added a boiling solution of 8 g. of potassium hydroxide in an equal volume of the same solvent. The yellow color at once changed to a deep red and potassium bromide separated. After boiling a minute and three-quarters the mixture was poured into 250 cc. of cold water. The yellow suspension was extracted with ether, and the ether solution washed with dilute hydrochloric acid to remove the red color. After evaporation, the residue was crystallized from 15 cc. of ethanol, giving 0.3 g. (33%) of long, slender, deep yellow needles of 2,3-diphenylnaphthoquinone with a m. p. 132–136°. A single recrystallization from hexane gave a product with a m. p. 138–140°; a mixed melting point with an authentic specimen[4] was the same. The residue was an intractable oil, the amount of which was increased by prolonged contact with the alkaline solution.

The conditions given seem to be the most favorable for a maximum yield of quinone. The latter was not affected by alcoholic alkali, indicating that the oils were produced by side reactions.

In a similar manner, 2,3-diphenyl-6,7-dimethylnaphthoquinone XXXII was formed from 3 g. of the dibromide XXXI in 25 cc. of dioxane, 12 g. of potassium hydroxide in 25 cc. of absolute ethanol, and boiling the resultant purple mixture for one and a half minutes. The yield was 0.6 g. (28%); it formed yellow needles.

Reductive acetylation gave the corresponding hydroquinone diacetates, IX (needles) and XXXIII (plates).

Summary

A number of tetrahydronaphthoquinones containing an angular phenyl group have been prepared.

These quinones have been reduced by various methods to dihydro and tetrahydro derivatives.

Dibromo derivatives are obtainable by the use of bromine. Upon treatment of these with alcoholic potash, hydrogen bromide is eliminated and 2,3-diphenylnaphthoquinones are obtained.

The angular phenyl group is rearranged, probably during the bromination reaction.

The evidence for the various structures and proposed mechanism of reactions are discussed.

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[CONTRIBUTION FROM THE DEPARTMENT OF PATHOLOGY OF THE UNIVERSITY OF CHICAGO]

The Unsaponifiable Residue of Human Liver. I. Preparation and Primary Chromatographic Fractionation[1]

BY D. WARREN STANGER, PAUL E. STEINER AND MIRIAM N. BOLYARD

The unsaponifiable residue of some human liver was shown by Steiner[2] and others to be cancerogenic when injected subcutaneously in mice. This residue was shown to be cancerogenic when prepared from some livers of persons who died of non-neoplastic diseases as well as from some livers of persons who died of cancer.[2a] The livers of the first group of persons are referred to as non-cancer livers while those from the second, as cancer livers, although the latter contained no tumor tissue or at most only traces of it. In a study of individual livers 21% of the cancer livers and 20% of the non-cancer livers were found to yield cancerogenic fractions. Even though the cancerogenic material was diluted by pooling each kind of liver it could still be demonstrated as shown by the experiment in which the unsaponifiable residue from 8 pooled cancer livers produced 12 tumors in 37 mice, and from 7 pooled non-cancer livers, 5 tumors in 35 mice.

In order, if possible, to isolate the cancerogen or cancerogens, a chemical study of this material was undertaken. In the present experiments the two kinds of liver, cancer and non-cancer, were studied in parallel to determine whether quantitative and qualitative differences exist which might yield clues as to the nature and importance of the cancerogenic substances.[3]

The unsaponifiable residue was prepared by saponification of the ground liver with alkali, solvent extraction of the non-saponified portion, and resaponification of this concentrate. The percentage of unsaponifiable material found in 248 cancer livers was 0.681 and in 176 non-cancer livers 0.657. This difference is very much smaller than that between 0.75 and 0.56 found by Steiner[4] for a series of 33 cancer and 11 non-cancer livers. In the small series the wide variation among individual livers may explain the difference in the averages.

