Alkoxy Derivatives of Dyrene: Identification and Carboxylesterase Inhibition

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Dyrene reacted with alkyl alcohols was analyzed by gas-liquid and thin-layer chromatography (glc and tlc). It was most reactive with methanol, particularly in the presence of NaOH or sodium methoxide. Dyrene also reacted with ethanol (30%) in a 0.03*N* HCl aqueous solution. Glc retention times of products increased as ethanol, propanol, or butanol was used in the reaction. Dyrene and alkoxydyrenes (new chemicals) produced fluorescent compounds when exposed to ultraviolet light. Further analyses were carried out by nuclear magnetic resonance and infrared spectroscopy. The reaction involved the replacement of chlorines with alkoxy groups. Unlike Dyrene, alkoxydyrenes were potent inhibitors of liver carboxylesterases. Pig and sheep liver esterases were inhibited by ≥ 0.1 μg and ≥ 2 μg of dimethoxydyrene (calculated in term of Dyrene), respectively; whereas chicken, turkey, beef, or monkey liver esterase was inhibited by ≥ 4 μg of dimethoxydyrene. Among the products, dimethoxydyrene was the strongest inhibitor of liver esterases.

Determination of the fungicide Dyrene [2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine] based on chlorinated *s*-triazine, *o*-chloroaniline, and chlorine were reported previously. Pyridine-alkali reaction (Zincke, 1904), based on active halogens of the *s*-triazine moiety, was used for determination of Dyrene (Barry and Lisk, 1959; Burchfield and Schuldt, 1958; Burchfield and Storrs, 1956a; MacDougall *et al.*, 1964), atrazine (Knüsli, 1964) and simazine and related chloro-*s*-triazines (Knüsli *et al.*, 1964; Ragab and McCollum, 1968). The aniline moiety diazotized after acid hydrolysis of Dyrene was used in another method (Meagher *et al.*, 1959). Chlorine hydrolyzed by NaOH was used to quantitate Dyrene in technical materials (Kane and Gillespie, 1960). Recently, Wales and Mendoza (1970) used the Dyrene-methanol reaction for confirmation of Dyrene present in plant extracts.

Dyrene incubated with methanol was shown inhibitory to liver carboxylesterase by starch-gel electrophoresis (Mendoza and Hatina, 1970). Burchfield and Storrs (1956b) showed *in vitro* that Dyrene reacted with enzyme preparations and compounds containing amino, sulfhydryl, and other nucleophilic functional groups, but the effect on enzyme activity was not studied.

Dudley *et al.* (1951) reported that *s*-triazine chloride reacted with methanol in the presence of NaOH or sodium methoxide to form alkoxy-*s*-triazine compounds. This type of reaction was further documented by Smolin and Rapoport (1959). Nevertheless, the reaction was never applied to analysis of Dyrene.

The following experiments were conducted to determine the Dyrene-alcohol products, the effect of ultraviolet light on Dyrene and Dyrene products, and the effect of Dyrene-methanol or ethanol products on liver carboxylesterases.

EXPERIMENTAL

The Dyrene standard, 99.6% pure, was dissolved in acetone (glass distilled), in hexane (glass distilled) with acetone as a cosolvent, or in alkyl alcohols (analytical grade). Concentrations of Dyrene in methanol or ethanol were 0.1, 0.003, and 0.0015*M*. Because of relative insolubility, Dyrene was studied at saturation point in propanols or butanols. Dyrene was stable in acetone or acetone-hexane combination.

Gas-liquid Chromatography (glc). An Aerograph Hi-Fi gas-liquid chromatograph equipped with an electrometer Model 500-C and an oven Model 550 was used. The chromatographic column was made of a coiled glass tubing, $4^1/_2$ ft long $\times$ $^1/_4$ in. o.d. packed with acid-washed Chromosorb W with silicones SE-30 and QF-1 (5:0.2:0.3 g) prepared according to the method of Mendoza *et al.* (1968a). Dyrene preparations were chromatographed under the following conditions: column, 190–195° C or 210–215° C with an N_2 flow rate of approximately 75 or 97 ml per min, respectively; 230° C injection block; 190° C detector; and an electrometer attenuation of 4- or 8-x at the electron capture voltage range of 1.

