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Animal Fats

2. THE COMPONENT ACIDS OF PYTHON FAT

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(Received 18 December 1952)

The need for more information on the composition of animal fats has been emphasized in the introduction to this series (Gunstone & Paton, 1953), and we now report the composition of python fat.

Previous reference to python fat is limited to an account by Tsujimoto & Kobayashi (1920) of a sample of python oil of acid value 0.6, saponification value 194.1, and iodine value 80.3, which yielded some ether-insoluble polybromides (2.1%) indicating the presence of highly unsaturated acids, probably of the C_{20} and C_{22} series. The characteristics of some other snake oils have been given by Pollard & McLaughlin (1950), but no analyses are reported. A few detailed analyses of the depot fats of Amphibia and Reptilia have been published, and from these few results some tentative generalizations have been drawn. More information is clearly needed.

EXPERIMENTAL

Source and preparation of the fat

The sample of python fat, kindly supplied by Mr G. T. Iles of Belle Vue, Manchester, had been obtained from a female reticulated python (*Python reticulatus*), 19 ft. long and 21 in. around its greatest girth. It had been fed on goats, ducks, hens and rabbits. The fat was probably attached to the intestines. The crude material (640 g.) was autoclaved to render it sterile, crushed and extracted with acetone. Removal of the solvent left the fat (580 g.) as a soft, white,

solid with the following characteristics; saponification equivalent 288.5, iodine value 73.0, free fatty acid 0.3% (as oleic). The mixed acids resulting on hydrolysis had saponification equivalent 275.9 and iodine value 76.1.

Method of analysis

The mixed acids were divided into four less complex fractions by crystallization under various conditions, the insoluble acids being recrystallized in each case. Crystallization was effected from acetone at -60° (twice), and from methanol at -40° and at -20° . Five fractions, *A*, *B*, *C'*, *C''* and *D* in order of increasing solubility were thus obtained, *C'* and *C''* being combined to form fraction *C*. The amounts and iodine values of each fraction are given in Table 1.

Concentrations of 10 ml. of solvent/g. of acids are used throughout.

Table 1. *Low-temperature crystallization of python fat*

Fraction	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>
Weight (g.)	54.06	66.61	26.14	35.26
Percentage of total	29.7	36.6	14.3	19.4
Iodine value	2.5	84.5	94.4	156.8

Each fraction was then methylated and fractionally distilled under reduced pressure, the iodine value and saponification equivalent of each fraction determined and certain fractions examined spectrographically, after

Table 2. *Fractionation of methyl esters of python fat*

Fraction	Wt. (g.)	Iodine value	Saponification equiv.	Fraction	Wt. (g.)	Iodine value	Saponification equiv.
A1	2.90	0.2	267.4	B1	2.79	26.4	268.7
A2	2.87	0.2	270.1	B2	2.85	70.4	290.2
A3	3.13	0.2	270.8	B3	3.20	83.6	294.6
A4	3.04	0.2	271.1	B4	3.24	84.8	295.2
A5	3.86	0.1	270.7	B5	2.95	85.3	295.0
A6	3.89	0.2	270.7	B6	3.04	85.3	294.9
A7	3.56	0.2	271.0	B7	3.36	85.3	294.8
A8	3.47	0.6	272.6	B8	3.42	85.6	295.4
A9	3.60	5.0	285.6	B9	3.13	85.4	295.6
A10	3.04	7.3	298.3	B10	3.16	85.2	295.1
A11	3.70	5.9	299.2	B11	3.12	85.2	295.4
A12	3.34	4.5	300.2	B12	3.34	85.3	295.7
A13	4.96	3.4	299.5	B13	3.54	85.1	295.6
A14*	2.96	9.7	324	B14	4.99	84.8	295.7
				B15	2.55	82.9	298.0
				B16†	1.25	67.5	300.5
C1	1.635	21.8	246.7	D1	2.65	68.0	255.0
C2	1.548	59.1	271.7	D2	2.73	95.2	271.5
C3	1.766	79.8	285.8	D3	3.11	128.9	285.8
C4	1.931	93.6	292.8	D4	3.70	151.9	294.6
C5	1.966	98.8	294.3	D5	2.80	154.8	294.6
C6	1.822	100.6	294.7	D6	2.97	154.7	294.4
C7	1.958	100.5	295.1	D7	3.23	153.9	295.2
C8	2.029	100.8	295.1	D8	3.35	152.7	294.7
C9	1.894	100.4	294.0	D9	3.18	152.8	295.6
C10	1.861	100.1	294.5	D10	3.17	177.8	306.4
C11	1.915	99.4	294.7	D11†	3.63	196.5	341
C12	2.276	98.6	301.5				
C13†	2.040	81.3	336				

