mediate in type between those of the higher land animals and those of the reptiles and amphibia so far reported, in which the palmitic acid content is somewhat reduced, being of the order of 10–20%, hexadecenoic acid being present in these fats to the extent of 5–15%.

Table 3 records the component acids of the rodents so far investigated. It is seen that the fats of the tame rabbit are on the whole similar to those of the other (tame) rodents. The palmitic acid content of the rabbit fats, however, is somewhat higher than that of the rat and guinea pig fats. The unsaturated C_{18} -acid content resembles that of the guinea pig rather than that of the rats, the guinea pig, however, elaborating a considerable amount of polyethenoid C_{20-22} acids, the amount being of the order of that present in the fats of Amphibia and reptiles. The saturated acids of the wild rabbit fat are likewise of the same order as those found in the fats of the tame rodents, but there is a marked difference in the proportions of the unsaturated acids present.

It appears, therefore, that the fats of the tame rodents fed on a suitable low-fat diet are generally similar, but judged from the only detailed data available there is reason to believe that the type of fat elaborated by the tame rodents is not typical of that of the wild varieties of the same species.

SUMMARY

1. The composition of the perinephric and interscapular fats of an experimental rabbit has been investigated.

2. The proportions of the component acids of both fats were very similar, the perinephric fat being somewhat the more unsaturated.

3. Both fats were broadly similar to those of other tame rodents, but differ appreciably from that recorded for a wild rabbit.

We wish to thank Prof. T. P. Hilditch, F.R.S., for his sustained interest in this investigation, and Prof. R. A. Morton, F.R.S., for placing the rabbit at our disposal.

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84. THE COLOURING MATTERS OF *PENICILLIUM NALGIOVENSIS* LAXA. PART 1. NALGIOVENSIN AND NALGIOLAXIN. ISOLATION, DERIVATIVES AND PARTIAL STRUCTURES

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Penicillium nalgiovensis Laxa was first described by Laxa (1932), who isolated it during an examination of the microflora of Ellischauer cheese, a form of Camembert cheese peculiar to the Nalžovy region of Southern Bohemia—hence the specific name. The production of this type of cheese was started about 1880 in an attempt to reproduce the flavour and aroma of Camembert cheese. During the ripening process, the surface of the cheese is covered with white, velvet-like mycelium which gradually becomes greenish-grey in colour. The surface eventually becomes flecked with reddish patches from which cultures of *P. nalgiovensis* can be isolated. According to Laxa this mould is one of the principal species associated with the ripening process. The species is

included by Raper & Thom (1949) in the *P. canescens* series of the *Asymmetrica-divaricata*. Growth of the species on cheese and in laboratory culture, particularly on media containing proteins, is associated with the production of considerable amounts of red pigment. The species has not been reported from any other source than Ellischauer cheese.

It was noted in this laboratory that the mycelium of *P. nalgiovensis*, grown on Raulin-Thom solution, gave strong red colours when moist portions of it were immersed in cold concentrated sulphuric acid or in aqueous sodium hydroxide, and pale to light red colours when immersed in aqueous sodium carbonate. On exhaustive extraction of the dried mycelium with light petroleum an orange solid

separated in the boiling solvent. Purification of this solid by crystallization from chloroform led to the isolation of a pure, hitherto undescribed, colouring matter for which the name *nalgiovensin* is proposed. Only a portion of the total colouring matters present in the mycelium is extracted with light petroleum. Subsequent extraction with ether or, preferably, with chloroform, yields further amounts of nalgiovensin and a second hitherto undescribed colouring matter for which the name *nalgolaxin* is proposed. Nalgiovensin constitutes about 1.0% of the dry weight of the mycelium of *P. nalgiovensis*, and nalgolaxin about 0.18%.

Nalgiovensin crystallizes from chloroform in long orange needles, and from glacial acetic acid in glistening orange plates, m.p. 199–200°, without decomposition. It has the molecular formula

contains one methyl group attached to carbon, one methoxyl group, and is dextrorotatory in chloroform solution $[\alpha]_{D}^{20} +48.1^\circ$; $[\alpha]_{D}^{20} +39.7^\circ$ (c, 0.1786). Nalgiovensin does not dissolve in aqueous 0.5N-sodium bicarbonate or in N-sodium carbonate, but dissolves readily in N-sodium hydroxide to give a deep-red solution with a slight bluish cast. It dissolves in cold concentrated sulphuric acid to an intense red solution which appears somewhat orange in shade by transmitted light. Its solution in glacial acetic acid is yellow in colour and does not show fluorescence in daylight.

The following functional derivatives of nalgiovensin were prepared. (a) *Triacetylnalgiovensin*, $C_{24}H_{22}O_9$, i.e. $C_{17}H_{10}O_2(OCH_3)(OCOCH_3)_3$, by heating with acetic anhydride and anhydrous sodium acetate. It forms fine yellow needles, m.p. 147–148°, and is laevorotatory in chloroform solution. On alkaline hydrolysis of this acetate nalgiovensin is regenerated. (b) *Nalgiovensin dimethyl ether*, $C_{20}H_{20}O_6$, i.e. $C_{17}H_{11}O_3(OCH_3)_3$, by heating with dimethyl sulphate and anhydrous potassium carbonate in acetone solution. It forms yellow needles, m.p. 187–188°, which are insoluble in aqueous N-sodium hydroxide, indicating the absence of a nuclear hydroxyl group, but dissolve in cold concentrated sulphuric acid to give an intense red solution with a slight bluish cast. It is dextrorotatory in chloroform solution. (c) *Monoacetylnalgiovensin dimethyl ether*, $C_{22}H_{22}O_7$, i.e.

by heating nalgiovensin dimethyl ether with acetic anhydride and anhydrous sodium acetate. It forms thin yellow-green rods, m.p. 152.5–153°, which are dextrorotatory in chloroform solution. (d) *Nalgiovensin trimethyl ether*, $C_{21}H_{20}O_6$, i.e.

by heating nalgiovensin dimethyl ether with methyl iodide and dry silver oxide in dry benzene solution. It forms fine yellowish-green needles, m.p. 177–178°, which are insoluble in aqueous N-sodium hydroxide, but dissolve in cold concentrated sulphuric acid to yield a solution indistinguishable in colour from that given by the dimethyl ether. It is dextrorotatory in chloroform solution. Methylation of nalgiovensin with dimethyl sulphate and 10% aqueous potassium hydroxide resulted only in the formation of the dimethyl ether.