Glc analysis was carried out on Dyrene dissolved in acetone, methanol, ethanol, propanol, 2-propanol, butanol, *sec*-butanol, or hexane before, or after, heating in a water bath. Aliquots of the reaction solution were diluted with acetone, methanol, or hexane to concentrations appropriate for glc or tlc. Dyrene incubated in methanolic NaOH and in 30% ethanol in 0.03*N* HCl aqueous solution was also studied. Likewise, Dyrene and its products were resolved by tlc and then analysed by glc before, or after, exposure to ultraviolet light.

Thin-layer Chromatography (tlc). Tlc plates coated with Kieselgel G-HR were prepared by the procedure previously reported (Mendoza *et al.*, 1968b). Dyrene preparations were spotted on the plate by means of calibrated disposable micropipets (Scientific Co., Broomall, Pa.) and were resolved in a glass tank containing benzene or 20 ml of acetone made to 100 ml with hexane. When the solvent front reached 15 cm, the plate was removed from the tank and air-dried. Two-dimensional tlc was also carried out in the same solvent system to determine the effect of ultraviolet light on Dyrene products.

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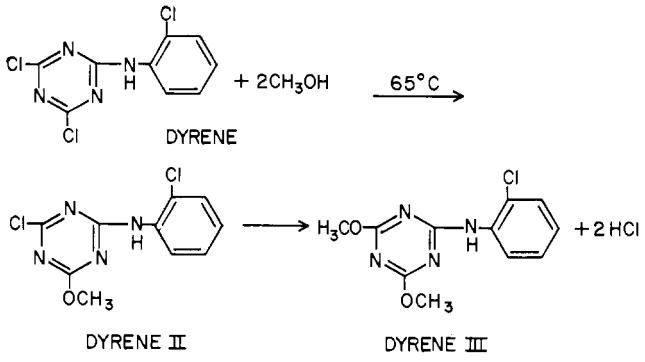


Figure 1. Diagram of Dyrene reaction with alkyl alcohol

Table I. Glc Retention Times of the Alkoxy Derivatives Relative to Dyrene (R_{Dyrene})

Chemicals	Column Temperature 190–195° C[a]	210–215° C[b]
Dyrene	1.00	1.00
Methoxydyrene	1.36	1.23
Dimethoxydyrene	1.92	1.54
Ethoxydyrene	....	1.49
Dimethoxydyrene	....	2.17

[a] Flow rate of 75 ml of N_2 per min (2 min per in. chart speed). [b] Flow rate of 97 ml of N_2 per min (2.5 min per in. chart speed).

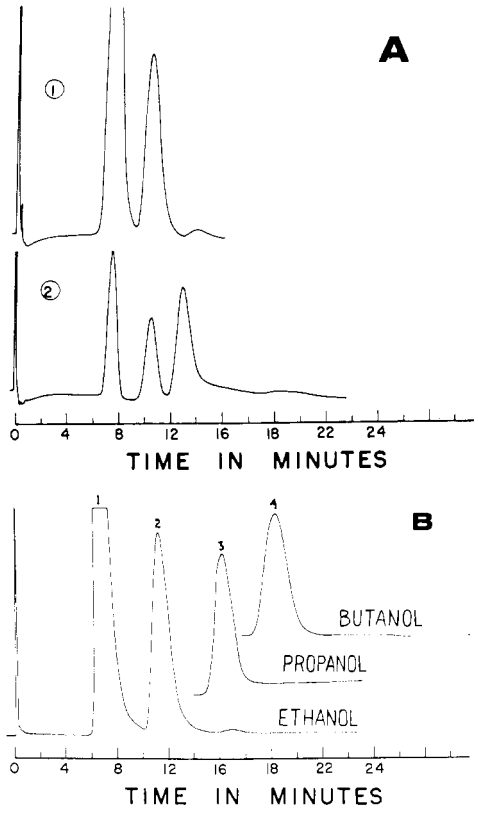


Figure 2. (A) Typical gas-liquid chromatogram of Dyrene incubated in methanol at 65° C for (1) 20 min and (2) 80 min. (B) A composite of gas-liquid chromatograms of Dyrene incubated with ethanol, propanol, and butanol showing the difference in retention times of the products represented by peaks 2, 3, and 4. Peak 1 with retention time of 7 min was Dyrene and was present in all heated solutions

Ultraviolet light exposure of Dyrene preparations on the plate before and/or after tlc resolution was carried out at various time intervals as discussed in the text. The plate was exposed 10 cm from the center of four 15-watt germicidal lamps (General Electric Co. Ltd.), which were mounted 7.5 cm apart in a wooden box.