* A14 acids, excluding unsaponifiable, iodine value 9.2, saponification equiv. 298.7.

† Weight of unsaponifiable B16, 0.03 g.; C13, 0.09 g.; D11, 0.39 g.

Table 3. *Ultraviolet-absorption measurements ($E_{1\text{cm}}^{1\%}$)*

Fraction	λ (m μ .) ... Iodine value	Unisomerized				Isomerized			
		234	268	300	315	234*	268†	300†	315†
B8	—	—	—	—	—	Nil	Nil	Nil	Nil
C acids	94.4	—	—	—	—	120.0	4.0	Nil	Nil
C2	62.3	—	—	—	—	14.7	4.0	Nil	Nil
C8	105.8	9.9	Nil	Nil	Nil	152.1	Nil	Nil	Nil
C13	—	41.0	16.1	6.9	5.1	80.2	31.4	10.5	9.0
D acids	156.8	—	—	—	—	468.6	77.6	15.3	12.3
D2	100.8	6.5	Nil	—	—	82.0	2.9	—	—
D7	161.3	9.0	Nil	—	—	627.1	31.6	Nil	Nil
D11	216.3	89.7	52.7	18.5	13.8	350.0	319.8	113.3	99.2

* Isomerized at 180° for 60 min.

† Isomerized at 170° for 15 min.

hydrolysis to the acids, both before and after alkali isomerization (Hilditch, Morton & Riley, 1945; Hilditch, Patel & Riley, 1951). Fractionation data are recorded in Table 2 and absorption measurements in Table 3.

Calculation of results

Fractions A and B were calculated by the standard methods described by Hilditch (1947, pp. 484–512); the methods used in calculating fractions C and D are briefly reported. The isomerization data for C8 (assumed to contain only C₁₈ esters) indicates the absence of saturated and triethenoid esters; this fraction is accordingly calculated as a mixture of mono- and di-ethenoid esters on the basis of the

observed iodine value. Fractions C6 to C11 are computed in the same way. C₁₈ esters appearing in fractions before C6 are assumed to have the same composition as in C6 and C₁₈ esters in fractions later than C11 as in this fraction (C11). The small absorption recorded at 234 m μ . for C2 is almost wholly due to the small quantity of C₁₈ esters present in this fraction which is therefore taken as a mixture of palmitic, hexadecenoic and unsaturated C₁₈ esters, as are fractions C3 and C4. The small amount of C₁₈ esters in C5 is taken to be wholly palmitic. The composition of the C₂₀ esters in fraction C12 is determined by the method of successive approximations, and the fraction is then partitioned between the C₁₈ and C₂₀ esters. The final fraction is similarly con-

sidered as a mixture of C_{18} and C_{20} esters along with unsaponifiable material.

Fraction *D* is calculated by methods similar to those described for fraction *C*. Assuming *D7* to be wholly C_{18} esters and free of stearate (shown to be absent from the *C* fraction) its composition in terms of mono-, di- and triethenoid acids is calculated from the spectroscopic data. The results indicate the presence of conjugated and of traces of non-conjugable isomers of the octadecadienoic acid (cf. Barker & Hilditch, 1950). Fractions *D5* to *D9* are all considered to have the same composition as *D7*. *D2* is a mixture of C_{16} and C_{18} esters; the composition of the latter is taken to be the same as in *D5* and that of the former is obtained by the successive approximation procedure. These values for the C_{16} and C_{18} esters are then used to calculate *D3* and *D4* as mixtures of these esters. Fraction *D1* is taken as myristic, tetradecenoic and mixed C_{16} esters with the same composition as in *D2*. Fractions *D10* and *D11* are calculated as already described for *C12* and *C13*.

