THE IRRITANT CONSTITUENT OF ANTI-LEPROTIC OILS

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A number of minor observations made in these laboratories during the last few years have served to call attention to the instability of chaulmoogric and hydnocarpic acids and of their derivatives. The acids themselves when exposed to diffused daylight gradually deteriorate into sticky varnishes. Aqueous solutions of "sodium hydnocarpate" (sodium salts of the lower-melting fractions of the total acids of *Hydnocarpus wightiana* oil) not only change color and become cloudy on standing, but the specific rotation and the pH both fall steadily. The chaulmoogric ester of hydroxymercuri-M-hydroxybenzaldehyde [1] which is completely soluble in certain oils when freshly made, loses this property on keeping, whereas other fatty acid esters of the same organic mercury complex can be kept for years without apparent change.

On the other hand, experience with the direct injection of *H. wightiana* oil, in place of derivatives such as the ethyl esters and the sodium salts, has brought out the fact that some samples of hydnocarpus oil are more irritant than others, and Muir [2] in particular has pointed out that oil which has been obtained from stale seeds, or has been stored exposed to air, heat and moisture, develops an irritant property additional to that characteristic of fresh oil.

Certain observations made in the course of an investigation of the still unidentified constituents of the total fatty acids of *H. wightiana* and sapucainha oils led the authors to consider that this instability of chaulmoogric and hydnocarpic acids might have some connection with the more irritant character of old or exposed hydnocarpus oil. The primary investigation referred to was diverted temporarily to this part of the problem, and the preliminary chemical and biological results are now presented.

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It seems clear from the somewhat voluminous literature that has been already accumulated on pain causation by the injection of hydnocarpus oil or its derivatives that there are at least two factors involved. There is (a) the irreducible minimum of pain caused by the injection of fresh, neutral oil of good quality or of carefully prepared ethyl esters of pure chaulmoogric acid, or pure hydnocarpic acid, or of mixtures of these pure acids. This factor is to be regarded as inherent.

The second factor (b) is artificial in origin and varies with the product under consideration. In the oil itself it is generally believed to be due to incipient rancidity or an irritant produced in the oil as a result of bad storage. In the ethyl esters it is taken to be a minute amount of irritating volatile aldehyde, or similar product, formed even in the most careful distillation of the mixed ethyl esters and removable by subsequent treatment with steam and final iodization ([3]).

In the case of the sodium salts the most popular explanation was excessive alkalinity until Jackson ([4]) showed conclusively that pain on injection of aqueous solutions of the sodium salts could not be correlated with the pH of the solutions.

It seemed clear to the writers that, in spite of the apparently varied nature of factors (b), pain causation might be due to a single component of the oil, and that it would be worth while to make a search for such a substance among the still unidentified constituents of a typical anti-leprotic oil. It was convenient to choose sapucainha oil for this purpose because we already had a large quantity of this oil under investigation, but the results obtained have been applied to and confirmed with *H. wightiana* oil, and there is no doubt of their general applicability to oils of this class.

PART I. CHEMICAL

The oil used was prepared in the laboratory by continuous extraction of sapucainha seeds with carbon tetrachloride, the last traces of solvent being removed from the oil by passage of a current of steam. The yield was 41.2 per cent. The oil gave the constants shown in Table 1, confirming its well known resemblance to *Hydnocarpus wightiana* oil ([5], [6], [7]).

The total fatty acids of sapucainha oil, prepared in the usual manner, were fractionated as far as possible by cold processes, since it appeared to be clear from the work of previous authors ([8], [9]) that

the residual acids could not be distilled as such, or as esters, without decomposition, and it was particularly desired to avoid the risk of producing irritating substances by such methods. The total acids were divided into two parts, A and B, by crystallization from alcohol.

