[CONTRIBUTION FROM THE CHEMISTRY LABORATORY OF THE UNIVERSITY OF MICHIGAN]

THE COUPLING OF DERIVATIVES OF POLYCYCLIC HYDROCARBONS WITH GLYCINE

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In recent years it has been shown that cancer may be produced in animals by means of chemical compounds, most of which are polycyclic hydrocarbons. From investigations of cell-free filtrates obtained from certain types of tumors in birds many investigators consider that the active agent in these cases is a submicroscopic organism or virus. From the results of these studies some investigators have considered that there is little connection between the modes of action of the virus and the hydrocarbons. Attention has been directed particularly to the different periods of time which elapse between the inoculation and the appearance of tumors. Thus, in contrast to an induction period of a week or two when an active tumor filtrate is used for inoculation, a matter of months may be required for the production of tumors by a chemical compound. It has been pointed out recently, however, that this phase of the subject requires further attention in view of the rapid production of tumors which has been observed with certain synthetic compounds, particularly with 9,10-dimethyl-1,2-benzanthracene, which has yielded tumors in five weeks when applied externally to the skin of mice.[1]

Recently, Wyckoff[2] has demonstrated that the active agent of the Shope papilloma is a protein of high molecular weight. It is apparent, then, that the two different lines of investigation have approached each other.[3] It is of interest, therefore, to study compounds prepared by combining a protein with an active carcinogenic compound or with a derivative of an active compound. The latter has already been accomplished by Creech and Franks,[4] who reported their results at the time our investigation was initiated. These investigators coupled 1,2,5,6-dibenzanthranylisocyanate, a derivative of the carcinogenic hydrocarbon 1,2,5,6-dibenzanthracene, with various proteins and with glycine and

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[1] BACHMANN, KENNAWAY, AND KENNAWAY, *Yale J. Biol. and Med.* **11**, 97 (1938).

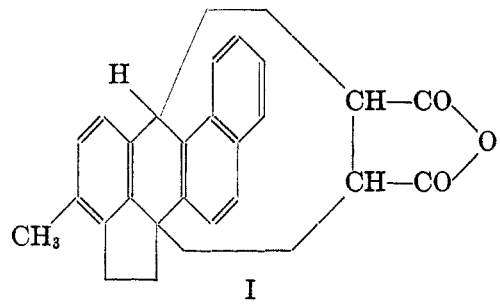
[2] WYCKOFF, *Compt. rend. soc. & biol.*, **125**, 5 (1937); *Science*, **86**, 92 (1937).

[3] See, for example, the excellent lecture by COOK, *Yale J. Biol. and Med.* **11**, 1 (1938).

[4] CREECH AND FRANKS, *Amer. J. Cancer*, **30**, 555 (1937).

studied the chemical and serological properties of the resulting compounds. Although the isocyanate and the protein compounds proved to be inactive, the compound obtained with glycine was capable of inducing tumors in mice in a relatively short time.

The successful preparation of compounds of the type of 3-methylcholanthrene-endo-succinic anhydride† (I) provided us with compounds which could be employed for coupling with the proteins in virtue of the active acid anhydride group in the molecule. These compounds are readily obtained by means of a Diels-Alder reaction involving the addition of maleic anhydride to the polycyclic hydrocarbon under the proper conditions of temperature and concentration.[5]