The separate results are finally gathered together in Table 4.

Identification of the acids present

The presence of the acids calculated to occur in this fat has been confirmed by the isolation of the acids, or of suitable derivatives, from appropriate ester fractions. Palmitic acid (m.p. and mixed m.p. 62–63°) and stearic acid (m.p. and mixed m.p. 69–71°) were isolated from fractions *A4* and *A10*, respectively. Oxidation of fractions *D2* and *B8* with $KMnO_4$ in dilute alkaline solution gave respectively dihydroxypalmitic acid (m.p. 123.5–125.5°, undepressed

when mixed with a similar sample from camel fat) and dihydroxystearic acid (m.p. and mixed m.p. 130–131°). Bromination of the acids from *D7* afforded tetrabromostearic acid (m.p. and mixed m.p. 112–113.5°) and a little hexabromostearic acid (m.p. 175–178°, raised to 181–182° on admixture with an authentic specimen). The bromination was carried out quantitatively following the procedure described by Markley (1947), the results indicating the presence of 77% of linoleic acid. This is higher than the value calculated from the isomerization data (67%) but the use of polybromide numbers is known to give only approximate results. This high value indicates that the octadecadienoic acid present in python fat is almost entirely the linoleic acid present in seed fats.

RESULTS AND DISCUSSION

The final results obtained in this analysis are recorded in Table 5, along with comparable data for some other amphibians and reptiles.

Klenk (1933) has commented on the fact that the acid components of these fats are intermediate between those of fats obtained from marine sources and from higher land animals, and this view has been accepted by Hilditch (1947). The present work shows python fat to be clearly intermediate between the marine and the higher land animal fats. This is shown particularly by the following values (figures in parenthesis are average values for marine and

Table 4. *Component acids of python fat*

	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>	Total	% (wt.)*	% (mol.)*
Myristic	0.19	0.07	0.62	0.40	1.28	1.3	1.6
Palmitic	16.77	1.87	0.77	0.24	19.65	19.7	21.2
Stearic	10.58	0.15	—	—	10.73	10.8	10.5
Arachidic	1.15	—	—	—	1.15	1.2	1.0
Tetradecenoic	—	—	0.15	0.30	0.45	0.5	0.5
Hexadecenoic	—	0.65	0.75	2.53	3.93	3.9	4.3
Hexadecadienoic	—	—	—	0.04	0.04	Trace	Trace
Octadecenoic	0.76	33.67	8.92	3.60	46.95	47.0	46.0
Octadecadienoic	—	—	1.79	8.85	10.64	10.7	10.5
Octadecatenoic	—	—	—	0.75	0.75	0.8	0.7
As Eicosenoic†	0.17	0.15	1.30	2.43	4.05	4.1	3.7
Unsaponifiable	0.07	0.02	0.06	0.23	0.38	—	—

* Excluding unsaponifiable material.

† Includes all unsaturated acids higher than C_{18} (average unsaturation –4.1H).

Table 5. *Component acids of amphibian and reptile fats*

(Values quoted are percentage by weight.)

	Python (a)	Frog (b)	Lizard		Tortoise (e)
			(c)	(d)	
Myristic	1	4	4	4	1
Palmitic	20	11	18	29	14
Stearic	11	3	7	10	4
Arachidic	1	—	—	—	—
Hexadecenoic	4	15	10	12	9
Octadecenoic	59	52	56	40	65
	(–2.4H)	(approx. –2.5H)	(–2.4H)	(–2.7H)	(–2.4H)
Higher unsaturated acids	4	15	5	5	7
	(–4.1H)	(approx. –6H)	(approx. –5H)	(–5.5H)	(approx. –4H)