Visualization Methods. Dyrene and Dyrene products resolved on tlc plates were observed with an ultraviolet light viewer or were sprayed with $AgNO_3$ solution (Mitchell, 1963) modified as follows:

(a) Stock solution—20 g of $AgNO_3$ (ACS grade) were dissolved in 100 ml of doubly-distilled H_2O and stored in a dark bottle.

(b) Spray solution—4 ml of solution (a), 100 ml of acetone, 10 ml of doubly-distilled H_2O, 6 ml of concentrated NH_4OH, and eight drops 2-phenoxyethanol were mixed. The mixture was stable approximately 1 week in a dark bottle. A fresh mixture was made when the solution turned dark and precipitates appeared.

Isolation of Products. Dyrene-methanol products were precipitated by flooding the reaction medium with distilled water. Glc was used to monitor the reaction so that a maximum amount of either of the Dyrene products could be recovered at a certain time.

Spectroscopy. Structures of Dyrene-methanol products were determined by using a nuclear magnetic resonance (nmr) spectroscope, Varian Model A-60A. Dyrene, Dyrene-methanol products, *o*-chloroaniline, and cyanuric chloride in KBr mulls were also analyzed with a Perkin-Elmer 221 infrared spectrophotometer.

Tlc-Enzyme Inhibition (Tlc-EI) Procedure. The tlc-EI procedure (Mendoza *et al.*, 1968b) was used to demonstrate the inhibitory property of Dyrene reaction products to liver carboxylesterases. Aqueous solutions of 2000 G liver extracts were prepared according to the recently published method (Mendoza *et al.*, 1969). Freeze-dried liver esterase (2000 G) was also used and was reconstituted before spraying with 0.05*M* Tris buffer: 530 mg of pig esterase powder per 72 ml of buffer. This concentration was comparable to the amount of solids in the diluted frozen extracts.

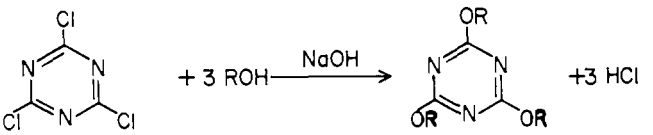
RESULTS AND DISCUSSION

Table I shows glc retention times of the alkoxy derivatives relative to that of Dyrene (R_{Dyrene}). Temperatures of 210° to 215° C were found satisfactory for quantitative detection of Dyrene. In this paper, Dyrene reaction products were referred to as Dyrene II (methoxydyrene), Dyrene III (dimethoxydyrene), Dyrene IV (ethoxydyrene) and Dyrene V (diethoxydyrene).

Effects of Different Alkyl Alcohols on Dyrene. Nmr spectra of Dyrene-methanol reaction products indicated that alkoxylation of the two carbons in the *s*-triazine ring occurred. The products were Dyrene II [2-chloro-4-(*o*-chloroanilino)-6-methoxy-*s*-triazine] and Dyrene III [2-(*o*-chloroanilino)4,6-dimethoxy-*s*-triazine] (Figure 1). Melting points found were 159–61° C (lit. 159–60° C) for Dyrene, 88–90° C for Dyrene II, and 96–8° C for Dyrene III.

The infrared spectra of Dyrene-methanol reaction products further confirmed that aniline and *s*-triazine moieties were intact as in Dyrene. The spectra of the Dyrene-methanol products did not correspond to cyanuric chloride and *o*-chloroaniline, indicating that Dyrene was not hydrolyzed to these compounds. This result was confirmed by tlc and glc. When methanol was evaporated from the solution, a loss of absorption at 3380 cm^{-1} region occurred; this may be due to the formation of HCl salts of alkoxydyrene.

At 65° C, Dyrene reacted more readily with methanol than with ethanol, propanol, and butanol. Majority of Dyrene, except in methanol, remained even after prolonged heating (Figure 2, A and B). The decrease in glc responses for and the increase in retention times of the products indicated the reaction reported by Dudley *et al.* (1951):



where R = CH_3, $-C_2H_5$, etc. The decrease in glc response was related to the removal of chlorines in the Dyrene molecule, whereas the increase in the retention times was associated with the increase in molecular weights of the alkoxy substituents. Under the conditions used, no reaction product was detected from Dyrene heated with acetone, 2-propanol, or *sec*-butanol.

