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Chemical Studies on a Pig Heart Muscle Lipid Which Stimulates the Enzymatic Reduction of Cytochrome-c[1]

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A pig heart lipid has been isolated which stimulates the enzymatic reduction of cytochrome-c. The analytical data on the lipid demonstrate that it consists of 3 or 4 components having similar properties and chemical structure. The lipid consists of long chain fatty acid monoesters part of which are keto esters. The fatty acids consist of a mixture of dienoic and trienoic acids and a saturated acid which has the chromatographic properties of stearic acid. The alcohol moiety of the lipid exists in part as a long chain unsaturated alcohol.

A number of studies[3-8] has suggested a role for lipids in the cytochrome system. The work of Nason and co-workers[9-11] has implicated tocopherol-like lipids in electron transport during biological oxidation. Investigations[12,13] have been carried out on the lipid and tocopherol content of purified preparations of cytochrome oxidase and cytochrome b-c_1. The lipid content of these preparations on a dry weight basis was 33 and 18%, respectively. However, both spectral and chemical analysis failed to demonstrate detectable amounts of vitamin E in either of the cytochrome preparations.

The study reported in this communication deals with the isolation of a biologically active lipid fraction from pig heart muscle. This lipid fraction was obtained from the lipid-hemin material reported previously[14] and has even greater activity with regard to stimulating the enzymatic reduction of cytochrome-c by an isoöctane extracted heart muscle preparation.[14] The lipid fraction (Experimental section, fraction 3) reported here is obtained by repeated column chromatography on silicic acid and represents only an extremely small portion of the total heart lipids. The fraction contains 3 to 4 components which have very similar properties and structure. Chemical, spectral, and chromatographic analyses demonstrate that the components are predominantly ketoesters of long chain fatty acids. The alcohols which are linked to the fatty acids have not been identified but one may be a long chain alcohol. Glycerol could not be detected in the alcohol fraction. The fatty acid content of the lipid fraction is 70%.

Chemical tests on the lipid fraction were as follows: a positive test was obtained with $KMnO_4$ (brown color), triphenyltetrazolium[15] (red color), 2,4-dinitrophenylhydrazine[16] (yellow-orange color), sulfuric acid–acetic anhydride 1:20 (test run in chloroform gave an orange color), and 1% phosphomolybdic acid in chloroform–ethanol 1:1 (blue-purple color). Five minutes heating at 100° was required for the last test. A negative test was obtained with ammoniacal silver nitrate. The lipid fraction was also free from P, cholesterol and sugar. Quantitative analysis of the lipid yielded an ester/ketone molar ratio of 2. The ester and ketone determinations were carried out as described previously.[17]

Spectral analyses also were made: the infrared spectrum of the lipid was in agreement with that of a long chain keto ester. Carbonyl bands found were 5.72 and 5.83 μ, the former being due to an ester carbonyl and the latter to a non-conjugated ketone carbonyl. The ultraviolet spectrum of the lipid showed a strong peak at 229 mμ. This absorption band was greatly diminished by reduction with hydrogen. The lipid fraction also yielded a sulfuric acid chromogen (orange color) which had an absorption peak at 306 mμ.

Chemical and chromatographic analysis of the fatty acids obtained by the alkaline hydrolysis of the lipid fraction showed that 4 acids were present. One fatty acid had the properties of stearic acid and another was similar to linolenic acid. The other two acids were unsaturated and were either dienoic or trienoic acids having a chain length of at least 20 carbon atoms. Upon reduction of the fatty acids with hydrogen only two saturated acids were observed chromatographically. One of these had the same R_f value as stearic acid and the other had a lower mobility which indicated that it is a C-20 or higher chain acid. The ultraviolet spectrum of the KOH–glycerol isomerized unsaturated fatty acids displayed a major peak at 232 mμ and a minor peak at 268 mμ thereby confirming the presence of dienoic and trienoic acids. The absence of an absorption band in the region 290–320 mμ indicated that little if any tetraenoic acid was present.

The combined chemical, spectral and chromato-

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graphic analyses demonstrate that the lipid fraction is a mixture of two or three unsaturated and one saturated long chain fatty acid monoesters and that one or more of these lipids is a ketoester.