It appeared probable from the colour reactions of nalgiovensin and of its derivatives, and from its solubility in alkalis that this colouring matter is a polyhydroxyanthraquinone. This opinion was confirmed and the general structure of nalgiovensin was indicated by the fact that when a chloroform solution of it was chromatographed on a magnesium carbonate column a single red band was formed. This band became bluish-red and was considerably diffused when the column was washed with absolute methanol. Almost identical colours are given by physcion, 4:5-dihydroxy-7-methoxy-2-methylantraquinone, structure (I) $R = CH_3$, under the same conditions.

The main features of the molecular constitution of nalgiovensin became clear as the result of oxidation experiments on nalgiovensin and its functional derivatives. Emodic acid trimethyl ether (4:5:7-trimethoxyanthraquinone-2-carboxylic acid) was obtained by the oxidation of nalgiovensin trimethyl ether with chromic acid in glacial acetic acid solution and also by the oxidation of nalgiovensin dimethyl ether with alkaline iodine in aqueous dioxan solution, in which oxidation no iodoform could be detected. On mild oxidation of nalgiovensin with chromic acid in glacial acetic acid solution at 60° there was obtained a small yield of 4:5-dihydroxy-7-methoxyanthraquinone-2-carboxylic acid (I), $R = COOH$, together with a much larger yield of an at present unidentified oxidation product having the molecular formula $C_{15}H_{14}O_6$. This substance which contains the same number of carbon and oxygen atoms as, but has two hydrogen atoms less than, nalgiovensin is, unlike nalgiovensin, optically inactive. We believe it to have structure (I) in which $R = C_3H_5O$ containing a CO group in place of CHOH.

On oxidation of triacetylnalgiovensin with chromic acid in a mixture of glacial acetic acid and acetic anhydride there is formed 4:5-diacetoxy-7-methoxyanthraquinone-2-carboxylic acid (diacetylemodic acid 7-methyl ether), i.e. the diacetate of (I), $R = COOH$, m.p. 214–215°. The structure of this oxidation product was proved by direct comparison of it with the oxidation product of diacetylphyscion which we prepared by a similar oxidation process. The two oxidation products proved to be identical. Their identity was confirmed by alkaline hydrolysis

of each of them. Both gave rise to 4:5-dihydroxy-7-methoxyanthraquinone-2-carboxylic acid (I), $R = \text{COOH}$, m.p. and mixed m.p. 322–323°. Chromic acid oxidation of carbon side chains attached to the anthraquinone nucleus, with the formation of the corresponding anthraquinone carboxylic acids, has been used frequently in the investigation of the molecular structure of naturally occurring poly-hydroxyanthraquinones, e.g. triacetylemodin gives triacetylemodic acid, $\text{CH}_3 \rightarrow \text{COOH}$ (Fischer & Gross, 1911; Eder & Hauser, 1925; Anslow, Breen & Raistrick, 1940); diacetylphyscion gives 4:5-diacetoxy-7-methoxyanthraquinone-2-carboxylic acid (diacetylemodic acid 7-methyl ether), $\text{CH}_3 \rightarrow \text{COOH}$ (Eder & Hauser, 1925); triacetylhelminthosporin gives triacetylhelminthosporic acid, $\text{CH}_3 \rightarrow \text{COOH}$ (Charles, Raistrick, Robinson & Todd, 1933); tetra-acetyl- ω -hydroxyemodin gives triacetylemodic acid, $\text{CH}_2\text{OAc} \rightarrow \text{COOH}$ (Anslow *et al.* 1940). If we accept that in the parallel oxidation of triacetylnalgiovensin to 4:5-diacetoxy-7-methoxyanthraquinone-2-carboxylic acid the reaction follows a similar course, certain important conclusions follow.

(1) That nalgiovensin has the same nuclear structure (I) as is present in physcion, i.e.

where $R = \text{CH}_3$ in physcion and $R = \text{C}_3\text{H}_7\text{O}$ in nalgiovensin.

(2) That the $\text{C}_3\text{H}_7\text{O}$ side chain in nalgiovensin must contain a hydroxyl group to account for the formation of triacetylnalgiovensin, nalgiovensin trimethyl ether and monoacetylnalgiovensin dimethyl ether, and may therefore be written $\text{C}_3\text{H}_6\text{OH}$. This conclusion is supported by the facts that in the oxidation of triacetylnalgiovensin one acetyl group is lost, just as is the case in the oxidation of tetra-acetyl- ω -hydroxyemodin to triacetylemodic acid, and that although nalgiovensin contains three hydroxyl groups which can be acetylated with acetic anhydride and sodium acetate, only two of them—almost certainly the nuclear hydroxyl groups—can be methylated with dimethyl sulphate and potassium carbonate, methylation of the third hydroxyl group—almost certainly that in the side chain—requiring the use of methyl iodide and silver oxide, a process which is known to methylate aliphatic hydroxyl groups, e.g. in sugars (Purdie & Irvine, 1901, 1903).

(3) That the $\text{C}_3\text{H}_7\text{O}$ side chain must contain an asymmetric carbon atom to account for the optical activity of nalgiovensin and its functional derivatives—an unusual property of naturally occur-

ring anthraquinones except of those conjugated ones occurring as glycosides.

(4) That the $\text{C}_3\text{H}_7\text{O}$ side chain must therefore have one of the following configurations:

- (a) $-\text{CHOH} \cdot \text{CH}_2 \cdot \text{CH}_3$;
- (b) $-\text{CH}_2 \cdot \text{CHOH} \cdot \text{CH}_3$;
- (c) $-\text{CH}(\text{CH}_3) \cdot \text{CH}_2\text{OH}$.

The evidence at present available favours either (a) or (b), since oxidation of side chain (a) to $-\text{CO} \cdot \text{CH}_2 \cdot \text{CH}_3$ or (b) to $-\text{CH}_2 \cdot \text{CO} \cdot \text{CH}_3$ would lead to loss of optical activity, whereas oxidation of (c) would lead to $-\text{CH}(\text{CH}_3) \cdot \text{CHO}$ which still contains an asymmetric carbon atom, and, as has been mentioned previously (see p. 564), mild oxidation of nalgiovensin with chromic acid in acetic acid solution at 60° gives a good yield of $\text{C}_{18}\text{H}_{14}\text{O}_6$ which is optically inactive.