Cholesterol was removed from the unsaponifiable residue by crystallization from solvents, by chilling, and finally by the use of digitonin. During the course of this work certain fractions of the residues were resaponified. The percentage of unsaponifiable material in the original livers was lowered to 0.533 for cancer and 0.491 for non-cancer. Of these final unsaponifiable residues 49.7% of the cancer and 53.6% of the non-cancer were found to be cholesterol.

The chromatographic fractionation of the cholesterol-free residue was carried out by adsorption of the material from petroleum ether solution on aluminum oxide. Five fractions were

(1) This investigation was aided by a grant from the Commonwealth Fund.

(2) (a) Steiner, *Science*, **92**, 431 (1940); *Cancer Research*, **2**, 425 (1942); **3**, 385 (1943); (b) Kleinenberg, Neufach and Schabad, *Am. J. Cancer*, **39**, 463 (1940); *Cancer Research*, **1**, 853 (1941); (c) Des Ligneris, *Am. J. Cancer*, **39**, 489 (1940); (d) Hieger, *ibid.*, **39**, 496 (1940); (e) Sannié, Truhaut and Guérin, *Bull. assoc. franc. étude cancer*, **29**, 106 (1941).

(3) Preliminary reports of work somewhat similar to this have appeared by Bürger and Plötner, *Deut. z. Verdauungs u. Stoffwechselkrankh.*, **3**, 180 and 183 (1940). Since only the very brief German abstracts are available in this country, no comparison of results is possible.

(4) Steiner, *Am. J. Path.*, **17**, 667 (1941).

separated: a colorless filtrate (A), a yellow filtrate (B), the elutriates from the lower and upper halves of the column (C-1 and C-2), and the elutriate from all of the aluminum oxide with the aid of acid (D). The weights of the residues obtained on evaporation of these fractions are given in Table I.

TABLE I

COMPOSITION OF CHOLESTEROL-FREE RESIDUE

	Cancer				Non-cancer			
Residue	Weight from 150 g.	%	Weight from 2.00 g.	%	Weight from 150 g.	%	Weight from 2.00 g.	%
A	27.9	18.6	0.375	18.7	30.0	20.0	0.407	20.4
B	48.0	32.0	.660	33.0	54.7	36.5	.740	37.0
C-1	27.6	18.4	.426	21.3	31.4	20.9	.437	21.9
C-2	21.7	14.5	.248	12.4	24.5	16.3	.273	13.6
D	12.8	8.5	.196	9.8	6.0	4.0	.137	6.8
	138.0	92.0	1.905	95.2	146.6	97.7	1.994	99.7

The results of chromatographic fractionations are comparable quantitatively only when care is taken to keep the weights of the sample and adsorbent and the volumes of the solvent and eluants constant. Since in the actual fractionations carried out, some of these factors were varied, it was questionable whether the percentage composition of the cancer and non-cancer residues in terms of residues A, B, C-1, C-2, and D were comparable. On the basis of the results obtained by chromatographing the large quantities, a method was worked out for a 2-g. sample and applied to both the cancer and the non-cancer cholesterol-free residues. Table I shows the results to be in good agreement with those obtained for 150 g. of the corresponding material.

Residue A was a water-white mobile oil. Analyses for carbon and hydrogen showed it to be a hydrocarbon fraction. The iodine number of residue A was 6.5 from cancer and 7.1 from non-cancer source. If all the unsaturated material present was due to traces of residue B, then residue A was 98% saturated hydrocarbon. This saturated hydrocarbon fraction has been observed by Channon[5b] and by Dimter.[6] Stryker[7] showed that its origin may in part be exogenous.

Residue B was an oil colored orange with small amounts of lipochrome pigments. It had an iodine number of 333 from cancer and 337 from non-cancer source. It was composed to the extent of more than 85% of the squalene-like substance first observed by Channon[5] and named hepene by Dimter,[6] This was shown by precipitation of 85% of residue B as hepene octahydrochloride when an ether solution of B was saturated with hydrogen chloride, even though the precipitation is not quantitative. It was further substantiated by the good agreement between the calculated iodine number (329) of hepene ($C_{45}H_{76}$) and that found for residue B.

