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A Polarographic Study of some Stilbene Derivatives of Biological Interest

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4-Aminostilbene and some related compounds have been shown to have an inhibitory action on the growth of young rats and on malignant tumours (Haddow, Kon & Harris, 1945). Certain of these substances are also potent carcinogens capable of eliciting tumours at sites remote from that of administration (Haddow, Kon & Harris, 1946). The ethylenic bond present in stilbene has been found to be electroreducible at the dropping mercury electrode (Laitinen & Wawzonek, 1942) and the present investigation was undertaken for the purpose of comparing the polarographic behaviour of compounds of this series showing differing biological activities. It was also considered that the data obtained in such experiments would be of value in assessing the suitability of the polarographic method of analysis in metabolic studies. Since stilboestrol is also a stilbene derivative (4:4'-dihydroxy- $\alpha\beta$ -diethylstilbene), and similar problems arise in connexion with investigations of its metabolism in the human subject (Warren, Goulden & Robinson, 1948), it was decided to include synthetic oestrogens of this type in the investigation.

EXPERIMENTAL

Materials

All the substances used in these experiments were pure compounds. A number of the stilbene derivatives containing basic substituents was kindly put at our disposal by Prof. G. A. R. Kon and Dr R. J. C. Harris. We are also greatly indebted to Prof. E. C. Dodds and Dr W. Lawson for generous gifts of stilboestrol derivatives.

Solvent and electrolyte

The solvent employed was a dioxan-water mixture (3 : 1 by vol.) (Laitinen & Wawzonek, 1942). Commercial dioxan (British Drug Houses Ltd. 'purified') was refluxed for 12 hr. with sodium and then fractionated. Repetition of the sodium treatment was sometimes necessary in order to achieve very negative decomposition potentials. Only recently purified dioxan was used since there was some deterioration on storage.

Laitinen & Wawzonek recommend the use of tetrabutylammonium iodide as a supporting electrolyte for the attainment of very negative discharge potentials. In the present experiments tetraethylammonium iodide was found to be quite suitable, and it was easily prepared by heating

together triethylamine and ethyl iodide in absolute ethanol on a steam bath. The quaternary salt is very sparingly soluble in ethanol and rapidly separates. The salt was collected and recrystallized from absolute ethanol. It is stable and not deliquescent. The base solution was a 0.1M solution of this electrolyte.

Polarograms

The current-voltage curves were recorded on a Tinsley ink-recording polarograph. Control curves were first recorded using the base solution alone. The curve for the base solution containing the substance under examination was then superimposed on the record of the control curve. In all the measurements the observed diffusion currents were corrected for the residual current given by the base solution. O_2 was removed from the solutions by flushing with a current of H_2 . The anode was a Hg pool of relatively large area and its potential was measured against a saturated calomel electrode (s.c.e.). The anodic potential was reproducible at -0.41 V. All cathodic potentials are also referred to the s.c.e.

The dropping Hg electrode had the following characteristics. At a pressure of 71.0 cm. of Hg the drop time (t) in the electrolyte solution used was 3.05 sec. (open circuit). The Hg flow (m) was 1.96 mg./sec. The calculated value of $m^{\frac{2}{3}}t^{\frac{1}{3}}$ is 1.887. The value of m was determined by direct weighing up to a negative potential of 2.4 V., and was found to be constant over this range. Direct measurements of t were also made over the same range, but the short drop times occurring at potentials more negative than 2.0 V. were checked independently by measurements of the diffusion current of nitrobenzene over the range -1.4 to -2.4 V. The effect of the decreased drop time reduced the product $m^{\frac{2}{3}}t^{\frac{1}{3}}$ from a value of 1.837 at -1.4 V. ($t=2.6$) to 1.566 at -2.4 V. ($t=1.0$), and the observed diffusion current of nitrobenzene showed a similar linear decrease over this range. All measurements were carried out at 25° .

RESULTS

In Table 1 the results obtained with stilbene derivatives not bearing substituents on the ethylenic carbon atoms are shown. Table 2 contains the measurements on compounds containing substituents in these positions. For each compound the range of concentration over which measurements were made is given together with the half-wave potential $E_{\frac{1}{2}}$ (v. s.c.e.), and the observed diffusion current i_d , after correction for the residual current. The half-wave potentials (in the tables negative signs have been omitted) have not been corrected for iR drop; with the type of cell used this correction would be small. The diffusion current shown by a mM-solution has been calculated from the measurements made at each concentration. The essential constancy of the half-wave potential with varying concentration of the substance, in those compounds that permitted its measurement, indicates that the electrode process is independent of the concentration of hydrogen ions, and justifies the use of unbuffered solutions in these measurements.