The main conclusions reached above were confirmed by the observation that, when nalgiovensin was reduced with hydriodic acid and red phosphorus in glacial acetic acid solution and the resulting anthranol was oxidized with chromic acid in acetic acid solution, there was formed a new anthraquinone, $\text{C}_{17}\text{H}_{14}\text{O}_5$. This substance has the following properties. (a) It no longer contains a methoxyl group and, unlike nalgiovensin, is readily soluble in *N*-sodium carbonate as would be expected when the CH_3O group in position 7 is replaced by a free hydroxyl group. (b) It is optically inactive, whereas nalgiovensin is dextrorotatory. This is readily explained by postulating that the $\text{C}_3\text{H}_6\text{OH}$ side chain in nalgiovensin is reduced to C_3H_7 in a manner analogous to that of the reduction, by the same reagents, of aloe-emodin, 4:5-dihydroxy-2-hydroxymethylanthraquinone, to chrysophanic acid, 4:5-dihydroxy-2-methylanthraquinone (Oesterle, 1911), and of ω -hydroxyemodin, 4:5:7-trihydroxy-2-hydroxymethylanthraquinone, to emodin, 4:5:7-trihydroxy-2-methylanthraquinone (Anslow *et al.* 1940). (c) It gives colour reactions which are indistinguishable from those given by emodin. (d) It forms a triacetyl derivative. We therefore formulate this reduction product as 4:5:7-trihydroxy-2-propylanthraquinone in which the nature of the propyl group, whether *n*-propyl from the side chains $-\text{CHOH} \cdot \text{CH}_2 \cdot \text{CH}_3$ or $-\text{CH}_2 \cdot \text{CHOH} \cdot \text{CH}_3$, or *iso*-propyl from the side chain $-\text{CH}(\text{CH}_3) \cdot \text{CH}_2\text{OH}$, is left undetermined for the present. We hope to obtain conclusive evidence on the nature of this propyl group and also on the exact structure of the $\text{C}_3\text{H}_7\text{O}$ side chain in nalgiovensin by means of synthetic experiments which are in progress at present and will be reported later.

The second colouring matter, nalgiolaxin, present in the mycelium of *P. nalgiovensis* has the molecular formula $\text{C}_{18}\text{H}_{15}\text{O}_6\text{Cl}$ and is, we believe, the first example of a derivative of anthraquinone containing

chlorine which has been recorded from any natural source. The presence of chlorine in nalgiolaxin would appear at first sight to be an anomaly since Raulin-Thom solution, on which the mould was grown (for composition see p. 567), does not contain any intentionally added source of chlorine. We are, however, informed by the manufacturers (Corn Products Co. Ltd.) of the glucose (dextrosol monohydrate) used in the medium that the chloride content, calculated as sodium chloride, of this grade of glucose lies between 0.035 and 0.04%, and is therefore approximately four to five times the amount required for the production of nalgiolaxin in the yields which were actually obtained. Further, as will be shown later, nalgiolaxin is a monochloro derivative of nalgiovensin, and, since chloroform and hydrochloric acid are used in the preferred method of extraction and purification of nalgiolaxin, it became necessary to prove that nalgiolaxin is not an artifact. This was done (for details see p. 572) by avoiding the use of any reagents containing chlorine in the isolation and purification process. The colouring matter so isolated proved to be identical with nalgiolaxin obtained by the preferred isolation procedure.

Nalgiolaxin crystallizes from ethanol in small yellow plates and needles, m.p. 248–248.5°, without decomposition. Like nalgiovensin it contains one methyl group attached to carbon, one methoxyl group and is dextrorotatory in chloroform solution: $[\alpha]_{D}^{25} + 40.3^\circ$. Its colour reactions are, in general, very similar to those of nalgiovensin and physcion, except that its solution in cold concentrated sulphuric acid is much bluer in tone and its red chromatogram on magnesium carbonate is also a shade bluer. Its solution in glacial acetic acid is yellow in colour and does not fluoresce in daylight.

Two functional derivatives of nalgiolaxin were prepared by the same methods as were used for the preparation of the corresponding derivatives of nalgiovensin. *Triacetylnalgiolaxin*, $C_{24}H_{21}O_9Cl$, i.e. $C_{17}H_9O_2Cl(OCH_3)_3$ (O.COCH₃)₃, lemon-yellow plates, m.p. 186.5–187°, is, like triacetylnalgiovensin, laevorotatory in chloroform solution. *Nalgiolaxin dimethyl ether*, $C_{20}H_{19}O_6Cl$, i.e. $C_{17}H_{10}O_3Cl(OCH_3)_3$, yellow needles, m.p. 206–207°, is, like nalgiovensin dimethyl ether, dextrorotatory in chloroform solution.

The close structural relationship between nalgiovensin and nalgiolaxin and the fact that they both possess the same anthraquinone skeleton structure was proved by reducing nalgiolaxin with hydriodic acid and red phosphorus and oxidizing the resulting anthranol with chromic acid. There was thus obtained the same optically inactive 4:5:7-trihydroxy-2-propylantraquinone as was formed on similar treatment of nalgiovensin. In both cases demethylation and reduction of the hydroxyl group in the side chain took place, and, in addition, in the case of

nalgiolaxin the chlorine in this substance was replaced by hydrogen. The removal of halogen (bromine) from the anthraquinone nucleus by means of hydriodic acid was used by Jacobson & Adams (1924) in their synthesis of emodin, so that our findings provide presumptive evidence that the chlorine atom in nalgiolaxin is present in the anthraquinone nucleus and not in the 3-carbon side chain.

This important point was proved by oxidizing triacetylnalgiolaxin with chromic acid in acetic acid-acetic anhydride solution. There was thus obtained a compound, $C_{20}H_{13}O_9Cl$, i.e.

Triacetylnalgiovensin, on similar treatment, gives a compound, $C_{20}H_{14}O_9$, i.e.

which, as previously stated, was identified as 4:5-diacetoxy-7-methoxyanthraquinone-2-carboxylic acid, i.e. the diacetate of (I), R=COOH. The two oxidation products have almost identical colour reactions, only differing in the fact that, while both of them are readily soluble in cold 0.5N-sodium bicarbonate, indicating the presence in each of a carboxyl group, the nalgiolaxin oxidation product gives a much redder solution, as might be expected from the presence in it of a nuclear chlorine atom. Hence we feel justified in postulating that the nalgiolaxin oxidation product is a nuclear monochloro derivative of 4:5-diacetoxy-7-methoxyanthraquinone-2-carboxylic acid, i.e. the diacetate of (II), R=COOH.