I

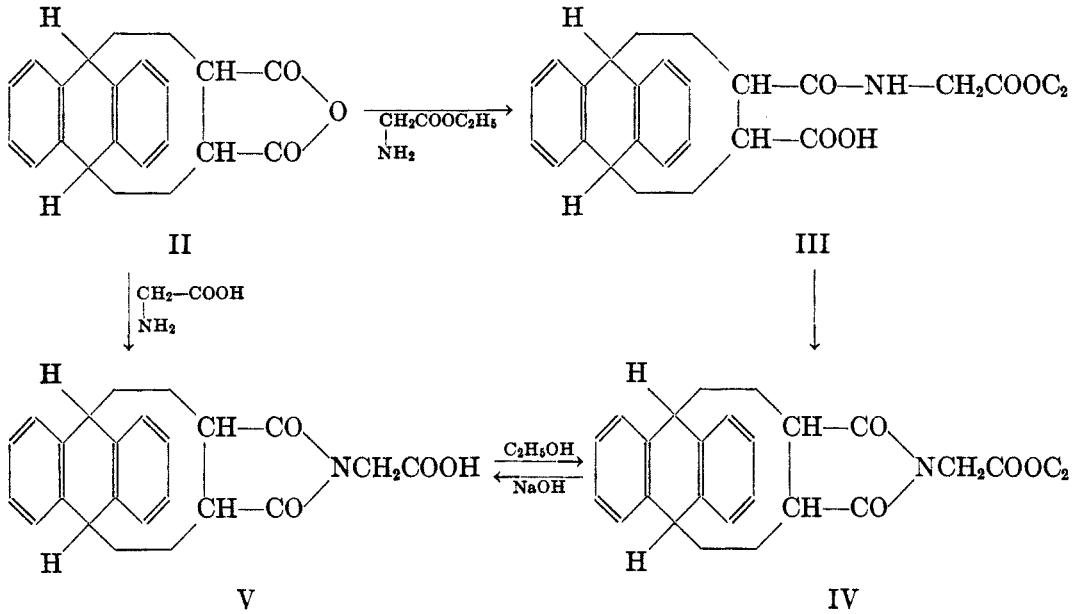
For purposes of orientation we studied the reaction between the endo succinic anhydrides and glycine, as the free amino acid and in the form of its ethyl ester, and the results are presented in this paper. In these compounds is illustrated the type of linkage which would be present in the protein derivatives, although probably not in the same relative position with respect to the carboxyl group. Anthracene- and 1,2-benzanthracene-endo-succinic anhydride were employed first in order to determine the most favorable conditions for reaction.

Anthracene-endo-succinic anhydride (II) reacts with glycine ethyl ester in benzene solution and forms the acid succinylglycine derivative (III). This compound is not very stable but exhibits a strong tendency to pass into the imido ester (IV). Thus, in organic solvents it slowly loses water; when heated it cyclizes readily; and hot dilute solutions of its sodium salt slowly precipitate the ester (IV). The latter ester may also

† The numbering system for the cholanthrene molecule is that employed in the index of *Chemical Abstracts*.

[5] (*a*) BACHMANN AND KLOETZEL, *J. Am. Chem. Soc.*, **60**, 481 (1938). (*b*) BACHMANN AND CHEMERDA, *ibid.*, **60**, 1023 (1938). (*c*) BACHMANN, J. ORG. CHEM., **3**, 434 (1938).

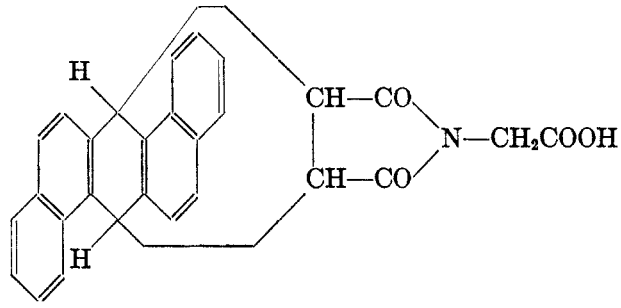
be prepared by interaction of II and glycine ethyl ester hydrochloride in pyridine.



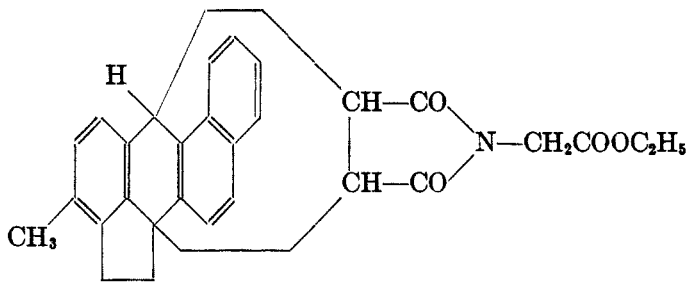
Moderate hydrolysis of IV gives the corresponding acid (V); more vigorous hydrolysis results in cleavage of the acid amide linkage as well and the formation of the dicarboxylic acid corresponding to the anhydride (II). The substituted glycine (V) has also been obtained from the reaction of II with glycine in aqueous alkaline solution. This acid (V) may be esterified to the ethyl ester, which is identical with the ester obtained by the methods described above.