Figure 3 shows the effect of heating Dyrene with methanol at 65° C. It also indicates that further reaction took place in the carrier solvent methanol prior to the glc analyses. Dyrene decreased slightly after 40 min heating and then abruptly, leaving only a trace after 100 min. The decrease in Dyrene coincided with the appearance of Dyrene II and Dyrene III. Dyrene III was less sensitive to electron-capture detection than Dyrene II, which was less sensitive than Dyrene.

Reaction of Dyrene with Methanol in the Presence of NaOH or Sodium Methoxide. In the presence of NaOH or sodium methoxide, Dyrene in methanol was readily converted to Dyrene II and then Dyrene III at approximately 25° C (Figure 4). This observation concurred with that of Dudley *et al.* (1951). Dyrene II and III concentrations were highest at 3 to 5- and 10 to 30-min periods, respectively. Only trace amounts of Dyrene were detected after 10 min. Dyrene II in methanol containing sodium methoxide diminished rapidly after 5 min; it was present in a trace amount after 30 to 40 min. However, Dyrene II in methanolic NaOH was still detectable 70 min after start of reaction.

Table II shows migration rates of reaction products, relative

Table II. Migration Rates of Reaction Products Relative to Dyrene (R_{Dyrene}) on 450 μ Thick Layer of Kieselgel G-HR at Approximately 25° C

Solvent system	Dyrene	Dyrene Products[a] II	III	IV	V
Benzene	1.00	0.58	0.23	0.70	0.44
20% acetone in hexane	1.00	0.90	0.79	1.07	1.07

[a] II = methoxy-; III = dimethoxy-; IV = ethoxy-; and V = diethoxydyrene.

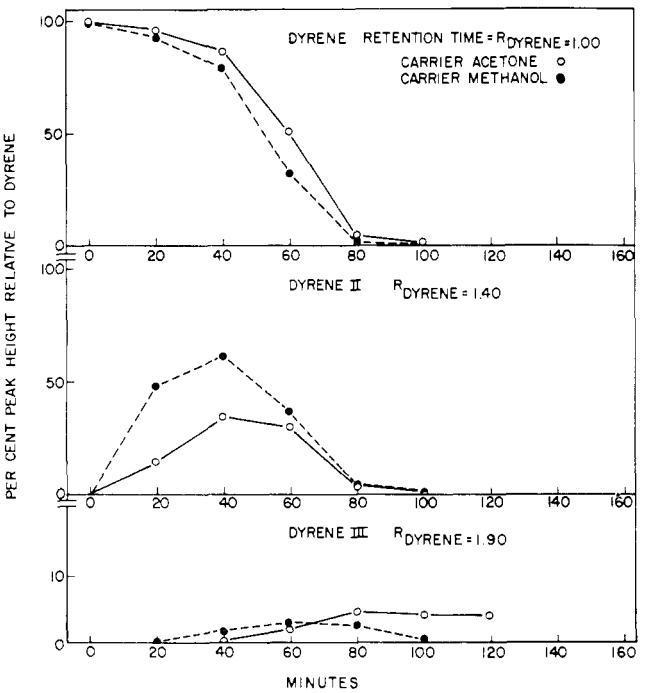


Figure 3. The reaction of Dyrene with methanol heated at 65° C and determined at different time intervals and the effect of acetone or methanol as a diluent carrier solvent for glc