We have at present no conclusive evidence of the exact position of the chlorine atom in the anthraquinone nucleus of nalgiolaxin. It must, however, be in one of the four available positions, i.e. 1, 3, 6 or 8. Further, if it were replaced by a hydroxyl group this group would be in one of the same four positions, and, if this hydroxyl group were in either position 1 or 8, but not in positions 3 or 6, the resulting polyhydroxyanthraquinone would be a derivative of quinizarin, 1:4-dihydroxyanthraquinone, and all known quinizarin derivatives give solutions in acetic acid which fluoresce in daylight (Raistrick, Robinson & Todd, 1934). Replacement of a nuclear halogen by hydroxyl was described by Ullmann & Schmidt (1919), who converted 1-chloro-4-hydroxy-2-methylantraquinone into 1:4-dihydroxy-2-methylantraquinone, i.e. 2-methylquinizarin, by heating with concentrated sulphuric acid and boric acid at 150–160°. The same reaction was also used for the replacement of a nuclear anthraquinone halogen atom by hydroxyl by Hayashi (1927) in the synthesis of 8-methylquinizarin, and by Anslow & Raistrick (1941) in the synthesis of catenarin, 1:4:5:7-tetrahydroxy-2-methylantraquinone. When nalgiolaxin was heated with concentrated sulphuric acid and boric acid the initially red solution changed colour

and became permanganate purple at 150–160°. A red colouring matter, at present not characterized, was recovered from the reaction mixture. Its solution in glacial acetic acid was orange-yellow in colour with a fine green fluorescence. It readily dissolved in aqueous N-sodium hydroxide and N-sodium carbonate to give blue-violet solutions. With concentrated sulphuric acid it gave a red-violet solution having a slight red fluorescence. These reactions are in marked contrast to the colours and non-fluorescent solutions given by nalgliolaxin in the same reagents and are very similar to those given by catenarin, 1:4:5:7-tetrahydroxy-2-methylanthraquinone, except that a solution of catenarin in cold concentrated sulphuric acid is much more blue-violet in colour. We believe that these observations, although at present purely qualitative, justify the tentative suggestion that the chlorine atom in nalgliolaxin is in either position 1 or 8, and that nalgliolaxin is therefore 1- or 8-monochloronalgiovinsin (II), in which $R = C_3H_7O$ as in nalgiovinsin.

EXPERIMENTAL

All melting points are uncorrected.

C, H, and Cl estimations were carried out by Drs Weiler and Strauss, Oxford, other estimations by one of us (J.Z.).

A 4 dm. tube was used for determination of optical rotations.

History of culture

The strain of *P. nalgiovinsin* Laxa used throughout this work was received in July, 1948, from Dr K. B. Raper of the Northern Regional Research Laboratory (N.R.R.L.), United States Department of Agriculture, Peoria, Illinois, U.S.A. This culture, N.R.R.L. 911 (Thom's No. 5337.2), was originally described by Laxa in 1932 and was received from him by Dr C. Thom in April, 1933. A full description of the strain is given by Raper & Thom (1949, p. 319).

Cultural conditions

P. nalgiovinsin does not sporulate heavily on beer-wort agar slopes. For this reason it was found desirable, for inoculation purposes, to sporulate the organism on sterile, moistened wheat bran. The bran spore inoculum was prepared as follows. Bran (5 g.) contained in a conical flask (250 ml.) was thoroughly moistened with tap water (4.5 ml.). The flask was plugged with cotton wool and autoclaved at 121° for 1 hr. After cooling, the bran was inoculated with *P. nalgiovinsin* and incubated in the dark at 24° for 10–14 days. The mould grew uniformly throughout the bran and sporulated fairly well. A spore suspension was prepared by shaking the sporulated bran culture with 150 ml. of sterile dilute agar solution (0.125%) and pipetting off the suspended mould spores. The residual bran was re-treated with a second portion of agar solution (100 ml.) which was combined with the first suspension. A sterile, perforated sleeve fitted on the end of the pipette prevented the inclusion of any bran particles. The mould spore concentration of the combined suspensions was approximately 1,000,000/ml.

Batches of a hundred 11. conical flasks were prepared, each containing 350 ml. of Raulin-Thom solution: i.e. glucose, 75.0 g.; tartaric acid, 4.0 g.; ammonium tartrate, 4.0 g.; $(NH_4)_2HPO_4$, 0.6 g.; $(NH_4)_2SO_4$, 0.25 g.; K_2CO_3 , 0.6 g.; $MgCO_3$, 0.4 g.; $FeSO_4 \cdot 7H_2O$, 0.07 g.; $ZnSO_4 \cdot 7H_2O$, 0.07 g.; distilled water, 1500 ml. After sterilization each flask was inoculated with 4 ml. of the spore suspension described above. The inoculated flasks were incubated at 24° in the dark. A thin white vegetative mat was visible by the 2nd day and was well formed by the 3rd day. On the 5th day the mycelium was light creamy-green in colour with a light-yellow reverse, and had become pale yellow-green with a yellow-brown reverse by the 9th day. When harvested, at the end of 28 days, the mycelium was heavily folded and quite brittle. It was dull greyish-green in colour with a dark-brown reverse.

In order to determine the optimum time for harvesting the cultures fifteen flasks were harvested after 7, 14, 21, 28 and 35 days' incubation. Determinations were made of pH (colorimetrically) and residual glucose (by polarimeter) in the culture filtrates, and weight of mycelium, dried to constant weight in a desiccator over conc. H_2SO_4 , and of crude nalgiovinsin isolated from the dried mycelium as described below. The results obtained are shown in Table 1.

Isolation and purification of nalgiovinsin

Batches of a hundred flasks were harvested at the end of 28 days' incubation. The brownish culture fluid was separated by decantation from the mould mycelium and was discarded. The mycelium was washed with distilled water, pressed as dry as possible in a tincture press and dried in a vacuum oven at 45–50°. The dried mycelium was ground to

Table 1. *Cultural characteristics of Penicillium nalgiovinsin* Laxa

Incubation period (days)	No. of flasks	pH	Glucose by polarimeter (%)	Mycelium wt. (g.)	Crude nalgiovinsin wt. (g.)
0	2	4.1	5.34	—	—
7	15	3.6	4.18	23.0	0.02
14	15	3.4	3.00	36.0	0.07
21	15	3.4	2.66	40.5	0.11
28	15	3.4	2.53	42.0	0.12
35	15	3.2	2.39	42.7	0.12

a fine powder in a coffee mill and was then distributed equally between five large Soxhlet thimbles and extracted in five Soxhlets with light petroleum (b.p. 40–60°) for 60–100 hr. During the extraction crude nalgiiovinsin separated from the boiling solution as an orange solid which is practically insoluble in boiling light petroleum. Approximately 45% of the total weight of nalgiiovinsin present in the mould mycelium is extracted under these conditions. Complete extraction of the pigment was accomplished only after subsequent extended extraction with CHCl_3 (see p. 571). After cooling, the crude nalgiiovinsin was separated by filtration and the orange-yellow light petroleum mother liquors, on removal of the solvent, left a small quantity of crude fat containing only traces of pigment. From a total of 989 flasks 2966 g. of dried mycelium were obtained yielding, on extraction with light petroleum, 8.97 g. of crude fat and 13.17 g. of crude nalgiiovinsin.