The reactions of 1,2-benzanthracene-endo-succinic anhydride and 1,2,5,6-dibenzanthracene-endo-succinic anhydride with glycine ethyl ester were quite analogous to that of the anthracene derivative. No attempt was made to isolate the intermediates analogous to III; instead, the crude products were warmed to increase the yields of the stable esters. The corresponding acids were best prepared by alkaline hydrolysis of the esters in dioxane solution. The product (VI) from 1,2,5,6-dibenzanthracene-endo-succinic anhydride is of particular interest in view of the carcinogenic activity of the disodium salt of 1,2,5,6-dibenzanthracene-endo-succinic acid.[6]

[6] Barry, Cook, Haslewood, Hewett, Hieger, and Kennaway, *Proc. Roy. Soc.* (*London*), **B117**, 331 (1935).



VI



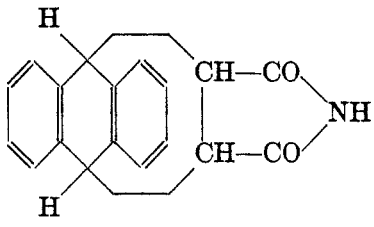
VII

3-Methylcholanthrene-endo-succinic anhydride (I) appeared to react more slowly with glycine ethyl ester than did the anhydrides mentioned above, unchanged anhydride usually being found along with its reaction products when these were formed under the mild conditions employed for preparing the anthracene derivatives. At higher reaction temperatures, dissociation of the anhydride into methylcholanthrene and maleic anhydride caused low yields of the ester (VII) and impure product. The use of excess glycine ester led to side reactions: the formation of a salt whose water solution slowly deposited the ester (VII); the glycine ester itself polymerizes quite rapidly[7] and the polymers also react with the anhydride to form very high-melting by-products.

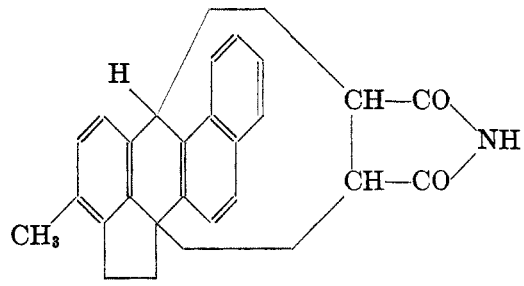
The imides of anthracene-endo-succinic acid and 3-methylcholanthrene-endo-succinic acid were also prepared. Anthracene-endo-succinimide (VIII) formed spontaneously in a warm aqueous solution of the ammonium salt of anthracene-endo-succinic acid. The 3-methylcholanthrene-endo-succinimide (IX) was prepared by reaction of the anhydride in dioxan solution with aqueous ammonia. The ease of imide formation

[7] CURTIUS AND GOEBEL, *J. prakt. Chem.*, [2], **37**, 159 (1888).

is characteristic of the endo succinic acid derivatives and is analogous to the ease of formation of their anhydrides.[5a]



VIII



IX

The derivatives of 3-methylcholanthrene and 1,2,5,6-dibenzanthracene are being tested for their carcinogenic activity in mice, the acids in the form of their water-soluble sodium salts, and the esters in organic solvents by external application and by subcutaneous injection.

Similar derivatives of other simple amino acids, as well as protein compounds formed from the hydrocarbon-endo-succinic anhydrides and crystalline egg albumin are being studied.