The ability of the lipid fraction to stimulate the enzymatic reduction of cytochrome-c also was tested.[18] It was found that at a level of 100 μg. this lipid was able to restore fully the activity of an isoöctane extracted pig heart preparation.[14] Furthermore, the hydrogenated lipid was almost as active as the unreduced material. Hence, the activity does not depend on unsaturation but rather resides in the long chain ketoester function. The fatty acids from the lipid fraction were about half as effective as the intact lipid. A number of lipids and associated compounds were tested[14,18] in order to elucidate the structural requirements for activity. These listed compounds were found to be the most active: vitamin E, vitamin K_1, phytol, unsaturated triglycerides, certain hydrocarbons, and the long chain ketoesters reported in this paper. Moderate activity was exhibited by methyl linoleate and methyl linolenate and by cholesterol esters of pig heart. On the other hand the following compounds were only slightly active or inactive: menadione, lysolecithin, linoleic acid, linolenic acid, and certain commercial antioxidants. Nason and Lehman[10] also have reported that certain triglycerides, lipid esters, and substances such as butter and milk are active. These data demonstrate that a long chain function is essential for activity but that the nature of the hydrocarbon side chain, such as the degree of branching and unsaturation, varies with different lipids.

Experimental

Preparation of the Starting Material.—Twenty-eight lb. of fresh pig heart ventricle muscle were freed of excess fat and the muscle ground in a meat grinder. A portion (5.8 kg.) of the ground muscle was extracted once with 6 liters and twice with 4 liters of acetone. The heart residue was then extracted in a Soxhlet apparatus for 18 hours with petroleum ether (b.p. 35–60°). The muscle residue (1.6 kg.) was treated with 4 liters of acetone–HCl (1.8 ml. of concentrated HCl per 98.2 ml. of acetone), allowed to stand for 1 hour at room temperature and filtered. The muscle was extracted three more times with 3-liter portions of acetone-HCl. The combined extracts were treated with an equal volume of petroleum ether and then with an equal volume of water. The hemins and lipid which occurred in the petroleum ether phase were washed three times with water and the solvent then evaporated off *in vacuo* under nitrogen to yield 50 g. of oil containing a mixture of hemin and lipid.

Column Fractionation of the Extracted Lipid–Hemin Mixture.—The lipid–hemin mixture was fractionated on silicic acid by the method of Morrison and Stotz.[19] The material was dissolved in 160 ml. of chloroform–hexane 1:1 and passed through a column which was prepared from a mixture of 300 g. of silicic acid (Mallinckrodt, 100 mesh, for chromatography) and 120 ml. of 0.15 *N* HCl. The lipid–hemin mixture separated into nine bands. The fastest moving band (band 1) consisted of only lipid. However, the lipid fraction reported in this paper was obtained from a lipid–hemin band (band 2) which ran just behind the fast moving band 1 mentioned above. Band 2 contained only a small amount of hemin and was well separated from the other 7 slower moving fractions which were rich in hemin. The eluate containing band 2 was evaporated to dryness *in vacuo* under nitrogen and the residue refractionated as described above. Band 2 moved much more slowly on the second column and required the addition of 5% methanol for elution. This treatment gave rise to four fractions. Only the fastest moving fraction (labeled A-1) was used for further study since it contained the highest biological activity. Fraction A-1 had absorption maxima at 229, 247 and 279 mμ. The yield of this lipid was 915 mg.

In order to study the lipid composition of this material it was fractionated on silicic acid by methods[20,12] shown to be effective for lipid chromatography. Eight hundred mg. of fraction A-1 was dissolved in 25 ml. of petroleum ether and fractionated as follows. Fifty grams of silicic acid was suspended in petroleum ether and the slurry poured into a column measuring 2.0 cm. internal diameter. After the adsorbent had settled and was well packed the lipid was introduced on the column and eluted successively by the solvents given in Table I. Biological activity was found only in fractions 3 and 7. Calibration curves of mono-, di- and triglycerides and of fatty acid methyl esters showed that fraction 3 corresponded to a monoester. If it is assumed that fraction 3 contains only monoesters, then the molecular weight of this lipid as deduced from the ester analysis is 532. Since this fraction has a fatty acid content of 70%, the average molecular weight of the alcohol moiety is about 160. Fraction 3 constitutes only 7.2% of the lipid fraction A-1. However, in terms of the total heart lipids, fraction 3 is a trace constituent. Indeed, this fraction constitutes only 0.0011% of the starting lipid–hemin material.