The crude nalgiiovinsin obtained by the above procedure melts at approximately 190° and was easily purified by successive crystallizations from CHCl_3 , yielding about 50% of its weight of pure nalgiiovinsin, m.p. 199–200°. Pure nalgiiovinsin could not readily be obtained by fractional crystallization of the dried CHCl_3 mother liquors, nor could these be satisfactorily purified by chromatography on MgCO_3 columns. They were, however, easily purified by acetylation and fractional crystallization from dilute acetic acid of the crude triacetylnalgiiovinsin. The pure triacetylnalgiiovinsin thus obtained, m.p. 147–148°, was then hydrolysed with aqueous NaOH to give pure nalgiiovinsin (see below).

General properties of nalgiiovinsin. Derivatives

Nalgiiovinsin crystallizes from CHCl_3 as beautiful, long, flat, orange needles, and from glacial acetic acid as glistening orange plates, m.p. 199–200°, without decomposition. (Found, on sample sublimed in a high vacuum at 160–170°: C, 66.1, 65.95; H, 4.9, 4.9; CH_3O , 9.3, 9.4; $\text{C}-\text{CH}_3$, 5.0; N, S, Cl, nil. $\text{C}_{18}\text{H}_{18}\text{O}_6$ requires C, 65.9; H, 4.9; $1\text{CH}_3\text{O}$, 9.45; $1\text{C}-\text{CH}_3$, 4.6%.) It is dextrorotatory in CHCl_3 solution: $[\alpha]_{5461}^{20} + 48.1^\circ$; $[\alpha]_{5790}^{20} + 39.7^\circ$ (c, 0.1786). It readily sublimes in a high vacuum at 160–170° without decomposition. It readily dissolves in warm CHCl_3 , glacial acetic acid and acetone, less readily in ethanol, and is only slightly soluble in light petroleum, b.p. 40–60°. It does not dissolve in 0.5N- NaHCO_3 or in N- Na_2CO_3 , but dissolves readily in aqueous NaOH to a red solution with a slight bluish cast. It dissolves in cold conc. H_2SO_4 to a beautiful red solution which appears somewhat orange by transmitted light. Its ethanolic solution gives an orange-brown colour with ethanolic FeCl_3 . Its solution in glacial acetic acid is yellow in colour and does not fluoresce in daylight. Its CHCl_3 solution gives a pure red band when chromatographed on columns of MgCO_3 (British Drug Houses Ltd., 'heavy'). The band becomes bluish-red and is considerably diffused when the column is washed with absolute methanol.

Triacetylnalgiiovinsin. Anhydrous sodium acetate (0.4 g.) was added to a solution of nalgiiovinsin (0.186 g.) in acetic anhydride (2 ml.), and the mixture was maintained at 100° for 2 hr. It was then cooled and poured into ice water. The yellow solid which separated (0.248 g.) was filtered, dried and crystallized from dilute ethanol to give fine, yellow needles of *triacetylnalgiiovinsin*, m.p. 147–148°. (Found, on sample dried in a high vacuum at 70° with no loss in wt.: C, 63.8, 63.65; H, 5.4, 5.1; CH_3O , 6.8, 6.8; CH_3CO , by hydrolysis, 27.6, 28.3. $\text{C}_{24}\text{H}_{22}\text{O}_9$ requires C, 63.4; H, 4.9; $1\text{CH}_3\text{O}$,

6.8; $3\text{CH}_3\text{CO}$, 28.4%.) Triacetylnalgiiovinsin is laevorotatory in CHCl_3 solution: $[\alpha]_{5461}^{20} - 8.9^\circ$; $[\alpha]_{5790}^{20} - 8.3^\circ$ (c, 0.6076).

Triacetylnalgiiovinsin (0.67 g.) was heated for 3 hr. with aqueous N-NaOH (45 ml.) on a boiling-water bath in an atmosphere of N_2 . The acetate quickly dissolved to give an intense wine-red solution. N- H_2SO_4 (50 ml.) was added and the resulting orange precipitate was coagulated by heating for 0.5 hr. and was then filtered, washed and dried (0.49 g.). The orange solid was sublimed and then crystallized from CHCl_3 , wt. 0.37 g., m.p. 199–200°. The colouring matter was still dextrorotatory in CHCl_3 solution: $[\alpha]_{5461}^{20} + 48.5^\circ$; $[\alpha]_{5790}^{20} + 40.4^\circ$ (c, 0.2192).

Nalgiiovinsin dimethyl ether. A mixture of nalgiiovinsin (0.54 g.), anhydrous K_2CO_3 (10.8 g.), redistilled dimethyl sulphate (5.4 ml.) and dry acetone (300 ml.) was boiled under reflux for 8 hr., during which period the colour of the solution changed from deep wine-purple to pure lemon-yellow. The hot solution was filtered and evaporated to 20 ml. *in vacuo*. Water (500 ml.) and 2N-HCl (50 ml.) were added. The yellow solid which separated was filtered, washed and dried (0.53 g.). It was dissolved in CHCl_3 (250 ml.) and traces of partially methylated material were removed by shaking the CHCl_3 solution with 0.5N-NaOH and then with water. On removal of the CHCl_3 there remained a yellow solid (0.51 g.) which was purified by sublimation in a high vacuum at 180° (0.49 g., m.p. 187–188°) followed by crystallization from aqueous ethanol. *Nalgiiovinsin dimethyl ether* was thus obtained as thin, branching yellow needles, m.p. 187–188°. (Found: C, 67.2, 67.2; H, 5.8, 6.0; CH_3O , 25.5, 25.5. $\text{C}_{20}\text{H}_{20}\text{O}_8$ requires C, 67.4; H, 5.7; $3\text{CH}_3\text{O}$, 26.1%.) Nalgiiovinsin dimethyl ether is dextrorotatory in CHCl_3 solution: $[\alpha]_{5461}^{20} + 35.9^\circ$; $[\alpha]_{5790}^{20} + 28.8^\circ$ (c, 0.4620). It is readily soluble in CHCl_3 , acetone and ethanol. It is insoluble in N-NaOH, but dissolves in cold conc. H_2SO_4 to an intense red solution with a slight bluish cast.