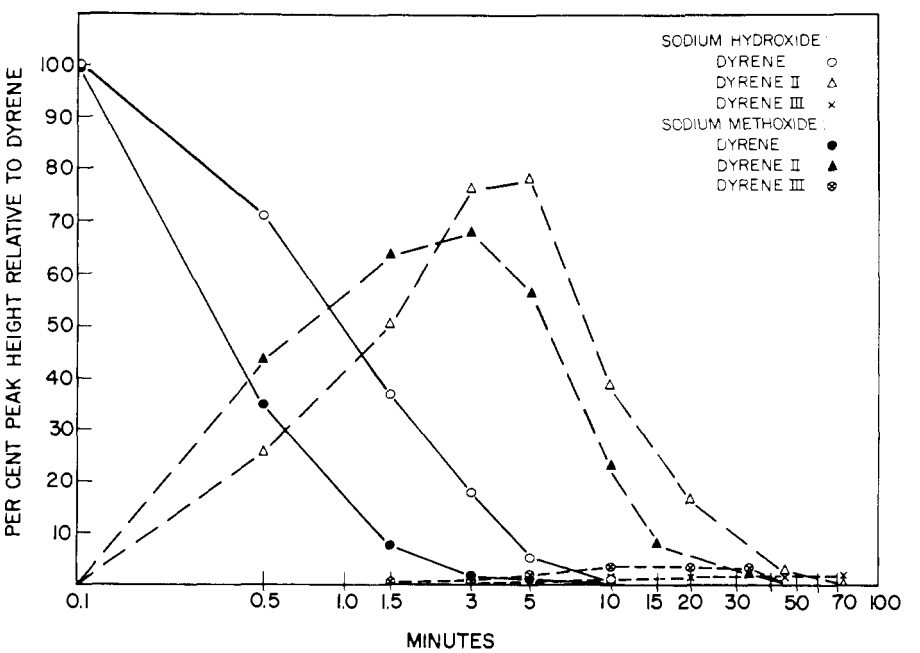


Figure 4. Reaction of Dyrene with methanol in the presence of NaOH or sodium methoxide at room temperature (or 25° C) determined at different time intervals

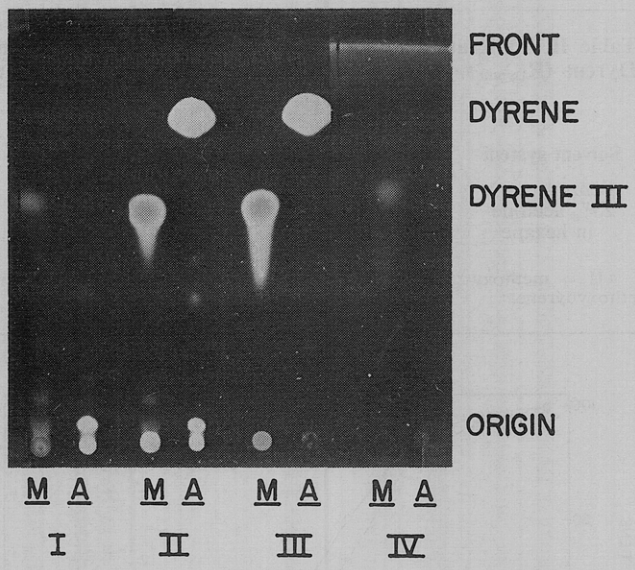
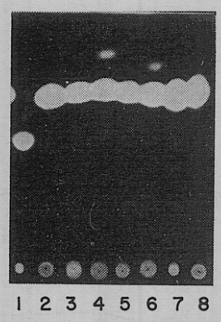


Figure 5. Effect of uv on Dyrene incubated at 65° C for 2 hr with methanol, *M*, or acetone, *A*. Positions *M* and *A* were irradiated (I) before, (II) before and after, (III) after, and (IV) neither before nor after resolutions



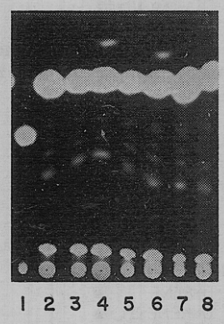


Figure 6. Typical thin-layer chromatograms of Dyrene incubated at 65° C for more than 1 hr (unless indicated) with different alcohols: (1) methanol, (2) ethanol (not heated), (3) *sec*-butanol, (4) butanol, (5) 2-propanol, (6) propanol, (7) acetone (not heated), and (8) ethanol

to that of Dyrene, in benzene and in 20% acetone in hexane systems. Benzene resolved the four reaction products.

Effect of Ultraviolet Light on Dyrene and Dyrene Products. Figure 5 shows the effect of ultraviolet light on Dyrene and Dyrene products. Dyrene appeared as dark spots under an ultraviolet light viewer (positions I *A* and IV *A*, not apparent in the photograph). Exposure to ultraviolet light for at least 15 min caused Dyrene to fluoresce intensely (position II *A* and III *A*). Irradiation of Dyrene before resolution produced two intensely fluorescent spots at and just above the origin, and several slightly fluorescent compounds (positions I *A* and II *A*). Dyrene-methanol products did not