Reduction of Fraction 3 with Hydrogen.—A portion (15.4 mg.) of fraction 3 was dissolved in 10 ml. of absolute methanol, 50 mg. of platinum oxide added, and the material reduced in a Parr apparatus under 60 lb. pressure of hydrogen for 3 hours at room temperature. After reduction, the methanol solution was filtered while hot to remove the catalyst and the filtrate concentrated to dryness *in vacuo* under nitrogen to yield 15.9 mg. of white solid. Before reduction fraction 3 occurred as a pale yellow oil. The reduced lipid gave a negative test with $KMnO_4$ but still gave a positive test with 2,4-dinitrophenylhydrazine.

TABLE I

COLUMN FRACTIONATION OF LIPID FRACTION A-1

Fraction no.	Eluting solvent Composition	Ml.	Lipid eluted [a] mg.	Ester analysis [b] μM	P analysis [c] μM
1	Petroleum ether	300	11	1.12	0.0
2	1% Et_2O in pet. ether	300	0.0	0.0	0.0
3	3% Et_2O in pet. ether	300	58	110	0.0
4	5% Et_2O in pet. ether	300	114	0.0	0.0
5	10% Et_2O in pet. ether	300	119	0.0	0.0
6	50% Et_2O in pet. ether	300	218	232	0.0
7	25% Methanol in Et_2O	300	183	115	63
8	Absolute methanol	300	21	8	8.5

[a] A total of 724 mg. of lipid was eluted. Thus the recovery of lipid put on the column was 90%. [b] Based on pure samples of lecithin, lysolecithin, monoglyceride and triglyceride. [c] The ester/P molar ratios of fractions 7 and 8 were 1.82 and 0.94, respectively, corresponding to a diester and monoester phosphatide.

Hydrolysis of Reduced Fraction 3.—Twelve mg. of fraction 3 was dissolved in 10 ml. of 1 *N* NaOH in methanol and refluxed for 2 hours. The methanol was removed and the residue acidified with 10 ml. of 3 *N* aqueous HCl and extracted with petroleum ether to yield 9.6 mg. of material. This material was shown to contain 12% (1.2 mg.) of neutral lipid (high molecular weight alcohol). Hence the fatty acid content of reduced fraction 3 was 70.5%.

Hydrolysis of Fraction 3.—Twenty-one mg. of fraction 3 was hydrolyzed in the same manner as described above. The yield of petroleum ether soluble material was 16.5 mg. of which 2.0 mg. was neutral lipid (high molecular weight alcohol). Hence the fatty acid content of fraction 3 was 69%.

Spectral Analysis of Fraction 3.—The infrared spectrum[21]

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of fraction 3 gave the following bands (in microns)[22]: 3.43vs, 3.51vs, 2.90 vw, 5.72vs, 5.83s, 6.81s, 6.94 ms, 7.25m, 7.70m, 7.82m, 8.05m, 8.17m, 8.33m, 8.42m, 8.52ms, 8.92m, 9.07w, 9.65w, 10.62ms, 11.27w, 13.14w, 13.70m, 13.87ms and 14.52w. The spectrum is in agreement with a non-conjugated long chain ketoester.

The ultraviolet absorption spectrum of fraction 3 in absolute methanol (0.8 mg. of lipid per ml. of methanol) showed a single peak at 229 mμ which was nearly abolished after reduction with hydrogen. The sulfuric acid chromogen of fraction 3 was prepared as described by Zaffaroni[23] at a concentration of 90 μg. per 3 ml. of sulfuric acid. A single peak was found at 306 mμ. The ultraviolet spectra were determined with a Beckman DU spectrophotometer.

Alkali Isomerization of the Fatty Acids of Fraction 3.—The fatty acids obtained by the alkaline hydrolysis of fraction 3 were isomerized with glycerol–KOH according to the procedure of Collins and Sedgwick.[24] The ultraviolet spectrum which was run at a concentration of 73 μg. per ml. of solution, showed a major peak at 232 mμ and a small peak at 268 mμ but no absorption band between 290 to 320 mμ. Pure samples of linoleic and linolenic acid were run simultaneously. Linoleic acid gave the characteristic diene peak at 232 mμ whereas linolenic acid showed the typical triene peak at 268 mμ and also a small peak at 232 mμ. The spectral results indicate that the fatty acids from fraction 3 are predominantly dienoic acids but that smaller amounts of trienoic acids are also present. However, little, if any, tetraenoic acid occurs in this fraction. The dienoic and trienoic acids are not exclusively linoleic and linolenic acids as shown by paper chromatographic studies but rather are either unsaturated acids having 20 or more carbon atoms or are unsaturated keto acids.