TABLE 1.—*Constants of sapucainha oil examined, compared with H. wightiana oil.*

Oil	Acid value	Saponification value	Iodine value, per cent	Specific rotation $[a] \frac{15°}{D}$	Specific gravity at 25°C
H. wightiana	6.0	196.0	95.0	+55.4°	0.9656
Sapucainha	21.7	199.7	101.3	+54.0°	0.9563

Fraction A.—This formed 56.5 per cent of the total acids. It had all the properties of a mixture of chaulmoogric and hydnocarpic acids ($[a] \frac{15°}{D} + 64°$ to $+ 65°$). Pure specimens of both these acids were prepared from it (chaulmoogric acid, melting point 68°, $[a] \frac{15°}{D} + 62.05°$; hydnocarpic acid, melting point 56°, $[a] \frac{15°}{D} + 67.7°$). Such a mixture is a familiar product in the examination of oils of this type and does not call for further comment. In the present instance it was used for the preparation of the "ethyl esters of the crystalline acids" (boiling point 198-215°/14 mm.) for the exposure and biological tests referred to below.

Fraction B.—This amounted to 43.5 per cent of the total acids and consisted of the crude residual acids. It was converted into dry sodium salts from which, after removal of unsaponifiable matter, the copper salts were precipitated by extraction, first with acetone and then with ether, into three fractions:

B1. Copper salts soluble in acetone,

B2. Copper salts insoluble in acetone but soluble in ether.

B3. Copper salt insoluble in acetone and ether.

The acids were regenerated from these copper salt fractions and examined; that from B2 consisted of crystalline acids and was added to fraction A and need not be further considered.

Copper salt, fraction B1.—This fraction, which represented about 26 per cent of the total acids, yielded an acid mixture of much more complex composition than the B2 fraction. It contained a considerable amount of chaulmoogric and hydnocarpic acids with some palmitic acid. The remaining constituents formed a brown, oily residue referred to below as the "syrupy acids." This, by repeated extraction, first with cold and then with hot light petroleum, was gradually resolved into three principal components.

1. A golden yellow, oily acid, melting point 10-15°C., which may still contain some dissolved chaulmoogric and hydnocarpic acids. It comprised about 5 per cent of the total acids. In view of the highly unsaturated character of components of the residual acids mentioned by previous workers ([10]), it is noteworthy that the iodine value of this fraction is only 99 per cent. It has $[a]$ $^{15°}_{D}$ $+ 28.6°$, and acid value 191. For biological examination it was converted into a sodium salt and ethyl esters (boiling point 175-215°/0.5 mm.).

2. A mixture of keto-acids, melting point 101-103°C., $[a]$ $^{15°}_{D}$ ± 0, iodine value *nil.* This amounted to about 4.5 per cent of the total acids. It was used in the biological experiments as the sodium salts and the ethyl esters (melting point 34-36°C., boiling point 215-230°/0.5 mm.).

3. A dark brown "tarry acid," identical with that recovered from the copper salt fraction B3 (see below), and added to it.

The syrupy acids are not to be regarded, therefore, as a single and chemically definite component of the sapucainha fatty acids, but as a mixture of the three substances just described, and it will be seen from the results of the biological tests that this product exhibits the irritant properties of the tarry acids modified by the bland nature of the oily acid constituent.

Copper salt, fraction B3.—The acid recovered from this fraction was an intractable black tar, the tarry acid; with that from copper salt B1 it formed about 9 per cent of the sapucainha fatty acids. There are two interesting points about this product: (1) it may prove to be identical with the acid resulting from the exposure of oils, or esters, of chaulmoogric and hydnocarpic acids to air and light, and (2) it is, like the exposure product just referred to, highly irritating on injection in the form of its ethyl esters. These esters cannot be distilled without decomposition even at 0.5 mm. pressure, and this decomposition results in still more irritating products. We have no doubt that these constitute the additional irritants found in some batches of ethyl esters of total fatty acids of hydnocarpus oil. It is also possible that, in the distillation of the ethyl esters of the total fatty acids, some ethyl ester of the tarry acid is carried over with the more readily volatile esters of chaulmoogric and hydnocarpic acids and itself forms an irritant.