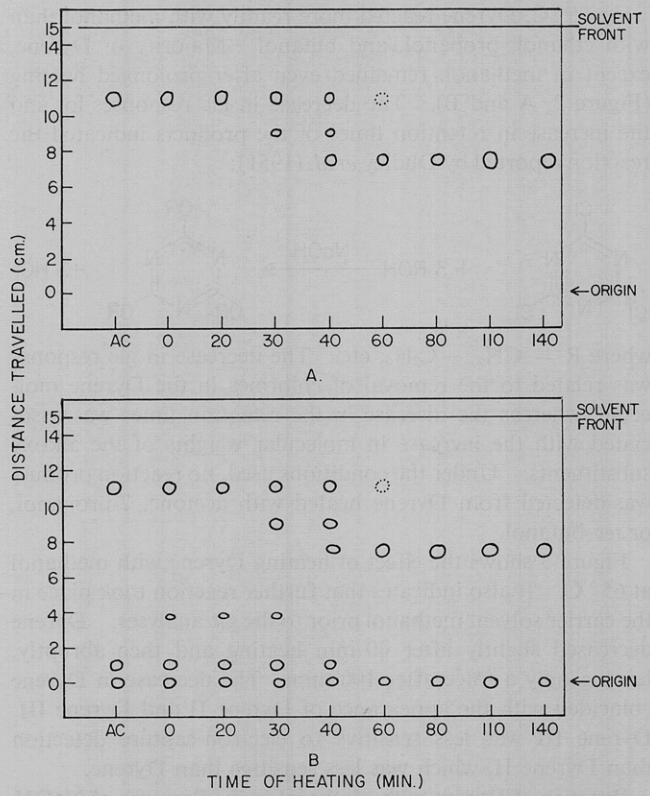


Figure 7. Effect of heating 0.2*M* Dyrene in methanol as shown by diagrams of plates on which Dyrene-methanol solutions were spotted and exposed to uv after (A) and before and after (B) resolutions. AC = Dyrene standard in acetone (control)

fluoresce (positions I *M* and IV *M*) unless they were exposed to ultraviolet light (positions II *M* and III *M*).

Two-dimensional tlc of Dyrene and its products showed that the additional spots were due to ultraviolet light and not the gel. Unchanged Dyrene had a faster migration rate than any of its products. Dyrene exposed to ultraviolet light for 1 hr before the first resolution produced a series of compounds that fluoresced when re-exposed to ultraviolet light for 15–60 min. In addition, subsequent irradiation converted the products to a final compound that was highly polar. Production of several fluorescent compounds after exposure of Dyrene to UV indicated the possibility of this process under the influence of sunlight.

Dyrene in Different Alcohols. Regardless of the reacting solvent used, Dyrene irradiated before resolution produced intensely fluorescent compounds at and just above the origin; (Figure 6, A and B) nonirradiated Dyrene appeared as a dark spot. Figure 5B shows that Dyrene (the largest and most intense spots) dissolved in acetone, ethanol, or propanol had products with similar migration rates. Fluorescent components of Dyrene in 2-propanol or *sec*-butanol had migration rates intermediate between those of Dyrene in butanol and in acetone, ethanol, or propanol. The complete conversion of Dyrene to Dyrene III after prolonged heating in methanol at 65° C confirmed that Dyrene was very reactive with methanol (Figure 6A and B, positions 1).

Only one spot corresponding to Dyrene was detected by tlc immediately after dissolution and after less than 20 min heating of 0.2*M* Dyrene in methanol (Figure 7). Likewise, a similar spot was observed with Dyrene in acetone, indicating that Dyrene had not undergone degradation. Dyrene II appeared after 20 min heating of the solution and persisted for another 20 min. At the 60-min period, aliquots showed

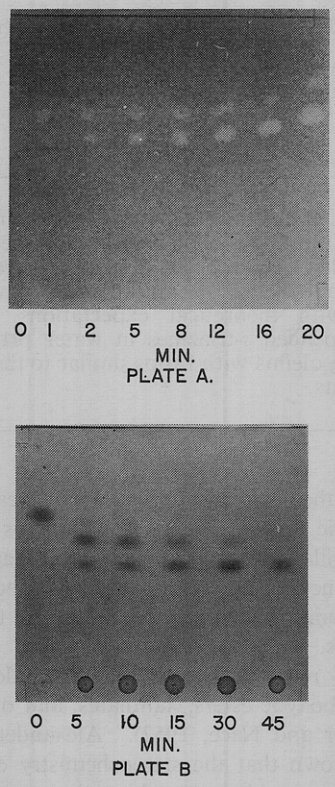


Figure 8. Chromatograms of Dyrene standard dissolved in acetone (0 min) and Dyrene reacted with methanol in the presence of NaOH. Each aliquot contained an equivalent of 4 μg (Plate A) or 2 μg (Plate B) of original Dyrene. Plate A was sprayed with pig liver homogenates and 5-bromoindoxyl acetate solution. The sites of Dyrene products appeared as white spots against an intense blue background. Plate B (250 μ thick layer of gel) was sprayed with an $AgNO_3$ solution. The compounds appeared as black spots after exposing to ultraviolet light