DISCUSSION

Two of the compounds included in this investigation have been examined by other workers (Laitinen & Wawzonek, 1942). For stilbene they reported $E_{\frac{1}{2}} = -2.14$ V. (v. s.c.e.) and $i_d/C = 4.04$. The corresponding values for this substance from Table 1 are -2.17 and 4.12 . Although the half-wave potential determined in the present experiments agrees with that found by Laitinen & Wawzonek, the agreement between the values for i_d/C is less good when allowance is made for the difference in capillary characteristics. At the voltage used by Laitinen & Wawzonek for measurement of the diffusion current of stilbene (-2.31 V.), $m^{\frac{2}{3}}t^{\frac{1}{3}}$ had a value of 1.828 in their experiments, while the corresponding value in the present experiments was 1.593. If correction is made for the difference between the two factors the value of i_d/C shown in Table 1 becomes 4.73 as compared with the value of 4.04 given by Laitinen & Wawzonek.

The same workers also measured triphenylethylene and their values of $E_{\frac{1}{2}} = -2.12$ and $i_d/C = 3.55$ may be compared with the data in Table 2. Again agreement between values for the half-wave potentials is good, but the value for i_d/C found in the present experiments is higher (3.86) and, when corrected for difference in capillary, considerably higher (4.31) than that found by the American workers.

The half-wave potentials of most of the compounds shown in Table 1 fall within a narrow range. In the stilbene series, substitution in one or both nuclei results in a slightly more negative half-wave potential. The substituted stilbene analogues containing a naphthalene nucleus (last four compounds in Table 1), however, give waves at potentials equal to that of stilbene or more positive. This is particularly striking in the case of α -4-dimethylamino-phenyl- β -2'-naphthylethylene. The second wave shown by these substances at approximately -2.50 V. is presumably due to reduction of the naphthalene nucleus, since reduction of this hydrocarbon gives a wave at this potential.

The general similarity of the values of i_d/C for most of the compounds in Table 1 indicates that there are no large differences in diffusion coefficients among these substances. In the cases of 4-amino-4'-hydroxystilbene and 4-amino-4'-hydroxy-2'-methylstilbene, which show rather lower values for i_d/C , it seems probable that these are due to changes in the nature of the potential-determining reaction at the dropping electrode rather than to reductions in diffusion rates. From the recorded current-voltage curves, plots were made of $\log i/(i_d - i)$ against voltage. In general the reciprocal slopes of these graphs were in approximate agreement with the theoretical value of 0.059 V.

for a reversible, potential-determining reaction involving one electron. However, the plots of the data for 4-amino-4'-hydroxystilbene and for 4-amino-4'-hydroxy-2'-methylstilbene gave slopes of 0.11 V.

in the 4 and 4' positions are very susceptible to oxidation and it is possible that small quantities of such oxidation products were present in the specimens used.

Table 1. *Polarographic data for stilbene derivatives not bearing substituents on the ethylenic carbon atoms*

Compound	$-E_{\frac{1}{2}}$ (V. v. S.C.E.)	i_d (μ amp.)	C (mm)	i_d/C
Stilbene	2.17	2.70	0.655	4.12
	2.17	5.40	1.310	4.12
4-Aminostilbene	2.30	2.12	0.646	3.28
	2.32	4.42	1.292	3.42
	2.31	6.68	1.938	3.44
4-Methoxystilbene	2.24	1.48	0.398	3.72
	2.24	3.04	0.796	3.82
	2.25	4.39	1.194	3.78
4-Hydroxystilbene	2.34	1.50	0.476	3.15
	2.34	2.27	0.714	3.18
	2.34	2.94	0.952	3.09
4-Dimethylaminostilbene	2.29	2.18	0.588	3.71
	2.29	4.39	1.176	3.73
	2.29	6.41	1.764	3.64
3-Dimethylaminostilbene	2.19	1.62	0.453	3.57
	2.19	3.29	0.906	3.63
	2.19	4.81	1.359	3.54
4-Amino-4'-hydroxystilbene	2.45	1.03	0.446	2.31
	2.44	2.00	0.892	2.24
	2.46	3.02	1.338	2.26
4:4'-Dimethoxystilbene	2.33	1.19	0.320	3.72
	2.31	2.34	0.640	3.66
	2.32	3.53	0.960	3.68
4-Dimethylamino-2'-methylstilbene	2.32	1.76	0.518	3.40
	2.30	3.60	1.036	3.48
	2.30	5.49	1.554	3.53
4-Dimethylamino-3'-methylstilbene	2.34	1.60	0.554	2.89
	2.33	3.68	1.108	3.32
	2.33	5.56	1.662	3.34
4-Dimethylamino-4'-methylstilbene	2.36	1.87	0.565	3.31
	2.35	3.67	1.130	3.25
	2.34	5.70	1.695	3.36
4-Dimethylamino-2':5'-diethylstilbene	2.37	0.90	0.303	2.97
	2.33	1.90	0.606	3.14
	2.34	4.43	1.515	2.92
4-Amino-4'-hydroxy-2'-methylstilbene	2.41	1.74	0.724	2.40
	2.44	2.49	1.086	2.30
2:4:6:2':4':6'-Hexamethylstilbene	2.47	1.08	0.752	1.44
	2.48	2.48	1.880	1.32
α -4-Dimethylaminophenyl- β -1'-naphthylethylene	2.12 2.50	1.32	0.322	4.10
	2.12 2.50	2.70	0.644	4.19
	2.10 2.50	3.94	0.966	4.08
α -4-Dimethylaminophenyl- β -2'-naphthylethylene	1.80 2.49	0.87	0.188	4.63
	1.82 2.49	1.90	0.470	4.06
α -4-Dimethylaminophenyl- β -1'-(2'-methyl)-naphthylethylene	2.18	0.73	0.239	3.05
	2.17	1.60	0.478	3.34
	2.17 2.54	3.75	1.195	3.14
α -Phenyl- β -1-(4-dimethylamino)naphthylethylene	2.08 2.53	—	—	—