Monoacetylnalgiiovinsin dimethyl ether. Nalgiiovinsin dimethyl ether (0.13 g.) was acetylated in the usual manner with anhydrous sodium acetate (0.26 g.) and acetic anhydride (2 ml.). The yellow solid (0.16 g.) which separated on dilution of the acetylation mixture with water was crystallized from ethanol (7 ml.). *Monoacetylnalgiiovinsin dimethyl ether* was thus obtained as thin yellow-green rods (0.09 g.), m.p. 152.5–153°. (Found: C, 66.0, 66.1; H, 5.65, 5.6; CH_3O , 23.4. $\text{C}_{22}\text{H}_{22}\text{O}_7$ requires C, 66.3; H, 5.6; $3\text{CH}_3\text{O}$, 23.4%.) It is dextrorotatory in CHCl_3 solution: $[\alpha]_{5461}^{22} + 4.8^\circ$; $[\alpha]_{5790}^{22} + 3.8^\circ$ (c, 0.2512). Its solution in cold conc. H_2SO_4 is almost indistinguishable in colour from that given by nalgiiovinsin dimethyl ether.

Nalgiiovinsin trimethyl ether. Nalgiiovinsin dimethyl ether (0.30 g.) was dissolved in dry benzene (100 ml.) and methyl iodide (20 ml.). Dry Ag_2O (5 g.) was then added in portions at hourly intervals (2 g. at 0 hr. and 1 g. at the end of the first, second and third hour) and the mixture was heated under reflux for a total of 8 hr. The solvents were removed *in vacuo* and the resulting solid was extracted with warm benzene (5 × 20 ml.). The filtered, yellow benzene solution was evaporated *in vacuo* leaving a crystalline yellow solid (0.31 g., m.p. 176.5–177°) which was purified by crystallization from ethanol. *Nalgiiovinsin trimethyl ether* was thus obtained as fine, yellow-green needles (0.26 g.), m.p. 177–178°. (Found, on sample sublimed in a high vacuum at 170°: C, 67.9, 67.8; H, 6.1, 6.1; CH_3O , 33.1. $\text{C}_{21}\text{H}_{22}\text{O}_8$ requires C, 68.1; H, 6.0; $4\text{CH}_3\text{O}$, 33.5%.) It is dextrorotatory in CHCl_3 solution: $[\alpha]_{5461}^{21} + 6.7^\circ$; $[\alpha]_{5790}^{21} + 5.8^\circ$ (c, 0.4531). Its solution

in cold conc. H_2SO_4 is almost indistinguishable in colour from that given by nalgiovensin dimethyl ether.

Attempts to prepare nalgiovensin trimethyl ether by other recognized methods failed. Methylation of nalgiovensin with a large excess of dimethyl sulphate and 10% (w/v) aqueous KOH at 45–50° or at 100° yielded nalgiovensin dimethyl ether. Attempts to methylate nalgiovensin dimethyl ether further, either in methanol solution with dimethyl sulphate and 10% aqueous KOH or in acetone solution with ethereal diazomethane, yielded only unchanged nalgiovensin dimethyl ether.

OXIDATION EXPERIMENTS WITH NALGIOVENSIN AND ITS DERIVATIVES

(a) With alkaline hydrogen peroxide

H_2O_2 (100 vol., 5 ml.) was added drop by drop to a solution of nalgiovensin (0.1 g.) in N-NaOH (7.5 ml.), and the whole was maintained in an atmosphere of N_2 . The wine-red solution was then slowly heated to 60°, without any evidence of a reaction occurring, and maintained at this temperature for 0.5 hr. The starting material was recovered unchanged by acidification with 2N-HCl (6 ml.) and subsequent sublimation of the recovered orange solid (m.p. and mixed m.p. 199–200°).

(b) Mild CrO_3 oxidation of nalgiovensin

A solution of CrO_3 (0.50 g.) in water (20 ml.) and glacial acetic acid (20 ml.) was quickly added with constant shaking to a solution of nalgiovensin (0.50 g.) in glacial acetic acid (200 ml.), the whole being maintained at 60°. The resulting brown solution was held at 60° for 0.5 hr. and was then poured into ice water (2 l.). The orange-yellow solid which separated was extracted from the solution with ether (3 × 1 l.). The golden-yellow ether solution was washed twice with water and was then extracted with 0.5N- NaHCO_3 (4 × 250 ml.). The reddish-orange NaHCO_3 extracts were washed twice with fresh ether, acidified with HCl and then re-extracted with CHCl_3 (3 × 50 ml.). The CHCl_3 solution was washed with water and evaporated to dryness, giving a red-orange solid (0.01 g.) which was crystallized from glacial acetic acid (8 ml.). 4:5-Dihydroxy-7-methoxyanthraquinone-2-carboxylic acid was thus obtained as orange needles, m.p. 320–321° (decomp.) and was identified by comparison with an authentic specimen (see § c) (m.p. 322–323° decomp., mixed m.p. 321–322° decomp.). The two specimens also gave indistinguishable colour reactions.

The neutral ether solution remaining after extraction with NaHCO_3 was washed with water and evaporated to dryness. The resulting orange solid (0.38 g.), m.p. 190.5–191.5°, was crystallized three times from ethanol giving beautiful, large, orange needles, m.p. 199–200°, mixed m.p. with nalgiovensin (m.p. 199–200°) being 178–185°. (Found: on sample sublimed in a high vacuum at 170–175°: C, 65.6; H, 4.3; CH_3O , 9.5, 9.2. $\text{C}_{24}\text{H}_{14}\text{O}_6$ requires C, 66.3; H, 4.3; $1\text{CH}_3\text{O}$, 9.5%.) This oxidation product, which is optically inactive in CHCl_3 solution (c, 0.4556), gives colour reactions with conc. H_2SO_4 and N-NaOH which are identical with those given by nalgiovensin (see p. 568).

(c) CrO_3 oxidation of triacetylnalgiovensin

Triacetylnalgiovensin (1.13 g.) was dissolved at 55–60° in a mixture of glacial acetic acid (23 ml.) and acetic anhydride (23 ml.). The temperature was maintained at 55–60°, while

a solution of CrO_3 (2.3 g.) in water (1.8 ml.) and glacial acetic acid (23 ml.) was gradually added with constant shaking over a period of 0.5 hr. The bath temperature was then raised to 65–70° and maintained at this level for 3 hr. The deep-green solution was poured into warm water (800 ml.) and left in the refrigerator overnight, when a yellow crystalline solid separated. The whole was extracted with CHCl_3 (3 × 150 ml.). The yellow CHCl_3 solution was first washed with water and was then extracted with 0.5N- NaHCO_3 (4 × 100 ml.) yielding (i) a CHCl_3 solution of neutral oxidation products, and (ii) an aqueous NaHCO_3 solution of acidic oxidation products.