(5) (a) Channon and Marrian, *Biochem. J.*, **20**, 409 (1926); (b) Channon, Devine and Loach, *ibid.*, **28**, 2012 (1934).

(6) Dimter, *Z. physiol. Chem.*, **271**, 293 (1941).

(7) Stryker, *Arch. Path.*, **31**, 670 (1941).

Residue C-1 was a reddish-orange, viscous oil with an iodine number of 253. Residue C-2 was a reddish-brown, very viscous oil with an iodine number of 149 from cancer and 157 from non-cancer source. Residue D was a dark brown, glassy solid with an iodine number of 87 from cancer and 97 from non-cancer source.

Investigation of these residues will be continued in an attempt to isolate pure substances and identify them.

Experimental Part

Saponification of the Liver.—Livers obtained at autopsy were ground and either frozen or used immediately. The liver was saponified in 10-kg. portions by heating for eight hours on the steam-bath with 750 g. of potassium hydroxide dissolved in 750 ml. of water. Three liters of 95% ethanol was added and the heating continued another eight hours. The cold saponification mixture was extracted once with 3- and six times with 2-liter portions of ethylene dichloride. The combined extracts were taken to dryness *in vacuo* at 55°. The residue, a dark reddish-brown oil or semisolid mass, was resaponified with 50 g. of potassium hydroxide in 500 ml. of 95% ethanol by refluxing for four hours. The cold saponification mixture was diluted with 500 ml. of water and extracted once with 1-liter, three times with 500-, and three times with 250-ml. portions of ethylene dichloride. The combined extracts were dried over anhydrous sodium sulfate, filtered, and taken to dryness *in vacuo* at 55°. The residue was a dark reddish-brown, oily, solid mass.

From 248 cancer livers, weighing 336.8 kg., an unsaponifiable residue of 2294 g. (0.681%) was obtained. From 176 non-cancer livers weighing 230.9 kg., a residue of 1516 g. (0.657%) was obtained.[8]

Removal of the Cholesterol.—The unsaponifiable fraction of the cancer livers was dissolved in boiling 95% ethanol and the cholesterol which crystallized out on cooling was collected on a filter and further purified by several recrystallizations from ethanol and from acetone; 550 g. of cholesterol I, m. p. 147.7–148.2°, was obtained. The residue I left by the evaporation of the combined mother liquors *in vacuo* weighed 1745 g. It was resaponified with 365 g. of potassium hydroxide in 3650 ml. of ethanol by the method described above. The unsaponifiable residue of 1365 g. thus obtained was extracted with boiling acetone. This left an unsaponifiable residue of 208 g. which was resaponified; an unsaponifiable residue of 87 g. was obtained. The acetone extract and this residue were worked up together for cholesterol II, 198 g., m. p. 148–148.5°. The combined mother liquors when evaporated left residue II, 1046 g.

The unsaponifiable fraction of the non-cancer livers furnished 320 g. of cholesterol I, m. p. 148–148.5°. The residue I, 1197 g., was resaponified with 250 g. of potassium hydroxide in 2500 ml. of ethanol. The unsaponifiable residue of 828 g. was acetone soluble. When worked up for cholesterol II it furnished 197 g., m. p. 147.5–148°. The combined mother liquors evaporated *in vacuo* left residue II, 617 g.

By chilling residues II in the ice box for several days, fine crystals separated. This material was collected by filtration and recrystallized from acetone and from petroleum ether as cholesterol III. From 983 g. of cancer residue II, 65 g. of cholesterol III, m. p. 147.5–148°, and 917 g. of residue III were obtained. From 552 g. of non-cancer residue II, 40 g. of cholesterol III, m. p. 147–147.2°, and 512 g. of residue III were obtained.