Although the nature of the available data precluded a high degree of accuracy in plotting these derived curves, there seems little doubt that the electrode process differed in these compounds. Stilbene derivatives bearing both amino and hydroxyl groups

Certain of the compounds in Table 1 are of especial interest since they possess carcinogenic properties. 4-Aminostilbene, 4-dimethylaminostilbene, 4-dimethylamino-2'-methylstilbene and α -4-dimethylaminophenyl- β -1'-naphthylethylene have all been

reported as inducing malignant tumours in rats (Haddow *et al.* 1945, 1946). From the data of Table 1 there is nothing to distinguish such biologically active substances from an inactive one such as stilbene.

with the waves developed by anthracene, naphthalene, and phenanthrene under the same conditions.

In general, the polynuclear aromatic hydrocarbon carcinogens give two waves at approximately -1.90 and -2.50 V. These waves are characteristic for the

Table 2. *Polarographic data for stilbene derivatives bearing substituents on the ethylenic carbon atoms*

Compound	$-E_2$ (V. v. S.C.E.)	i_d (μ amp.)	C (mm)	i_d/C
α -Ethylstilbene	2.30	1.57	0.408	3.84
	2.31	3.14	0.816	3.84
	2.31	4.65	1.224	3.80
$\alpha\beta$ -Diethylstilbene (m.p. 71.5–72.5°)	NW	—	—	—
$\alpha\beta$ -Diethylstilbene (m.p. 74–75°)	(2.59)	—	—	—
Stilboestrol (4:4'-dihydroxy- $\alpha\beta$ -diethylstilbene)	NW	—	—	—
Dienoestrol (3:4-di-(4'-hydroxyphenyl)hexa-2:4-diene)	NW	—	—	—
4:4'-Dihydroxy- α -methyl- β -ethylstilbene	NW	—	—	—
4:4'-Dihydroxy- α -methyl- β -cetylstilbene	NW	—	—	—
4:4'-Dihydroxy- α -phenyl- β -ethylstilbene	NW	—	—	—
2:2'-Dihydroxy- $\alpha\beta$ -diethylstilbene	NW	—	—	—
4-Methoxy- $\alpha\beta$ -diethylstilbene	NW	—	—	—
4:4'-Diacetyl- $\alpha\beta$ -diethylstilbene	1.77	1.34	0.172	7.80
	1.78	2.71	0.345	7.85
	1.79	4.07	0.517	7.85
4:4'-Dihydroxy- α - <i>n</i> -propylstilbene	2.52	2.80	1.890	1.47
4:4'-Dihydroxy- α - <i>n</i> -amylstilbene	2.53	1.80	1.570	1.15
4:4'-Dihydroxy- α -isopropylstilbene	(2.61)	—	—	—
Triphenylethylene	2.12	1.22	0.328	3.72
	2.09	2.53	0.656	3.86
	2.10	3.80	0.984	3.86
Triphenylmethylethylene	2.24	1.20	0.330	3.64
	2.25	2.45	0.660	3.72
	2.24	3.59	0.990	3.63
α -4-Hydroxyphenylstilbene	2.17	0.86	0.266	3.23
	2.17	1.75	0.532	3.29
	2.17	2.68	0.798	3.35
α -Phenyl- $\beta\beta$ -di-4-hydroxyphenylethylene	2.28	1.04	0.320	3.25
	2.29	2.28	0.640	3.56
α -Ethyl- β -4-hydroxyphenylstilbene	2.36	2.20	0.467	4.72

Compounds showing no reduction at the dropping electrode are indicated by NW (no wave). Two compounds appeared to give indications of waves at very negative potentials and these values are included in parentheses.