EXPERIMENTAL

Reaction of anthracene-9,10-endo-α,β-succinic anhydride with glycine ethyl ester.— To a suspension of 12 g. of powdered anthracene-endo-succinic anhydride in 90 cc. of warm benzene was added 8 g. of glycine ethyl ester. Warming and swirling the solution for ten minutes caused all of the anhydride to dissolve. The clear warm solution was allowed to stand fifteen hours without further heating. Washing with water caused the benzene solution to solidify to a jelly-like mass. It was diluted with much ethyl acetate and extracted with 5% sodium carbonate solution—thus separating the acidic product described below. The benzene-ethyl acetate solution yielded 6.5 g. of product melting at 183–185°. Three recrystallizations from ethyl acetate gave small white prisms of *anthracene-9,10-endo-α,β-succinoglycine ethyl ester* (IV) melting at 187–188°.

Anal. Calc'd for $C_{22}H_{19}NO_4$: N, 3.90. Found: 4.04.

Acidification of the sodium carbonate extract precipitated 4.8 g. of a white powdery acid which melted at 118–121° to a cloudy liquid. After slight effervescence the melt resolidified, and melted again to a clear liquid at 174–176°. Two recrystallizations from ethyl acetate (without much heating) gave the material as small colorless prisms which melted at 154–156°, resolidified partly by 162°, and remelted at 175–177°.

Anal. Calc'd for $C_{22}H_{21}NO_6$: N, 3.69. Found: N, 3.51.

Neutral. equivalent, Calc'd: 379. Found: 369.

A sample (0.1 g.) of the acid was heated in a bath until it melted and resolidified. The resulting solid was dissolved in ethyl acetate and crystallized, giving fine prisms melting at 186–187° which gave no depression in melting point when mixed with the imido ester (IV). The acid therefore appears to be *anthracene-9,10-endo-α,β-(acid succinyl)-glycine ethyl ester* (III).

Treatment of the acid in ether with diazomethane did not give a stable methyl ester; instead, this treatment converted the acid into the imido ester (IV), melting at 187–188°.

When a benzene solution of 4.14 g. of anthracene-endo-succinic anhydride and 2.3 g. of glycine ethyl ester was refluxed for thirty minutes, then concentrated, 4.0 g. (75 per cent. of the theoretical yield) of the imido ester was obtained; but none of the acid (III) could be found.

The same ester (IV) was obtained in 74 per cent. yield when a mixture of 4.14 g. of anthracene-endo-succinic anhydride, 2.1 g. of glycine ethyl ester hydrochloride, and 20 cc. of dry pyridine were heated on a steam bath for two hours.

Anthracene-9,10-endo-α,β-succinoglycine (V).—A suspension of 2 g. of the powdered ethyl ester in 35 cc. of 2% sodium hydroxide and 5 cc. of ethanol was refluxed for fifteen minutes. Acidification of the resulting clear solution precipitated 1.7 g. of an acid melting at 220–250°. Recrystallizations from acetic acid and from ethyl acetate gave 1 g. of small colorless prisms melting at 270–271°.

Anal. Calc'd for $C_{20}H_{15}NO_4$: N, 4.20. Found: N, 4.18.

The acid is more readily obtained by the reaction of anthracene-endo-succinic anhydride with glycine. A solution of 2.4 g. of the anhydride in 14 cc. of hot pyridine was added to a solution of 1.1 g. of glycine and 1.1 g. of sodium carbonate in 5 cc. of water. The mixture was held at 90° for one hour, diluted and acidified. Crystallization of the precipitate from acetone gave 2.2 g. of anthracene-endo-succinoglycine melting at 269–270°. Quite similar results were obtained when the reaction was repeated using 30 cc. of dioxan in place of the 14 cc. of pyridine.

Shaking warm solutions of anthracene-endo-succinic anhydride in benzene or ethyl acetate with alkaline solutions of glycine gave no satisfactory yield of the substituted glycine; most of the anhydride was recovered unchanged.

A suspension of 0.5 g. of anthracene-endo-succinoglycine in 10 cc. of absolute ethanol containing 12 drops of sulfuric acid was warmed over a steam bath for two hours. Cooling the resulting clear solution deposited 0.3 g. of the ethyl ester (IV) melting at 185–186°.