The cholesterol remaining in residues III was removed with digitonin. A boiling solution of 20 g. of digitonin in

(8) In a more recent preparation the combined ethylene dichloride extracts after the second saponification were washed with 250 ml. of water before drying. From 270 kg. of cancer liver 1608 g. (0.596%), and from 255 kg. of non-cancer liver 1506 g. (0.591%) of unsaponifiable residue was obtained.

200 ml. of 95% ethanol was added to 60 g. of residue III in 100 ml. of ethanol heated to boiling and the mixture kept at the boiling point for five minutes. When cool the suspension was centrifuged and the ethanol solution was decanted and filtered through a Pyrex Büchner funnel with an M fritted disc. The precipitate of cholesterol digitonide was washed twice with ethanol and once with ether by centrifuging and then transferred to the funnel and washed with ether until the filtrate was colorless. The ethanol and ether filtrates were combined and evaporated *in vacuo* leaving residue IV. The cholesterol was obtained from the digitonide by Bergmann's[9] modification of Schoenheimer's method. The cake of cholesterol digitonide was broken up, dried at 60°, dissolved in 125 ml. of pyridine, and kept at 100° for one hour. The pyridine was removed by vacuum distillation and the residue dried *in vacuo* for one hour at 100°. The dry material was powdered and extracted in a Soxhlet for three hours with ether. Evaporation of the ether left the cholesterol IV as a residue. The digitonin from which the cholesterol had been removed was used again. From 917 g. of cancer residue III, 70 g. of cholesterol IV and 848 g. of residue IV were obtained. From 512 g. of non-cancer residue III, 41 g. of cholesterol IV and 463 g. of residue IV were obtained.

Cancer cholesterol I and II, 748 g., and residue II, 1046 g., together weighed 1794 g., which was the weight of the total unsaponifiable residue. If all of the 1046 g. of residue II had been used for further treatment, 144 g. of cholesterol would have been obtained from it. Thus a total of 892 g. of cholesterol would have been obtained from 1794 g. of residue; 49.7% of the cancer unsaponifiable residue was cholesterol. By the same method it was calculated that 608 g. (53.4%) of cholesterol would have been obtained from 1134 g. of non-cancer residue. On the basis of these final weights of unsaponifiable material the percentage in the original liver was 0.533 for cancer and 0.491 for non-cancer liver.

Fractionation of the Cholesterol-free Unsaponifiable Residue by Chromatographic Adsorption.—For fractionation 150 g. of residue IV was used. The chromatographic adsorption was best carried out with 75-g. portions of the residue dissolved in 1000 ml. of petroleum ether. This was adsorbed on a column of aluminum oxide 4.6 × 24 cm. The column was prepared by suspending 350 g. of the aluminum oxide standardized according to Brockmann by E. Merck, Darmstadt, in 500 ml. of petroleum ether in a flask and partially evacuating to remove entrapped air. This suspension was transferred to the tube with additional amounts of petroleum ether. The settling of the suspended particles under gravity was sufficient to secure uniformity of packing. The solution to be chromatographed was allowed to flow through the column under gravity alone and was followed by 1500 ml. of petroleum ether to develop the chromatogram. Fractions were obtained as follows: a colorless filtrate (A), an orange filtrate (B), elutriate from the lower and from the upper half of the column (C-1 and C-2), and elutriate from all of the aluminum oxide with the aid of acid (D). For C-1 and C-2, elution with a mixture of four volumes of methanol and one of ether was used, and for D this same mixture to which 1% of concentrated hydrochloric acid had been added. Further chromatography of fraction B resulted in a colorless filtrate combined with A, an orange filtrate (B), and the elutriate from the entire column combined with C-1. When these fractions were evaporated to dryness *in vacuo*, there were obtained the following residues: a water-white, mobile oil (A), an orange oil (B), a reddish-orange, viscous oil (C-1), a reddish-brown, very viscous oil (C-2), and a dark brown, almost glassy solid (D). Table I shows the amounts of each residue obtained from 150 g.