(a), Present work. (b), Klenk (1933). (c) and (e), Klenk, Ditt & Diebold (1935). (d), Hilditch & Paul (1937).

higher land-animal fats, respectively); palmitic acid, 20% (12–15%, 25–30%); stearic acid, 11% (very small, values rising to 30%); and also in the mean unsaturation of the C_{18} unsaturated acids, $-2.4H$. (Unsaturation more than $-3.0H$, slightly greater than $-2.0H$.) The content of hexadecenoic acid (4%) and of unsaturated acids higher than C_{18} (4%) are closer to the lower values typical of the higher land animals than to the higher values recorded for fish oils. The figures given in Table 5 show a general agreement between python fat and the other results given for frog, lizard and tortoise fats. It is of interest to note that fat from a frog, the only amphibian listed here, is more similar to that of the fishes than are the reptile fats.

Many analyses of animal fats, detailed in other respects, do not distinguish between unsaturated acids with the same carbon content; instead the average unsaturation is recorded. Following the use of low-temperature crystallization and of spectrographic methods as standard procedures in fat analysis, it has been possible to obtain greater insight into the nature of the unsaturated and, more particularly, the polyethenoid acids. Thus octadecadi- and octadecatri-enoic acids have been shown to occur in several animal fats. In python fat the unsaturated C_{18} acids include oleic (47.1%), linoleic (10.7%) and linolenic (0.8%) acids, the nature of the polyethenoid acids being indicated by the isolation of tetrabromostearic acid (in high yield) and of hexabromostearic acid. Calculation of the spectrographic data actually indicates the presence of traces (1–2% of the C_{18} diene acids) of isomeric octadecadienoic acids. These quantities are not considered large enough to be significant. Relatively larger quantities of these isomers have been reported in hippopotamus fat.

The lack of information concerning the detailed structure of polyethenoid acids higher than C_{18} still makes it difficult to distinguish individual acids among this group which are consequently grouped together as 'eicosenoic' acid. This group of acids has a mean unsaturation of $-4.1H$ and the spectrographic data (on fractions *C*13 and *D*11) indicate the presence of tetraethenoid acids; less unsaturated acids must also be present.

In calculating the results from spectrographic data obtained after alkali isomerization, empirical constants are used. These constants result from the study of pure acids obtained from seed fats by physical means (Hilditch *et al.* 1951) and thus apply to the wholly *cis*-acids. Under the standard conditions of isomerization, different constants would be obtained with acids having wholly or partly a *trans*-structure. The use of the published constants for animal fats therefore presupposes that the polyethenoid acids are identical with those from seed fats or at least that they behave identically on

alkali isomerization. It is considered that the octadecadi- and octadecatri-enoic acids in python fat are essentially linoleic and linolenic acids so that the error introduced by using these constants is very small, if any. Further evidence that the octadecadienoic acid in another animal fat (crocodile) is ordinary linoleic acid has been obtained in a different way (Gunstone & Russell, 1952).

Our attention has been drawn to some recent comparative studies of bile salts which appear to lead to certain generalizations similar to those resulting from the study of component fatty acids. Haslewood & Wootton (1950) state that the nature of the bile salts appears to be a characteristic property of the species. Evolutionarily more primitive vertebrates contain C_{27} acids or alcohols, whilst more 'modern' vertebrates have C_{24} acids. In addition certain species, or groups of related species, elaborate unique bile salts which, according to Haslewood & Wootton (1950), is to be expected among more primitive species. The Boidae (boa and python) appear to belong to this last class in that their bile salts contain appreciable quantities of pythocholic acid (Haslewood & Wootton, 1951). More relevant to the discussion in this paper is the observation that the Crocodylidae (alligator and crocodile) which belong to the wider group of Amphibia and Reptilia under discussion contain a C_{27} acid, trihydroxycoprostanic acid, among their bile salts (Haslewood, 1952). The same acid has also been obtained from frog bile (Haslewood, 1952). The structure of this acid (II) clearly shows it to be intermediate in constitution between scymnol (I) (present in the bile of Elasmobranch fishes) and cholic acid (III) (present among the bile acids of higher land animals).