(i) *Treatment of 'neutral' CHCl_3 solution.* On removal of the CHCl_3 there remained a yellow oil (0.96 g.), which later solidified, of which 0.83 g. was successively re-oxidized three times yielding 0.47 g. of neutral fraction after the second oxidation, 0.31 g. after the third and 0.23 g. after the fourth oxidation. These neutral fractions consist of a mixture of neutral oxidation intermediates. In a second experiment the crude neutral fraction (0.18 g., m.p. 155.5–156.5°) obtained from the oxidation of 0.26 g. of triacetylnalgiovensin was repeatedly crystallized from aqueous ethanol and then from absolute ethanol finally giving beautiful, long, yellow needles (0.11 g.), m.p. 165–166°. (Found: C, 61.3, 60.95; H, 4.35, 4.6; CH_3O , 6.9, 6.7. $\text{C}_{24}\text{H}_{20}\text{O}_{10}$ requires C, 61.5; H, 4.3; $1\text{CH}_3\text{O}$, 6.6. $\text{C}_{24}\text{H}_{22}\text{O}_{10}$ requires C, 61.3; H, 4.5; $1\text{CH}_3\text{O}$, 6.6%.) The oxidation product, which appears to be optically inactive in CHCl_3 (c, 0.152), dissolves in cold conc. H_2SO_4 to a red solution indistinguishable in colour from that given by nalgiovensin with the same reagent.

(ii) *Treatment of NaHCO_3 solutions.* The four NaHCO_3 extracts were quickly and separately acidified with 2N-HCl and extracted with CHCl_3 . On removal of the CHCl_3 from the four extracts there remained 0.09 + 0.20 + 0.06 + 0.03 g. = 0.38 g. of yellow solid. This was fractionally crystallized from ethanol and then from 50% aqueous acetic acid from which there separated first a small amount of a monoacetate and then finally a diacetate in long, thin, yellow needles, m.p. 214–215°, the overall yield of which was about 50% of the crude acid oxidation product. The diacetate was identified as 4:5-diacetoxy-7-methoxyanthraquinone-2-carboxylic acid (diacetylemodic acid-7-methyl ether). (Found: C, 60.2, 60.3; H, 3.85, 3.8; CH_3O , 7.7. Calc. for $\text{C}_{20}\text{H}_{14}\text{O}_9$: C, 60.3; H, 3.5; $1\text{CH}_3\text{O}$, 7.8%.) It did not depress the m.p. (214–215°) of an authentic specimen prepared by oxidation of diacetylphyscion (see p. 570) and previously described by Eder & Hauser (1925). Both specimens gave the following reactions: 0.5N- NaHCO_3 , yellow solution; N- Na_2CO_3 , or N-NaOH, red solution with a slight bluish cast; conc. H_2SO_4 , intense reddish-orange solution.

The identity of the oxidation diacetate was confirmed by hydrolysis to the parent anthraquinone. The diacetate (0.07 g.) was heated with aqueous 0.5N-NaOH (35 ml.) on a boiling-water bath for 2 hr. in an atmosphere of N_2 . The intense wine-red solution was acidified with 2N-HCl (12 ml.) and heated for 0.5 hr. to coagulate the gelatinous orange precipitate which was filtered, washed and dried (0.045 g.). It was crystallized from glacial acetic acid as orange needles or as heavy reddish needle clusters, m.p. 322–323° (decomp.) alone or in admixture with 4:5-dihydroxy-7-methoxyanthraquinone-2-carboxylic acid prepared from diacetylphyscion (see p. 570). Both specimens were readily soluble in cold 0.5N- NaHCO_3 to a reddish-orange solution and both gave reactions with N- Na_2CO_3 , N-NaOH and cold conc. H_2SO_4 which were indistinguishable from those given by the diacetyl compound (see previous paragraph). The acid is

sparingly soluble in CHCl_3 , ethanol and glacial acetic acid from which solvent it crystallizes with acetic acid of crystallization which is extremely difficult to remove. (Found, on sample dried in a high vacuum at 85° with no loss in wt.: C, 57.3, 57.2; H, 4.0, 3.75. $\text{C}_{18}\text{H}_{10}\text{O}_7 + \text{C}_2\text{H}_4\text{O}_2$, i.e. $\text{C}_{18}\text{H}_{14}\text{O}_9$, requires C, 57.8; H, 3.8. On sample sublimed in a high vacuum at $230\text{--}235^\circ$, m.p. $322\text{--}323^\circ$ (decomp.): C, 61.4; H, 3.6. Calc. for $\text{C}_{18}\text{H}_{10}\text{O}_7$: C, 61.15; H, 3.2%.)

Preparation of 4:5-diacetoxy-7-methoxyanthraquinone-2-carboxylic acid from physcion. A sample of the lichen *Xanthoria parietina* L. Fr. was collected from limestone walls at Oranmore, near Galway, Eire. The dried lichen (56.8 g.) was extracted with cold CHCl_3 (6×400 ml.). The dark solid (0.62 g.) remaining on removal of the solvent was purified by sublimation in a high vacuum at 160° (0.40 g.) and crystallization from glacial acetic acid (14 ml.). Physcion (4:5-dihydroxy-7-methoxy-2-methylantraquinone) was thus obtained as flat, orange needles (0.31 g.), m.p. $206\text{--}207^\circ$, not depressed on admixture with an authentic specimen (m.p. $206\text{--}207^\circ$). The lichen physcion gives colours with cold conc. H_2SO_4 and N-NaOH which are indistinguishable from those given by nalgiovinsin.

Physcion (0.20 g.) was acetylated with anhydrous sodium acetate (0.4 g.) and acetic anhydride (2 ml.) in the usual manner. Diacetylphyscion (0.26 g.), m.p. $185\text{--}187^\circ$, was obtained crystalline when the reaction mixture was poured into water. This acetate (0.26 g.), without any further purification, was dissolved in a mixture of glacial acetic acid (5.2 ml.) and acetic anhydride (5.2 ml.) at $55\text{--}60^\circ$, and oxidized at $65\text{--}70^\circ$ for 3 hr. with CrO_3 (0.52 g.) dissolved in water (0.41 ml.) and glacial acetic acid (5.2 ml.). Oxidation of the methyl group in physcion to COOH was complete in one oxidation (cf. oxidation of triacetylnalgiovinsin). The crude oxidation product was purified exactly as described in § c, ii, p. 569, and 4:5-diacetoxy-7-methoxyanthraquinone-2-carboxylic acid (0.10 g.) was thus obtained as long, thin, yellow needles, m.p. $214\text{--}215^\circ$. Eder & Hauser (1925) give the m.p. of this acid as $214\text{--}215^\circ$. This acetylated acid (0.09 g.) was hydrolysed with 0.5 N-NaOH (40 ml.) in N_2 (see p. 569). The resulting 4:5-dihydroxy-7-methoxyanthraquinone-2-carboxylic acid was obtained as heavy reddish needle clusters (0.05 g.), m.p. $322\text{--}323^\circ$ (decomp.), by crystallization from glacial acetic acid. Eder & Hauser (1925) give the m.p. of this acid as 300° . Their preparation was crystallized from pyridine, the product containing pyridine of crystallization. The pyridine was removed by heating at 120° , the acid so obtained melting at 298° raised to 300° by sublimation *in vacuo* at an unspecified temperature. Our preparation contained acetic acid of crystallization, and when sublimed in a high vacuum at $250\text{--}260^\circ$ the sublimate melted at $304\text{--}305^\circ$. Part of the same specimen sublimed at $230\text{--}235^\circ$ gave a sublimate melting at $322\text{--}323^\circ$.