predominantly Dyrene III, a small amount of Dyrene, and none of Dyrene II. Probably, Dyrene II was less sensitive to tlc detection or was rapidly converted to Dyrene III. Only Dyrene was detected after 80 to 140 min. This result agreed with that obtained by tlc-$AgNO_3$ procedure and glc.

Tlc-EI Procedure. Dyrene-methanol or methanolic NaOH products strongly inhibited pig liver carboxylesterases sprayed on tlc plates (Figure 8A). (Dyrene was used at the rate of 2 μg of Dyrene per 5 μl of 0.01*N* NaOH or 2.5 μl of 0.02*N* NaOH, both in methanol.) Dyrene III, the most stable product, was the strongest inhibitor. Parent compound Dyrene was not inhibitory to the enzymes studied. The ultraviolet light and $AgNO_3$ reagent (Figure 8B) confirmed that the inhibitors were Dyrene II and III. The inhibitory property of alkoxydyrene was not altered by the ultraviolet light. Pig liver carboxylesterase detected $\geq$0.1 μg of Dyrene III calculated in terms of Dyrene. Similar preparations of sheep liver carboxylesterase detected $\geq$2 μg of Dyrene III; chicken, turkey, beef, or monkey carboxylesterases, $\geq$4 μg. The freeze-dried homogenate of the same pig liver also detected $\geq$0.1 μg of Dyrene III.

When the reaction was carried out in 0.02*N* NaOH-methanol solution at approximately 25° C, enzyme inhibitors corresponding to Dyrene II and III were obtained within 1 min after dissolution of Dyrene. Dyrene II disappeared completely after 60 min, whereas Dyrene III persisted even after 240 min.

When 0.003*M* Dyrene was redissolved with methanol alone at 65° C, Dyrene II was visible under ultraviolet light as early as the first 60-min period. The first sign of inhibition of esterases by Dyrene II and III was observed only after 180 min. No further reaction was observed even after 240 min. However, when 0.1*M* of Dyrene in methanol was incubated at 65° C, enzyme inhibition by Dyrene II and III was detectable in 20- and 30-min periods. In methanol, Dyrene at 0.1*M* required approximately 1 min heating at 65° C to complete dissolution. At a 60-min period, only Dyrene III was detected. The concentration of Dyrene in methanol was, therefore, critical to the reaction.

At 35° or 65° C, 0.003*M* Dyrene in ethanolic NaOH produced what were tentatively identified as Dyrene IV and Dyrene V. Dyrene V was a stronger inhibitor of pig liver esterase than Dyrene IV, but it was a much weaker inhibitor than Dyrene III. At 0.003*M*, Dyrene reacted with 30% ethanol in 0.03*N* HCl aqueous solution, or with ethanol alone, produced Dyrene IV only. The products were confirmed by glc, tlc, and by tlc-glc combination, *i.e.*, tlc spots corresponding to Dyrene and Dyrene products were confirmed by glc. The biological significance of the Dyrene transformation to potent enzyme inhibitors and of Dyrene reaction particularly with ethanol in an aqueous solution in the presence of HCl merits further investigation.

ACKNOWLEDGMENT

We thank J. C. Cavagnol and C. A. Anderson, Chemagro Corp., Kansas City, Mo., for providing the Dyrene standard; G. A. Neville and W. P. Cochrane for advice in spectroscopy; J. B. Shields and Ray O'Brien for the technical assistance; Jannie van Eÿk and Harold Baird for illustrations; and K. A. McCully, Bozidar Stavric, and H. L. Trenholm for criticism of the manuscript.

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Received for review April 6, 1970. Accepted July 15, 1970. Presented at the 83rd Annual Meeting of the Association of Official Analytical Chemists, Washington, D.C., October 1969.