SUMMARY

1. A quantitative study of the component acids of python fat is reported for the first time.

2. The results are similar to those previously reported for other amphibians and reptiles, thus providing additional evidence in support of the generalization that fats of this type are intermediate in composition between fish fats and land-animal fats.

3. Palmitic (19.7%), stearic (10.8%), oleic (47.1%) and octadecadienoic (10.7%) acids are major components, the octadecadienoic acid being almost entirely linoleic acid. Myristic, arachidic, tetradecenoic, hexadecenoic, octadecatrienoic and higher unsaturated acids are present as minor components.

We wish to thank Mr G. T. Iles of Belle Vue, Manchester, for supplying us with the sample of python fat used in this investigation, and for the information concerning the animal; also Prof. J. W. Cook, F.R.S., for drawing our

attention to the work on bile salts; the University of Glasgow for a scholarship held by one of us (R.P.P.) and Imperial Chemical Industries Ltd., for a grant towards the purchase of apparatus.

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Enzymic Hydrolysis of Adenosine Phosphates by Cobra Venom

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Zeller (1948, 1950*a*) found that snake venoms liberate one molecule of inorganic orthophosphate (ortho-P) from adenosinetriphosphate (ATP), and concluded that the venoms contain an enzyme which hydrolyses ATP to adenosinediphosphate (ADP) and ortho-P. In an attempt to use the enzyme of cobra venom for the preparation of ADP from ATP Zeller's observation of the formation of one molecule of ortho-P from ATP was confirmed but no ADP was found. A further study of the reaction revealed that the enzyme, in fact, hydrolyses ATP to adenosine, inorganic pyrophosphate (pyro-P) and ortho-P, and that ADP is not an intermediate in this reaction.

EXPERIMENTAL

Materials and methods

Adenosine phosphates. The barium salt of ATP was prepared from rabbit muscle (see LePage, 1949) and converted into the sodium salt as described by Eggleston & Hems (1952). The concentration of ATP in the stock solution was about 6.5 mg./ml. The preparation contained some ADP, ortho-P and pyro-P; the quantities of these impurities are stated for each experiment. A sample of adenosinemonophosphate (AMP) (free acid) was supplied by Roche Products Ltd., through the courtesy of Dr F. Bergel and Dr A. L. Morrison. It showed no other phosphates or

adenosine derivatives when tested by the chromatographic procedure described below. The concentration of the stock solution was 14 mg./ml. AMP, neutralized to pH 8.4. Barium-ADP was obtained from Schwarz Laboratories Inc., New York. It was doubly decomposed with Na_2SO_4 and was also found to be free from phosphates and other adenosine derivatives when tested chromatographically.

Cobra venom. A sample of dried cobra venom (*Naja naja*) was kindly supplied by the late Dr C. H. Kellaway, F.R.S. A stock solution of 5.4 mg./ml. was used in all experiments.

Antiserum. A sample of dried cobra 'antivenine' was also supplied by Dr Kellaway. This is a dried serum prepared by Lamb (1902, 1904) from the blood of rabbits which had received, about every 10 days for 4 or 5 months, subcutaneous injections of cobra venom. This serum forms a precipitate with cobra venom which is maximal when the ratio of venom to antiserum is 1 to 4.

Incubation and sampling. Stock solutions of cobra venom (0.2 ml.), adenosine phosphate (0.2 ml.) and 0.1 M-barbitone buffer (Michaelis, 1930), pH 8.4 (0.4 ml.), were mixed and incubated at 30°. When antiserum was included it was dissolved in the buffer solution. The pH chosen is near the optimum for the venom ATP-ase (Zeller, 1948, 1950*a*) and 5-nucleotidase (Gulland & Jackson, 1938). A sample (0.1 ml.) was taken immediately after mixing and transferred to 30% (w/v) aqueous trichloroacetic acid solution (0.05 ml.). Other samples were collected at intervals and treated in the same way. They were stored in the 'deep freeze' till they were analysed.