Paper Chromatographic Analysis.—Paper chromatography of fraction 3 and the petroleum ether extractable material obtained after the alkaline hydrolysis of fraction 3 was carried out in three solvent systems. The first system (solvent I) was methanol–water 9:1 and utilized Whatman no. 1 filter paper impregnated with 10% mineral oil as described by Ashley and Westphal.[25] The second system (solvent II) was acetic acid–water 9:1[26] and also utilized filter paper impregnated with 10% mineral oil. The third system which was developed in this Laboratory consisted of isoöctane–acetone–acetic acid 95:4:1 and required silicic acid impregnated paper which was prepared as described previously.[27]

Fraction 3 gave one major spot (R_f 0.61) and a trace spot (R_f 0.42) in solvent III. The major spot moved in a manner similar to methyl linoleate (R_f 0.58) whereas the trace spot moved the same as stearic acid (R_f 0.42). In this system monopalmitin and tripalmitin had R_f values of 0.00 and 0.72, respectively, whereas oleyl–stearyl diglyceride had an R_f value of 0.59.

The petroleum ether extractable material from the hydrolysis of fraction 3 gave 4 spots in solvent I (R_f values of 0.40, 0.54, 0.67 and 0.84). All spots except the fastest moving one gave a positive test with brom thymol blue. Hence the three slower moving components are fatty acids and the fastest moving component is believed to be a long chain alcohol. Furthermore, all spots except the slowest moving one gave a positive test with $KMnO_4$. Moreover, after reduction with hydrogen, the spots having R_f values of 0.54 and 0.67 were transformed into a saturated fatty acid which had an R_f value of 0.33. The R_f values of stearic, linoleic and linolenic acids in this system were 0.39, 0.59 and 0.67, respectively.

In solvent II, the petroleum ether extractable material gave 5 spots having R_f values of 0.10, 0.19, 0.30, 0.48 and 0.79. All components except the fastest moving one gave a positive test with brom thymol blue and all components except the two slowest moving ones gave a positive test with $KMnO_4$. After reduction with hydrogen the components having R_f values of 0.30 and 0.48 were transformed into a saturated fatty acid having an R_f value of 0.10. The R_f values of stearic, linoleic and linolenic acids in this system were 0.17, 0.39 and 0.48, respectively.

The chromatographic analysis indicates that two fatty acids of fraction 3 are stearic and linolenic acid. Another component moves similarly but not identically with linoleic acid. The finding that the unsaturated acids are converted, after reduction, to a saturated acid having an R_f value less than that of stearic acid demonstrates that the major part of the unsaturated acids contain 20 or more carbon atoms and hence cannot be linoleic or linolenic acids. The fastest moving component which fails to give a positive test with brom thymol blue is believed to be an unsaturated high molecular weight alcohol.

(22) The abbreviations have the following designation: s, strong; vs, very strong; m, moderate; w, weak; ms, moderately strong, vw, very weak.

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Synthesis of Potential Anticancer Agents. XI. $N^{2,6}$-Alkyl Derivatives of 2,6-Diaminopurine[2]

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Several $N^{2,6}$-alkyl derivatives of 2,6-diaminopurine have been prepared by the stepwise reaction of aliphatic amines with 2,6-dichloropurine. The 6-alkylamino-2-chloropurines failed to react with aqueous ammonia, but the desired 2-amino-6-alkylaminopurines were successfully prepared from 2-amino-6-methylthiopurine. During the course of this work an improved procedure for the preparation of 2,6-dichloropurine was developed.

As a part of our general program to exhaustively investigate purines in search of more effective anticancer agents, we have prepared a number of alkyl derivatives of 2,6-diaminopurine. 2,6-Diaminopurine itself has been shown to possess some anticancer activity[3] and is known to be incorporated into nucleic acids as guanylic acid.[4] Although a number of 6-alkylaminopurines have been prepared, the synthesis of only a few $N^{2,6}$-alkyl-2,6-

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