The tarry acid when first isolated has an acid number 213 (approximately) and apparent saponification number 285 (approximately). After recovery from the saponification product it has an

acid number of 260. On esterification with ethyl alcohol it yields two products with the following characters:

(A) Insoluble in light petroleum; acid number about 37; saponification number about 252; ethoxyl about 12.3 per cent.

(B) Soluble in light petroleum; acid number about 19; saponification number about 249; ethoxyl about 18.1 per cent.

These data can be explained if it may be assumed that the tarry acid is essentially a mixture of a hydroxy-dibasic acid of molecular weight about 418, with the corresponding lactone acid. The first will have two free carboxyl groups (acid number, calc. 268); the lactone acid will contain one free carboxyl group (acid number, calc. 140) and one lactone group (apparent saponification number 280). Further, that on esterification the free carboxyl group is first esterified yielding the petroleum-insoluble product (A), which is a monoethyl ester (ethoxyl, calc. 10.5 per cent) still containing the lactone group (apparent saponification number, calc. 262), and the petroleum-soluble product (B), in which both carboxyl groups have been esterified giving rise to a diethyl ester of a hydroxy-acid (ethoxyl, calc. 19.0 per cent, saponification number, calc. 236).

For comparison with the tarry acid the only satisfactory ''exposed product'' yet available is that from the ethyl esters of the mixed crystalline acids (Fraction A, p. 151) after exposure to light and air during the summer of 1933. This should correspond approximately with the petroleum-insoluble monoethyl ester (A) referred to above. The two are compared in Table 2.

TABLE 2.—*Constants of ethyl esters of (1) crystalline acids before and after exposure, and (2) tarry acids, petroleum insoluble and soluble fractions.*

Constant	Ethyl esters of crystalline acids		Ethyl esters of tarry acids	
	Before exposure	After exposure	Petroleum insoluble	Petroleum soluble
Acid value	0.7	40.3	37	19
Saponification value	189.0	274.0	252	249
Ethoxyl, per cent.	14.9	8.3	12.3	18.1
Iodine value, per cent.	88.0	15.0	40	43
Specific rotation $[a]_D^{15°}$	+59.0	+0.3	*	+1.5
Specific gravity D.18/18	0.9093	1.032	—	1.0180

* Too dark to read.

Having regard to the intractable character of these products and the difficulty of bringing two such materials, isolated in dif-

ferent ways, to the same degree of purity we think it not unreasonable to assume as a working hypothesis that these two products consist largely of the same constituent, and we hope by further work to establish this conclusion definitely. Its practical importance is obvious since both these products are highly irritant, as is shown below, and if they prove to be identical it should not be difficult to find means of avoiding the formation in these oils of the tarry acid, or, if it is formed, of either eliminating it or converting it into an innocuous product.

Effects of heat upon the crystalline acids.—In contradistinction to the tarry acid the other components of the total fatty acids, viz, the crystalline acids (fraction A, p. 151), the keto-acids (p. 152) and the oily acid (p. 152) all yield ethyl esters which distil without apparent decomposition under reduced pressure, and it is unlikely that they become irritant as a result of such distillation. It was, however, considered desirable to investigate this point so far as the ethyl esters of the mixed crystalline acids are concerned. For this purpose the esters, prepared in the usual way, were distilled at atmospheric pressure instead of reduced pressure. There was no obvious decomposition. The esters so distilled were subjected to a current of steam, which carried over traces of oil that on injection proved to be quite bland. The amount was too small to be examined chemically, but it probably consisted merely of unchanged esters.

