XIX. THE ACTION OF SOME *ENDO*SUCCINIC ACIDS DERIVED FROM POLYCYCLIC HYDROCARBONS ON THE RED BLOOD CORPUSCLES OF THE MOUSE

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(*Received 17 December 1938*)

Strong [1936] and Strong & Francis [1937] have described a fall in the haemoglobin content of the blood of female mice of strains which are liable to the development of spontaneous mammary carcinoma. The experiments of Parsons and her collaborators have shown that general irradiation of mice or rats with sub-lethal doses of X-rays increases and accelerates tumour production on subsequent treatment of the animals with a carcinogenic compound [Mayneord & Parsons, 1937; Clarkson *et al.* 1938; Parsons, 1938]. This change produced by the X-rays is one affecting the haemopoietic system; the red blood cells are diminished (in rats) and iron is deposited in the lymph glands.

Parsons and her fellow-workers have also shown that the same signs of blood destruction are present in mice undergoing treatment with a carcinogenic substance. Application of the prussian blue reaction to sections of the lymph glands of these animals before the appearance of tumours revealed the presence of iron. In a forthcoming publication the present author will adduce quantitative estimations of iron in support of these microscopical observations.

In view of these results it was considered of interest to carry out some simple *in vitro* experiments on the action of the compound used by Parsons and co-workers. The experiments were ultimately extended to embrace a series of similar compounds derived from carcinogenic and closely related non-carcinogenic hydrocarbons. The choice of the hydrocarbons to be used was necessarily restricted to those which yield adducts with maleic anhydride. For example, phenanthrene and 3:4-benzphenanthrene were excluded by this condition and, so far, all efforts to obtain an adduct from 3:4-benzpyrene have been unsuccessful.

Materials

The compound used by Parsons and co-workers was sodium 1:2:5:6-dibenzanthracene-9:10-*endo*-$\alpha\beta$-succinate [Cook, 1931]. Diels & Alder [e.g. 1931] and Clar [1932] have shown that hydrocarbons of the anthracene type form adducts with maleic anhydride. More recently Bachmann & Kloetzel [1938] have shown that the reaction is a balanced one and have described a procedure for obtaining good yields of the adducts from several important carcinogenic hydrocarbons.

In the present work the solutions of the Na salts of the *endo*succinic acids were prepared as follows. The anhydrides were prepared by boiling the hydrocarbon (1 part) in xylene or benzene (100 parts) with maleic anhydride (10 parts). After distillation of the solvent the anhydride was hydrolysed by warming for a few minutes with conc. KOH, the alkaline solution diluted with a large volume of water, warmed to effect solution of the K salt and filtered from unchanged

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hydrocarbon. On acidification of the filtrate the free acid was precipitated and collected. Solutions of the Na salts of the *endo*succinic acids were prepared by warming a slight excess of the acid with a measured volume of $N/10$ NaOH and filtering from the excess acid. Sufficient NaCl was added to the filtrate to make the final concentration 0·85 % (the effect of the *endo*succinate on the tonicity of the solution may be neglected since its concentration is low and its mol.wt. is high).

In a few cases this procedure had to be modified as the free acid on precipitation rapidly dehydrated to the anhydride which was insoluble in dilute NaOH. In these cases advantage was taken of the fact that the Na salts are very sparingly soluble in presence of NaCl and, after hydrolysing the anhydrides with conc. NaOH, the salt of the *endo*succinic acid was precipitated by saturating the solution with NaCl. The Na salt was collected and well washed with sat. NaCl and finally with 0·85 % NaCl to remove excess alkali.

The solubilities of the Na salts in 0·85 % NaCl vary considerably. In the group consisting of the derivatives of 1:2:5:6-dibenzanthracene (*cis* compound), 1:2:3:4-dibenzanthracene, 5:6-*cyclo*penteno-1:2-benzanthracene, 9:10-dimethyl-1:2-benzanthracene, cholanthrene and methylcholanthrene, the solubility in cold saline is not very great and the stock solutions consisted of saturated solutions of their salts. Anthracene, naphthacene, 1:2-benzanthracene and the remaining methyl-substituted 1:2-benzanthracenes employed gave Na salts which were relatively easily soluble and stock solutions of these were prepared containing 2 mg. per ml. (0·2 %). The *trans*-acid from 1:2:5:6-dibenzanthracene also gave a readily soluble Na salt.

In all cases the solutions used for the haemolytic tests had a *p*H of approximately 7·2.

The compounds used in this investigation are given in the following list under the name of the parent hydrocarbon. With one exception all the *endo*-succinic acids used have the normal (*cis*) configuration. Reference is made below to their method of preparation and in the case of six members of the series which have not been hitherto described, analytical specimens of the anhydrides were isolated and the results of their elementary analyses are given. All melting points are uncorrected.

Anthracene. Prepared from anthracene by the method of Diels & Alder [1931]. The Na salt is easily soluble in saline.

Naphthacene. The procedure given by Clar [1932] was followed. The Na salt is readily soluble.

