicating an apparent conjugation of 11 and 18 %, respectively.

On analyzing samples no. 18 A, B and E for alkali-hydrolyzable conjugates, apparent conjugation values of 2, 12 and 1 %, respectively, were found.

As a check, 500-µl aliquots of samples no. 18 A, B and E were fractionated by thin-layer chromatography, the entire area below the 2,4-D zone (located by treating the standard segment of the plate with the silver reagent) being eluted and the eluate hydrolyzed by acid and then analyzed according to the standard procedure. In sample no. 18 B, 5 µg of 2,4-D were detected in the hydrolyzed fraction, corresponding to a conjugation of about 10 %, the values for the other two samples being below 0.5 µg of 2,4-D. The conjugate or conjugates were not isolated in a pure form.

For the purpose of comparison, also plasma samples from pigs no. 18 A, B and E were analyzed for acid- and alkali-hydrolyzable conjugates. No significant differences were observed, the apparent conjugation consistently being below 5 %.

C. *Stability of 2,4-D ester*

Pigs and rats were given 2,4-D butyl ester orally, and extracts of urine, plasma and tissues were analyzed for free and esterified 2,4-D. The results are presented in Table 2.

Table 2. Free and esterified 2,4-D in body fluids and tissues of pigs and rats given 2,4-D butyl ester. Values given are the means of duplicate determinations.

Animal no.	Sample	2,4-D found, µg/ml or g	
		Free	Esterified
Pig 9 A[a])	Plasma	190	1.5
Pig 9 A[b])	„	35	0.8
	Red blood cells	4	0.5
	Urine	250	1.1
	Liver	17	0.3
Rat 4 G[c])	Plasma	13	0.6
	Red blood cells	1.5	0.2

[a]) 3 doses of 50 mg/kg.
[b]) 23 „ „ 50 „
[c]) Single dose of 100 mg/kg.

Invariably, only trace amounts of ester-like compounds were found.

In control experiments, chromatographically separated fractions of urine and plasma extracts of pig no. 9 A were analyzed for hydrolytically liberated 2,4-D. The samples were extracted according to the standard procedure, but omitting the acid hydrolysis of urine and the alkali treatment at evaporating plasma extracts. Extract aliquots equivalent to 5 ml of urine or plasma were chromatographed, the entire area above the 2,4-D zone was eluted with methanol and the eluate hydrolyzed by alkali and analyzed by the standard procedure.

The 2,4-D levels found in this way were below 0.5 μg/ml, both in urine and in plasma.

DISCUSSION

Plasma protein binding. The observed elution of 2,4-D along with the plasma protein, in the gel filtration experiments using buffer as the eluant, suggests a certain, although weak protein binding of 2,4-D. The high K_D value of free 2,4-D in these experiments (2.2) indicates adsorption of the compound to the gel. The existence of a 2,4-D-protein interaction was confirmed by the considerably enhanced mobility of 2,4-D observed when 50 % plasma was used as the eluant, the K_D value decreasing to 0.95.

The protein binding of phenoxy acids has not been reported previously. The phenomenon has been demonstrated with drugs, however, the observed binding varying within wide limits for different compounds (see for instance *Barlow et al.* 1962). Since only the unbound fraction of a compound in plasma is able to participate in diffusion equilibria, an interaction of this kind will modify the distribution of the compound in the organism and therefore also its pharmacological effects. The effect of plasma protein binding on distribution has been treated quantitatively by *Martin* (1965).

Conjugation. The hydrolysis studies provide evidence of significant conjugation of 2,4-D in only a few of the samples of pig urine examined, the degree of conjugation being below 20 %, and in none of the plasma samples. From one urine sample a fraction, being more polar than 2,4-D and yielding 2,4-D in a quantity corresponding to about 10 % of the total, was separated chromatographically.

Apparently, phenoxy acid conjugation in the animal organism

has not been reported previously. Early studies, cited by *Williams* (1959), indicated unsubstituted phenoxy acetic acid to be excreted unchanged in urine by man, dogs, rats and rabbits. This was shown to be true also for the *o*- and *p*-chloro derivatives in the rabbit *(Levey & Lewis* 1947). Recently, 2,4-D, as well as 4-chloro-2-methyl- and 2,4,5-trichlorophenoxyacetic acids, were shown to be eliminated, completely and intact, in urine of cattle (*Lisk et al.* 1963; *Bache et al.* 1964; *St. John et al.* 1964). Similar results were obtained with sheep, using radioactive 2,4-D (*Clark et al.* 1964).