Chromatographic Analysis of a 2-G. Sample.—A 2-g. sample of residue IV in 30 ml. of petroleum ether was chromatographed on a column 1.4 × 7.7 cm. made from 10 g. of aluminum oxide suspended in 15 ml. of petroleum ether. When all of the solution had been added, the chromatogram was developed with 45 ml. of petroleum ether. Colorless filtrate A, orange filtrate B, and elutriates C-1 from the lower and C-2 from the upper half of the column with the mixture of methanol and ether were obtained as described above. The residue left on evaporation of filtrate B *in vacuo* was dissolved in 30 ml. of petroleum ether and rechromatographed on a column 1.7 × 10.2 cm. made from 20 g. of aluminum oxide in 30 ml. of petroleum ether. The chromatogram was developed with 35 ml. of petroleum ether. The colorless filtrate was combined with A, the orange filtrate and the elutriate from the lower 8.2 cm. first with petroleum ether and then with the mixture of methanol and ether were called fraction B, and the elutriate from the upper 2 cm. was combined with fraction C-1. The entire 30 g. of aluminum oxide was eluted with glacial acetic acid, the elutriate evaporated to dryness, extracted with ether, and the ether extract evaporated leaving residue D. Table I shows the results of the analyses.

Determination of Iodine Numbers.—The Rosenmund–Kuhnhenn method as modified by Yasuda[10] was used for determination of iodine numbers. Care was taken to keep the excess of pyridine sulfate dibromide reagent between 25 and 35%. Iodine numbers were for cancer residue A, 6.5; B, 333; C-1, 253; C-2, 149; D, 87; and for non-cancer residue A, 7.1; B, 337; C-1, 253; C-2, 157; D, 97.

Analysis of Residue A.—Determinations of carbon, hydrogen, and ash were made on a sample of cancer and of non-cancer residue A. *Anal.* Found: (cancer residue A) C, 85.41; H, 13.29; ash, 2.35; (non-cancer residue A) C, 85.51; H, 13.03; ash, 1.85.

Hepene Octahydrochloride.—Samples of 0.5000 g. of residues A and B dissolved in 4 ml. of dry ether were saturated with dry hydrogen chloride at 0 to −5°. In the case of residues A only slight darkening of the solution was produced and only a trace of precipitate weighing less than 0.5 mg. was obtained. In the case of residues B the solutions turned dark brown and heavy precipitates were thrown down which were collected in crucibles with fritted discs and washed with a little cold ether. The almost pure white residues were dried *in vacuo* over sodium hydroxide pellets. From the 0.5000-g. sample of cancer residue B, 0.6168 g. of the insoluble hepene octahydrochloride was obtained, which corresponds to 0.4188 g. of hepene (83.76%). Similarly from non-cancer residue B, 0.6262 g. was obtained which corresponds to 0.4259 g. of hepene (85.04%). Analysis for chlorine was made by using the Stepanow[11] method to obtain the chlorine as chloride ion and the Volhard method for its determination.

Anal. Calcd. for $C_{45}H_{76}\cdot 8HCl$: Cl, 31.21. Found: (cancer) Cl, 30.92; (non-cancer) Cl, 30.85.

Summary

1. The unsaponifiable fraction has been prepared from human cancer and non-cancer livers and separated into cholesterol and cholesterol-free residue.

2. The cholesterol-free residues have been separated into five fractions by chromatographic adsorption and slight differences in the percentage composition between cancer and non-cancer residues shown.

3. The fractions have been characterized by their iodine numbers; one has been shown to be saturated hydrocarbon and another largely hepene.

4. The investigation of these residues will be continued in an attempt to isolate pure substances and identify them.

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(9) Bergmann, *J. Biol. Chem.*, **132**, 471 (1940).

(10) Yasuda, *ibid.*, **94**, 401 (1931).

(11) Stepanow, *Ber.*, **39**, 4056 (1906).