1:2-Benzanthracene. Prepared according to Clar's directions. Na salt comparatively easily soluble.

1:2:5:6-Dibenzanthracene. The normal (*cis*) compound was prepared according to Bachmann's improved method. Conversion into the stereoisomeric *trans*-compound was effected in the manner described by Diels & Alder [1931] for the corresponding anthracene adduct. The *cis*-acid was converted into the *cis*-dimethyl ester [Bachmann & Kloetzel, 1938] and the latter rearranged by boiling in alcoholic solution with Na. The *trans*-ester which is formed as an intermediate is hydrolysed by the excess of $NaOC_2H_5$ to the *trans*-Na salt. The free *trans*-acid was liberated from the Na salt by acidification. A specimen of this acid on recrystallization from dilute acetic acid melted at 255–257° and gave a depression on mixing with a specimen of the pure *cis*-acid (M.P. 230°). The solubility of the Na salt of the *trans*-acid is very much greater than that of the *cis*-acid.

For further characterization a specimen of the *trans*-acid was converted into the dimethyl ester by means of diazomethane. The ester crystallized from

methyl alcohol in prisms, M.P. 179–180°. (The corresponding *cis*-ester has M.P. 230–231°). Found: C, 79·5; H, 5·6%. $C_{28}H_{22}O_4$ requires: C, 79·6; H, 5·25%.

1:2:3:4-Dibenzanthracene. The adduct was prepared in xylene. The xylene solution was cooled in the ice-chest overnight and the solid which separated was collected and recrystallized twice from xylene. Clusters of thick needles were obtained, M.P. 250–251°. Found: C, 82·85; H, 4·3%. $C_{26}H_{16}O_3$ requires: C, 83·0; H, 4·3%. The Na salt of the acid is sparingly soluble in saline.

*5:6-*cyclo-*Penteno-1:2-benzanthracene.* The free acid was prepared by the general procedure described above. On boiling a solution of the free acid in acetic anhydride for a short time and then cooling the solution the anhydride slowly crystallized in stout needles. After two recrystallizations from acetic anhydride the crystals melted at 245–246°. Found: C, 81·5; H, 5·0%. $C_{25}H_{18}O_3$ requires: C, 81·7; H, 5·0%. The Na salt of this acid is sparingly soluble in saline.

Cholanthrene. The adduct was prepared in benzene. The solid which separated on cooling the benzene solution was recrystallized from ethyl acetate. The anhydride crystallized in rectangular plates melting at 219–220°. Found: C, 81·5; H, 4·7%. $C_{24}H_{16}O_3$ requires: C, 81·8; H, 4·6%. The Na salt is sparingly soluble.

Methylcholanthrene. A specimen of the anhydride supplied by Dr W. E. Bachmann was converted into the Na salt of the acid. The salt is sparingly soluble.

9:10-Dimethyl-1:2-benzanthracene. A specimen of the anhydride was furnished by Dr Bachmann. The Na salt is sparingly soluble.

3-Methyl-1:2-benzanthracene. The free acid was prepared in the usual way. Treatment with acetic anhydride and recrystallization from the same solvent yielded the anhydride as prisms melting at 257–258°. Found: C, 80·9; H, 4·8%. $C_{23}H_{16}O_3$ requires: C, 81·15; H, 4·7%. The Na salt is relatively easily soluble.

5-Methyl-1:2-benzanthracene. Prepared as for the 3-methyl compound. The anhydride crystallized from acetic anhydride in fine needles melting at 252–253°. Found: C, 81·1; H, 4·8%. $C_{23}H_{16}O_3$ requires: C, 81·15; H, 4·7%. Na salt relatively easily soluble.

10-Methyl-1:2-benzanthracene. The anhydride prepared from the free acid by boiling with acetic anhydride crystallized from this solvent in prisms melting at 262–264°. Found: C, 81·0; H, 5·0%. $C_{23}H_{16}O_3$ requires: C, 81·15; H, 4·7%. The Na salt is relatively easily soluble.

Other methyl-substituted 1:2-benzanthracenes. Solutions of the sodium salts of the *endo*succinic acids of 4-, 6-, 2'-, and 3'-methyl-1:2-benzanthracenes and also of 2':7-dimethyl-1:2-benzanthracene were prepared without isolating the intermediate anhydrides. The Na salts of these acids are all relatively easily soluble in 0·85% NaCl.

TECHNIQUE OF THE HAEMOLYSIS EXPERIMENTS

Mice were killed by breaking the neck and the blood from the heart drawn off with a syringe as quickly as possible. The blood was discharged into a small volume of 0·85% NaCl, centrifuged, the red cells washed twice with saline and suspended in a small volume of 0·85% NaCl.

The aqueous solutions of the Na salts were set up in 4 small tubes; the first contained the "stock" concentration (2 mg. per ml. in the case of the more soluble salts and a saturated solution in the case of those salts which were not soluble to this extent), and the remaining 3 aliquot dilutions of the first. A fifth tube contained only 0·85% NaCl as a control. To each of the 5 tubes two drops

of the red blood cell suspension were added and the tubes inverted once or twice to ensure mixing. The tubes were stoppered and observed at intervals for 24 hr. at room temperature.