Anthracene-9,10-endo-α,β-succinimide (*VIII*).—One-half gram of anthracene-endo-succinic anhydride was dissolved in 20 cc. of hot 1*N* sodium hydroxide, diluted to 100 cc., and acidified to precipitate the acid. The washed precipitate was dissolved in 20 cc. of ammonium hydroxide (the anhydride itself would not dissolve in ammonium hydroxide) and heated over a steam bath. The clear solution slowly turned cloudy then precipitated 0.45 g. of the granular imide. It was difficultly soluble in hot toluene, from which it separated as colorless prisms melting at 303–304.5° with decomposition.

Anal. Calc'd for $C_{18}H_{13}NO_2$: N, 5.09. Found: N, 4.90.

1,2-Benzanthracene-9,10-endo-α,β-succinoglycine ethyl ester.—A suspension of 0.85 g. of powdered 1,2-benzanthracene-endo-succinic anhydride (m.p. 241–242°, dec.) in 50 cc. of warm benzene was treated with 0.5 g. of glycine ethyl ester dissolved in 5 cc. of benzene. After warming for one hour on a steam bath, the solution was washed with dilute hydrochloric acid and concentrated to 15 cc. The white powder which separated was recrystallized from ethyl acetate, giving 0.55 g. of platelets of the imido ester melting at 226–227°. The ester is soluble in warm benzene, ethyl acetate, or dioxan; very slightly soluble in ethanol. It gives no color with cold concentrated sulfuric acid.

Anal. Calc'd for $C_{26}H_{21}NO_4$: N, 3.40. Found: N, 3.30.

1,2-Benzanthracene-9,10-α,β-succinoglycine.—A solution of 0.3 g. of the ethyl ester

in 16 cc. of dioxan was warmed to 75°, 3 cc. of 2*N* sodium hydroxide was added, the mixture shaken for five minutes, then allowed to stand at 40° for four hours. Dilution with water gave no precipitate. Acidification gave 0.2 g. of crude acid melting at 217–224°, which, after recrystallization from acetic acid then from acetone, gave 0.12 g. of colorless microscopic prisms melting at 242–244°, dec.

Anal. Calc'd for $C_{24}H_{17}NO_4$: N, 3.65. Found: 3.40.

*3-Methylcholanthrene-6,12*b-*endo-α,β-succinoglycine ethyl ester* (*VII*).—To a suspension of 1 g. of powdered 3-methylcholanthrene-endo-succinic anhydride in 80 cc. of hot benzene was added 0.68 g. of glycine ethyl ester in three portions, with ten minutes' warming on the steam bath between additions. After standing for forty-five minutes at 65–70°, the resulting clear solution was washed with dilute hydrochloric acid and water, and concentrated to 10 cc. The crude white product (0.9 g., m.p. 145–190°) partly dissolved in 25 cc. of warm ethyl acetate, leaving 0.2 g. of residue which did not melt below 270°, at which point it began to decompose. Fractional crystallization of the ethyl acetate solution gave 0.2 g. of recovered anhydride and 0.4 g. of methylcholanthrene-endo-succinoglycine ethyl ester melting at 180–181°. The ester is soluble in hot benzene, ethyl acetate, acetone, and dioxan; very slightly soluble in ethanol. It crystallized from ethyl acetate in colorless microscopic plates, melting at 181–182° (bath preheated to 170°) to a clear liquid which decomposes after a few seconds.

Anal. Calc'd for $C_{29}H_{25}NO_4$: N, 3.10. Found: N, 3.18.

Powdered methylcholanthrene-endo-succinic anhydride (0.3 g.) was found to dissolve slowly at 25° in 10 cc. of benzene containing 0.3 g. of glycine ethyl ester, but the solution could not be crystallized. Washing with water extracted some unchanged ester and a salt which, during two days' standing, changed into 0.1 g. of the ester melting at 180–181°. The benzene solution yielded high-melting substances and a very small amount of the ester melting at 180–181°.