TABLE 3.—*Constants of the ethyl esters of the crystalline acids after heating in various ways.*

Treatment of ethyl esters	Acid value	Ester value	Iodine value	Specific rotation $[a] \frac{15°}{D}$
1. Distilled at 198-215°/14 mm........	0.7	188	88	+59°
2. Distilled at 330-350°/760 mm.....	4.0	174	86	+54.5°
3. Lot No. 2, after steaming	3.0	178	87	+54.8°

The constants of the esters before and after these treatments are compared in Table 3. It is clear from these that the slight change in the esters produced by over-heating is of the same character as that resulting from exposure to light (increase in the acid value and fall in specific rotation), and that these changes are not further affected by steaming. It will obviously be desirable to investigate the effect on the ethyl esters, of long continued heating,

but for the present purpose it is sufficient to record the results of biological tests on these esters after distillation at atmosphere pressure and the same product after (a) steaming and (b) iodization.

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PART II.   BIOLOGICAL [1]

METHOD OF TESTING FOR IRRITANT PROPERTIES

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The test for irritant properties was carried out by the injection of the material into the skin of the guinea-pig, in the manner now common in the testing of antitoxic sera.   The drug, either undiluted or diluted with some bland solvent, was injected into the dermis after removal of hair by clipping.   The use of depilatory pastes was avoided as the skin becomes unduly sensitive to the necrotic action of the drug.   The doses injected were 0.05 cc. to 0.1 cc.   The needle used was short and stiff, 1 cm. in length excluding the nozzle, and 0.045 cm. in diameter (Burroughs, Wellcome & Co., No. 214).   A syringe with a tightly fitting plunger was used, one in which no leak-back occurs when the nozzle is blocked by the finger and the piston is compressed.   Series of eight injections on each side of a guinea-pig were made, which permitted comparison of the action of more than one sample in the same animal.

The diluents used were ethyl oleate and commercial "liquid paraffin."   Both of these were absorbed, usually without more than a slight trace of redness and with no necrosis.   However, one sample of ethyl oleate developed, on keeping, some substance—possibly free fatty acid—which caused necrosis of guinea-pig skin.   Another sample produced necrosis in one guinea-pig out of three.   Some of the fractions of sapucainha acids were not miscible with liquid paraffin, and for these fresh ethyl oleate was used.   In each experiment a control injection of the diluent alone was made.

The result of the injection of liquid paraffin is a very slight redness and thickening, which occurs during the first twenty-four hours and then rapidly fades.   The blandest members of the chaulmoogric acid series studied, *viz.*, iodized ethly esters, produced very little more reaction than the diluent itself.   With the more irritating members of the series a greater degree of vascular reaction was followed by thickening, necrosis and the formation of a black scab which sloughed off.   The most irritant members of this series produced complete necrosis of an area up to one centimeter or more

[1] J. W. Trevan and A. M. P. Attwood are responsible for all experiments on animals.

TABLE 4.—*Results of biological tests of substances studied, in terms of necrosis after intradermal injection of guinea pigs.*

Substance tested	Degree of necrosis caused by concentration of substance, per cent.[a]									Minimal necrosing concentration.[b]
	100	50	25	12.5	6.25	3.0	1.5	0.75	0.375	
FREE ACIDS[c]										
1. Total acids	⋯	++++	⋯	⋯	+	+	⋯	0	⋯	1.5
2. Oily acids	⋯	++++	⋯	⋯	++	++	⋯	+	⋯	1.5
3. Syrupy acids[d]	⋯	++++	⋯	0	⋯	⋯	⋯	⋯	+	0.375
ETHYL ESTERS										
4. Of total acids	±	⋯	0	0	⋯	⋯	⋯	⋯	⋯	100
5. Of crystalline acids, A										
(a) Distilled at 198/14 mm.	++	0	⋯	⋯	⋯	⋯	⋯	⋯	⋯	75
(b) Distilled at 350/700 mm.	++	0	⋯	⋯	⋯	⋯	⋯	⋯	⋯	75
(c) Substance 5b, steamed	++	0	⋯	⋯	⋯	⋯	⋯	⋯	⋯	75
(d) Substance 5b, iodized	0	0	⋯	⋯	⋯	⋯	⋯	⋯	⋯	Neg. at 100
(e) Substance 5a, exposed	0	⋯	++ 0+	0 0	+	±	⋯	⋯	⋯	3
6. Of oily acids	0	⋯	0	0	⋯	⋯	⋯	⋯	⋯	Neg. at 100
7. Of keto-acids[d]	⋯	⋯	+	0	⋯	⋯	⋯	⋯	⋯	19
8. Of syrupy acids, undistilled	++	++	0	0	⋯	⋯	⋯	⋯	⋯	38
9. Of syrupy acids, distilled	++	++	++	0 ++	0 0 ±	⋯	⋯	⋯	⋯	10
10. Of tarry acids[d]	⋯	++	++	++	⋯	+	⋯	⋯	⋯	6.25
SODIUM SALTS[e]										
11. Of total acids	⋯	⋯	⋯	⋯	⋯	++++	0	0	0	2.25
12. Of oily acids	⋯	⋯	⋯	⋯	⋯	++++	0	0	0	2.25
13. Of keto-acids	⋯	⋯	⋯	⋯	⋯	++++	± ±	± 0	⋯	2.25
14. Of syrupy acids	⋯	⋯	⋯	⋯	⋯	+	±	0	0	1.5
15. Of tarry acids	⋯	⋯	⋯	⋯	⋯	⋯	⋯	⋯	⋯	1.5