It is interesting to compare the data for these substances with those for other carcinogens. Wawzonek & Laitinen (1942) showed that, although aromatic compounds possessing a high degree of resonance, such as benzene, are not reduced at the dropping mercury electrode, aromatic polynuclear hydrocarbons showing a diminished degree of resonance are reducible. In experiments which have been confirmed and extended by the present authors, they established the polarographic behaviour of such carcinogens as 3:4-benzpyrene, methylcholanthrene, 1:2:5:6-dibenzanthracene, and 1:2-benzanthracene. The polarographic waves resulting from reduction of these compounds in aqueous dioxan were compared

reduction of an anthracenic structure (9:10 addition) followed by that of the resulting naphthalenic nucleus (1:4 addition). The polarographic reduction resembled, in the relative ease and site of attack, such reactions as the addition of alkali metals or reduction by ethanol and alkali metals.

From the data in Table 1 it is clear that the susceptibility of the ethylenic bond of stilbene derivatives to reactions involving addition at this site is intermediate between that shown by the anthracenic and naphthalenic bonds of the hydrocarbon carcinogens. Possibly, with stilbene derivatives, as with polycyclic aromatic hydrocarbons, this degree of susceptibility is an important feature in their

adsorption on to enzymes. From a comparative point of view the stilbene ethylenic bond is analogous to the 1:2 double bond in pyrene ($E_1 = -2.10$) rather than to the 9:10 double bond in phenanthrene ($E_1 = -2.44$).

All but one of the compounds shown in Table 2 are α - or $\alpha\beta$ -substituted stilbene derivatives. The exception is dienoestrol in which there is a conjugated hexadiene system. In general, monosubstitution on the α -carbon atom results in reducible derivatives with half-wave potentials approximately the same or slightly higher than that of stilbene itself. This applies to both α -alkyl and α -aryl derivatives. The nature of the substituent groups (alkyl or aryl) is, however, of decisive importance in disubstituted derivatives. Stilbene derivatives with alkyl groups on both α and β carbon atoms are, in general, not reducible at the mercury cathode. In the exceptional case of $\alpha\beta$ -diethylstilbene, two specimens were available; one had m.p. $71.5-72.5^\circ$, the other m.p. $74-75^\circ$. These are probably the *trans* and *cis* forms, respectively, of this hydrocarbon (Goulden, unpublished). Although the constitution of these two substances has not yet been finally established, the evidence at present available suggests that the *trans* form is not reducible. The higher-melting form gave some evidence of a very ill-defined wave at approximately -2.6 V. Apart from this one rather doubtful exception, $\alpha\beta$ -dialkyl derivatives of the stilbene and stilboestrol series were not reducible. The large wave shown by 4:4'-diacetyl- $\alpha\beta$ -diethylstilbene is presumably produced by reduction of the keto groups, since reduction of acetophenone under the same conditions gave a wave at about the same potential (-1.67 V.). Disubstitution with one alkyl and one aryl group produces reducible derivatives, e.g. triphenyl-methylethylene and α -ethyl- β -(4-hydroxyphenyl)-stilbene.

One object of the present work was to explore the possibility of using polarographic methods of analysis in the detection and estimation of stilbene derivatives in metabolic experiments. The results indicate at least one serious practical difficulty. The half-wave potentials lie between about -2.2 and -2.6 V., i.e. more negative than the discharge potentials of ions of the alkali metals or of ammonium (c. -2.2 V.). The presence of small amounts of these ions makes it impossible to detect or measure the reduction of stilbene derivatives. Preliminary experiments on blood and urine from rats treated with 4-dimethylaminostilbene showed that the preparation of extracts suitable for polarographic measurements (i.e. free from Na^+ , K^+ and NH_4^+) was difficult, and in no case was a satisfactory measurement obtained.

SUMMARY

1. Polarographic data are presented on 37 compounds of the stilbene and stilboestrol series.
2. Although certain of the stilbene derivatives possessed carcinogenic potency, no differentiation could be made between these compounds and related non-carcinogenic stilbenes.
3. Stilbene derivatives of the types, α -alkyl, α -aryl, or α -alkyl- β -aryl, are reducible at the dropping mercury electrode. Derivatives of the type $\alpha\beta$ -dialkyl are not reducible.
4. $\alpha\beta$ -Dialkylstilboestrols and dienoestrol are not reducible.
5. The highly negative half-wave potentials of stilbene derivatives make the use of polarographic methods difficult for the detection and estimation of these substances in biological materials.

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