A solution of 0.6 g. of methylcholanthrene-endo-succinic anhydride in 5 cc. of dry pyridine at 65° (decomposition occurs above 75°) was treated with portions of powdered glycine ethyl ester hydrochloride until 0.3 g. had been added. The yellow solution was kept at 70° for four hours, cooled, diluted with water, and extracted with benzene. The washed benzene solution yielded 0.42 g. of recovered anhydride and 0.1 g. of methylcholanthrene-endo-succinoglycine ethyl ester melting at 179–180°.

*3-Methylcholanthrene-6,12*b-*endo-α,β-succinoglycine.*—A mixture of 0.21 g. of the ethyl ester in 8 cc. of dioxan and 1.5 cc. of 2*N* sodium hydroxide was held at 50–55° for nine hours, with occasional shaking. Dilution with water gave a faint precipitate of unchanged ester; acidification deposited a crystalline powder, which crystallized from ethyl acetate in short, microscopic prisms (0.15 g.) melting at 233–234.5° with decomposition.

Anal. Calc'd for $C_{27}H_{21}NO_4$: N, 3.31. Found: N, 3.12.

*3-Methylcholanthrene-6,12*b-*endo-α,β-succinimide* (*IX*).—A solution of 0.1 g. of 3-methylcholanthrene-endo-succinic anhydride in 8 cc. of dioxan was treated with 3 cc. of concentrated ammonium hydroxide during three hours, the solution being kept hot by a steam bath. Evaporation to 4 cc. and cooling deposited 0.07 g. of white imide melting at 250–252°, dec. It was difficultly soluble in hot benzene, from which it separated as white microscopic plates melting at 252–253°, dec.

Anal. Calc'd for $C_{25}H_{19}NO_2$: N, 3.85. Found: N, 4.09.

The above three methylcholanthrene derivatives, the ester, the acid, and the imide give similar characteristic clear-red solutions with cold concentrated sulfuric acid. The corresponding anhydride (I) does not dissolve in cold sulfuric acid, but warming gives a red color, then decomposition.

1,2,5,6-Dibenzanthracene-9,10-endo-α,β-succinoglycine ethyl ester.—A suspension of 2.2 g. of powdered 1,2,5,6-dibenzanthracene-endo-succinic anhydride (m.p. 232-233°, prepared by the method of Bachmann and Kloetzel[5a] and by heating the acid described by Cook[8] with acetic anhydride) in 100 cc. of hot benzene was treated with 1.7 g. of glycine ethyl ester in 5 portions at intervals of a few minutes. The resulting clear solution was heated over a steam bath for two hours, then washed with water and concentrated. Recrystallization of the crude product (2.3 g., m.p. 215-217°) from ethyl acetate gave 2.2 g. of platelets of the ester melting at 220-221°.

Anal. Calc'd for $C_{30}H_{23}NO_4$: N, 3.03. Found: N, 2.90.

1,2,5,6-Dibenzanthracene-9,10-endo-α,β-succinoglycine (VI).—A solution of 0.35 g. of the ester in 30 cc. of dioxan was mixed with 2.2 cc. of 1*N* sodium hydroxide. After heating at 90° for eight hours, dilution with 50 cc. of water caused the solution to deposit 0.25 g. of pure unchanged ester. Acidification of the filtrate from this ester gave 0.09 g. of the acid melting at 251-253°. Much more complete hydrolysis was effected by adding 1 cc. portions of 2*N* sodium hydroxide at intervals of several hours to the refluxing dioxan solution of the ester, but the purity of the acid obtained was less. The acid crystallized from acetic acid in small colorless plates melting at 252-253°, dec.

Anal. Calc'd for $C_{28}H_{19}NO_4$: N, 3.23. Found: N, 3.24.

SUMMARY

The maleic anhydride addition products of four polycyclic hydrocarbons have been converted into derivatives of *N*-succinoglycine. Such derivatives of 3-methylcholanthrene and 1,2,5,6-dibenzanthracene were desired for testing their carcinogenic activity.

[8] Cook, *J. Chem. Soc.*, **1931**, 3273.