In the control tubes the upper limit of the sinking red cells was quite sharply defined, and later these formed a compact deposit. In the experimental tubes the first sign of haemolysis was often a diffuseness in the upper limit of the falling red cells and the fluid above had a yellow tinge. Where the compound was very actively haemolytic, lysis occurred before any appreciable settling of the red cells had taken place and the first observable change was the increasing transparency as compared with the opaque suspensions in the control tubes.

Results

Using the simple technique outlined above the haemolytic actions of the 18 compounds previously mentioned were tested. Broadly speaking the activities of the compounds fall into three groups. In the 1st are those compounds which haemolysed rapidly and gave clear red transparent solutions within a matter of minutes. The members of this group are all sparingly soluble salts so it was not possible to arrange them in any order of activity as the saturated solutions employed were not necessarily of equal concentrations. In the 2nd group are those compounds which showed a lower degree of activity, and an approximate estimate of the relative potencies of these compounds could be obtained since the stock solutions of all of them contained 2 mg. per ml. In the 3rd group are three compounds which showed no evidence of haemolytic action even though left in contact with the red cells for 24 hr.

Table I gives a summary of the results for these three groups.

Table I

	Haemolytic activity
Group I. (Saturated solutions)	
*endo*Succinates from:	
1:2:5:6-Dibenzanthracene (*cis* and *trans*)	Strongly haemolytic
1:2:3:4-Dibenzanthracene	,, ,,
5:6-*cyclo*-Penteno-1:2-benzanthracene	,, ,,
Cholanthrene	,, ,,
Methylcholanthrene	,, ,,
9:10-Dimethyl-1:2-benzanthracene	,, ,,
Group II. (Solutions 0·2% and less)	
*endo*Succinates from:	
2′:7-Dimethyl-1:2-benzanthracene	+ + + + + +
5-Methyl-1:2-benzanthracene	+ + +
6-Methyl-1:2-benzanthracene	+ + +
10-Methyl-1:2-benzanthracene	+ + +
3′-Methyl-1:2-benzanthracene	+ + +
3-Methyl-1:2-benzanthracene	+ +
2′-Methyl-1:2-benzanthracene	+ +
4-Methyl-1:2-benzanthracene	+
Group III. (Solutions 0·2% and less)	
*endo*Succinates from:	
Anthracene	Inactive
Naphthacene	,,
1:2-Benzanthracene	,,

Discussion

The first point of interest in considering the results is any possible correlation between the haemolytic activities of the *endo*succinic acids and the known carcinogenic activities of the parent hydrocarbons. (It should be noted that the

water-soluble derivatives themselves have been tested for cancer-producing action in only three cases—the derivatives from 1:2:5:6-dibenzanthracene (*cis* and *trans*) and methylcholanthrene; all these produced sarcomas in mice.)

In Group I of the Table, 5 of the 6 hydrocarbons are potent carcinogens, the exception being 1:2:3:4-dibenzanthracene which in this laboratory produced no tumours when applied to the skin of a series of 20 mice, of which only 2 lived for more than a year, the last mouse dying on the 487th day. In an earlier experiment 3 epitheliomas were obtained in 3 out of 70 mice with an impure specimen of the hydrocarbon, the earliest tumour appearing after 619 days. No reports on this compound from other laboratories have been found in the literature. In Group II, the 2′:7-dimethyl-, 2′-methyl- and 3′-methyl-1:2-benzanthracenes have not yielded tumours in any experiments recorded as yet. The 5 remaining hydrocarbons in this group are definitely carcinogenic, but, with the exception of the 10-methyl derivative, inferior in carcinogenic potency to the active hydrocarbons of Group I. In Group III anthracene and naphthacene have not given tumours in any experiments as yet reported, while 1:2-benzanthracene produced 1 epithelioma when applied to the skin of 30 mice, although negative results have been obtained in another series of 50 mice.

In general it may be said, for the 18 compounds tested, that the water-soluble salt derived from a hydrocarbon which is known to be carcinogenic is haemolytic but that the converse is not necessarily true and hydrocarbons which have not been shown to be carcinogenic may give rise to haemolytic *endo*-succinic acids.

Summary

The Na salts of the *endo*succinic acids derived from 17 polycyclic hydrocarbons have been examined for haemolytic action on mouse red blood cells. Haemolytic action was shown by the water-soluble derivatives from all hydrocarbons with a pronounced carcinogenic activity. Haemolysis was also shown by the derivatives of some hydrocarbons which have not been found to be carcinogenic.

I am indebted to Dr L. D. Parsons, who suggested this investigation, for much help with the haemolysis experiments and to Dr W. E. Bachmann and to Prof. J. W. Cook for the gift of compounds. It is a pleasure to express my thanks to the Sir Halley Stewart Trust for a Fellowship held during this work and to the British Empire Cancer Campaign for generous grants which have supported this investigation.

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