[a] Except where otherwise stated the diluent was liquid paraffin.
[b] The estimated lowest per cent concentration that would cause necrosis in the dose used.
[c] The crystalline acids, keto-acids and tarry acids were too insoluble in the free state for injection.
[d] Ethyl oleate used as solvent.
[e] Used in aqueous solution.

in diameter. If the drug was diluted before injection the area of the scab diminished in size and the scab might take four to six days to form. The highest dilution at which slight necrosis occurs in six days has been used to compare the various substances studied.

The results are summarized in the Table 4, where the plus signs represent the average amount of necrosis in the different guinea-pigs caused by each concentration of the substance tested. Variations in sensitivity of the animals occasionally cause discrepancies. At the limiting dilution one guinea-pig may show a very small necrosis and another none. This is taken as the end point for the figures in the last column. Where the break is complete from necrosis to no necrosis, the end point is taken between the two dilutions. When the animal gave no reactions this is indicated by O. Dilutions not tested are indicated by dashes.

It is clear from the results of these tests that the crystalline acids and the oily acid are bland products when administered as esters. In comparison with the syrupy acids the oily acid is relatively inert as an irritant, even in the free state. Distillation of the ethyl esters of the crystalline acids at atmospheric pressure instead of at 14 mm. does not increase their irritant quality, while the latter can still be reduced materially by iodization. It should be noted, however, that iodized esters of the crystalline acids are less stable than iodized esters of the total acids. On exposure to light the color of the iodized esters gradually diminishes and the product becomes highly irritant; this change is retarded if not entirely inhibited by cold storage in the dark. The ethyl esters of the keto-acids are clearly less bland than those of the crystalline and oily acids, but in the form of the sodium salts they are not more irritant than the sodium salts of these fractions, although the difference between any of the soaps is comparatively small. The ethyl esters of the syrupy and the tarry acids and the exposed ethyl esters are far more irritant than any of the other products, and this property is enhanced in the ethyl esters of the syrupy acids when they are distilled and thereby partly decomposed.

SUMMARY

The constituent fractions of the acids of sapucainha oil have been separated by cold processes as far as possible and tested for irritant properties.

A crystalline acid fraction (chaulmoogric, hydnocarpic and palmitic acids), an oily acid (5 per cent), and a keto-acid (4.5 per cent), gave bland ethyl esters and sodium salts.

The only product to exhibit marked irritant properties was a tarry acid fraction (9 per cent), which appeared to consist essentially of a lactonic acid.

The ethyl esters of the crystalline acids were not rendered irritant by distillation at 350°/760 mm., but on long exposure in thin layers to light and air they change in physical and chemical character and become highly irritant, possibly owing to the production of the lactonic acid above mentioned.

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