The results of the present study, as well as those of previous investigations, however, are compatible with the observation that strong acids, of pK_a 3 or below, are usually excreted without conjugation in the animal organism (*Williams*).

Stability of 2,4-D ester. The observation that substantial amounts of 2,4-D acid only, and not of 2,4-D ester (Table 2), could be detected in body fluids and tissues of pigs and rats given 2,4-D butyl ester orally points to a rapid hydrolysis of the ester in the animal organism. These results agree with those of similar studies with an ester of 2-(2,4,5-trichlorophenoxy)propionic acid given orally to a cow, a high proportion of the dose being eliminated renally as the free acid (*St. John et al.*).

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SUMMARY

Gel filtration on Sephadex was applied to the study of plasma protein binding af 2,4-dichlorophenoxyacetic acid (2,4-D). Equilibrated with horse plasma and eluted with buffer pH 7.4, 2,4-D was recovered to 2 % in the plasma protein fraction (K_D 0), free 2,4-D being eluted at a K_D value of 2.2. When 50 % plasma in buffer was used as the eluant the mobility of 2,4-D increased considerably (K_D decreasing to 0.95), thus confirming the existence of a 2,4-D-protein interaction.

Hydrolysis experiments in conjunction with thin-layer chromatography revealed no conjugation of 2,4-D in plasma of pigs and rats given 2,4-D amine orally and only slight conjugation, not exceeding 20 %, in urine of pigs, given 2,4-D amine orally.

2,4-D butyl ester administered orally to pigs and rats was shown to be rapidly hydrolyzed to 2,4-D acid, only trace amounts of ester being detectable in body fluids and tissues.

ZUSAMMENFASSUNG

Studien über dem tierischen Metabolismus von Phenoxyessigsäureherbiziden.

Die Plasmaproteinbindung der 2,4-Dichlorphenoxyessigsäure (2,4-D) wurde mittels Gelfiltrierung auf Sephadex untersucht. Bei Eluierung von 2,4-D, mit Pferdeplasma äquilibriert, unter Verwendung eines Puffers von pH 7,4 wurde 2 % der zugesetzen 2,4-D im Plasmaproteinanteil wiedergefunden (K_D 0). Freie 2,4-D wanderte mit einem K_D-Wert von 2,2. Das Vorhandensein einer Wechselwirkung zwischen Plasmaprotein und 2,4-D wurde durch Eluieren mit 50 %-igem Plasma in Puffer von pH 7,4 bestätigt, wobei eine wesentlich höhere Wanderungsgeschwindigkeit der 2,4-D festgestellt wurde (K_D 0.95).

Mittels Hydrolysenversuche und anschliessender Dünnschichtchromatographie war nach oraler Zufuhr von 2,4-D-amin an Schweinen und Ratten keine Paarung von 2,4-D im Plasma wahrzunehmen. Eine geringgradige Paarung, unter 20 %, wurde im Harn der Schweine nachgewiesen.

Nach oraler Eingabe des 2,4-D-butylesters wurde bei Schweinen und Ratten eine schnelle Hydrolyse des Esters beobachtet.

SAMMANFATTNING

Studier över metabolismen av fenoxiättiksyreherbicider i djur.

Plasmaproteinbindning av 2,4-diklorfenoxiättiksyra (2,4-D) studerades med hjälp av gelfiltrering på Sephadex. Vid användning av buffert pH 7,4 som elueringsmedel eluerades 2,4-D, ekvilibrerad med hästplasma, till 2 % med plasmaproteinfraktionen (K_D 0), medan fri 2,4-D vandrade med K_D 2,2. Förekomsten av en plasmaproteinbindning av 2,4-D bekräftades genom eluering med 50 %-ig plasma i buffert pH 7,4, varvid en väsentligt ökad vandringshastighet för 2,4-D konstaterades (K_D 0,95).

Genom hydrolysförsök i förening med tunnskiktskromatografi kunde ingen konjugering av 2,4-D konstateras i plasma från grisar och råttor, som tillförts 2,4-D-amin oralt. En obetydlig konjugeringsgrad, mindre än 20 %, påvisades i urin från grisar.

Efter oral tillförsel av 2,4-D-butylester till grisar och råttor påvisades en snabb hydrolys av estern till 2,4-D-syra.

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