(d) *Alkaline iodine oxidation of nalgiovinsin dimethyl ether*

The method used was essentially that described by Fuson & Bull (1934). 10% aqueous NaOH (1 ml.) was added to a solution of nalgiovinsin dimethyl ether (0.1 g.) in dioxan (10 ml.). The iodine reagent (KI, 10 g.; I_2 , 5 g.; water, 40 ml.) was added dropwise with constant shaking. There did not appear to be any decolorization and an excess of the reagent was added (7 ml.). The dark solution was heated and held at 60° for 1 hr. The excess I_2 colour was then discharged by the dropwise addition of 10% aqueous NaOH (approx. 3 ml.)

and the yellow solution was diluted with water to 125 ml. After holding in the refrigerator overnight, the yellow solid which separated was filtered and dried (0.04 g.). It did not contain iodoform, and since it was shown to be a K salt it was decomposed by refluxing for 0.75 hr. in 50% aqueous acetic acid (30 ml.). On cooling, flat yellow needles (0.02 g.) separated, m.p. $268\text{--}269^\circ$ (decomp.), alone or in admixture with authentic emodic acid trimethyl ether, m.p. $268\text{--}269^\circ$ (decomp.). (Found: C, 60.3; H, 4.4. $\text{C}_{18}\text{H}_{14}\text{O}_7 \cdot \text{H}_2\text{O}$ requires C, 60.0; H, 4.5%.) Emodic acid trimethyl ether crystallizes with 1 mol. water of crystallization.

The alkaline oxidation filtrate from the above K salt was extracted with CHCl_3 yielding 0.03 g. of unchanged nalgiovinsin dimethyl ether. The CHCl_3 -extracted alkaline solution was acidified with HCl and was aerated for a short time at 60° to eliminate the last traces of CHCl_3 and some of the liberated I_2 . The yellow solid which separated on cooling was filtered, washed with water, dried (wt. 0.03 g.) and crystallized from ethanol (15 ml.) yielding 0.015 g. of emodic acid trimethyl ether in fine yellow needles, m.p. $267\text{--}268^\circ$ (decomp.). (Found: C, 60.05; H, 4.5. $\text{C}_{18}\text{H}_{14}\text{O}_7 \cdot \text{H}_2\text{O}$ requires C, 60.0; H, 4.5%.) In a repeat oxidation of 2 min. duration no iodoform was observed, 70% of the starting material was recovered unchanged and about 30% of emodic acid trimethyl ether was isolated.

Authentic emodic acid trimethyl ether was prepared by methylation of authentic emodic acid with dimethyl sulphate and aqueous KOH essentially as described by Eder & Hauser (1925). The crude methylation product was purified by crystallization from ethanol. It forms thin yellow needles, m.p. $268\text{--}269^\circ$ (decomp.). Eder & Hauser (1925) give the m.p. as 270° (decomp.). The oxidation acid and authentic emodic acid trimethyl ether gave the following indistinguishable colour reactions: with cold conc. H_2SO_4 an intense red solution with a slight bluish cast; readily soluble in 0.5 N-NaHCO₃ and N-Na₂CO₃ to a yellow solution unchanged on standing; readily soluble in aqueous N-NaOH and 10% NaOH giving a yellow solution which becomes reddish on standing for 1 hr.; insoluble in 10% KOH, slightly soluble in 5% KOH, readily soluble in 1% KOH.

(e) *CrO₃ oxidation of nalgiovinsin trimethyl ether*

Nalgiovinsin trimethyl ether (0.23 g.) dissolved in glacial acetic acid (4.6 ml.) and acetic anhydride (4.6 ml.) was oxidized exactly as described for triacetylnalgiovinsin (see § c) with CrO_3 (0.46 g.) in water (0.37 ml.) and glacial acetic acid (4.6 ml.). The oxidation appeared to proceed more quickly than that of triacetylnalgiovinsin. The acid fraction obtained (0.02 g.), on crystallization from ethanol (3 ml.), yielded thin yellow needles, m.p. $267\text{--}268^\circ$ (decomp.), not depressed on admixture with authentic emodic acid trimethyl ether. It also gave the same colour reactions (see § d).

REDUCTION OF NALGIOVINSIN

A mixture of nalgiovinsin (0.50 g.), glacial acetic acid (10 ml.), red phosphorus (0.50 g.) and HI (2 ml. sp.gr. 1.7) was refluxed for 5 hr. The mixture was cooled and poured into water (500 ml.). Traces of I_2 were destroyed by the addition of a slight excess of aqueous NaHSO₃, and the suspended solid was separated by filtration, washed, dried and extracted with warm CHCl_3 . After removal of the solvent the resulting

yellow solid was reduced a second time with fresh quantities of reagents. The filtered CHCl_3 solution, after the second treatment, was evaporated to low bulk. The *anthranol* crystallized as microplates (0.43 g.), m.p. 185°.

A small sample (0.01 g.) was recrystallized from CHCl_3 -light petroleum (b.p. 40–60°) giving pale-yellow plates, m.p. 188°. It gave colour reactions typical of an emodin-type anthranol. It dissolved in cold conc. H_2SO_4 to a yellow solution which slowly became dark green on standing or more rapidly on warming. It readily dissolved in N-NaOH to a yellow solution which, on shaking, passed through a series of colours giving finally a stable red solution with a slight bluish cast. It did not dissolve in cold $\text{N-Na}_2\text{CO}_3$ except on long standing.

The anthranol (0.42 g.), m.p. 185°, without further purification, was dissolved in glacial acetic acid (95 ml.). The solution was maintained at 60° and a solution of CrO_3 (0.42 g.) in water (6 ml.) and glacial acetic acid (6 ml.) was quickly added with constant shaking. The mixture was held at 60° for 0.5 hr. and was then poured into ice water (500 ml.). The brown solid which separated was filtered, washed and dried (0.43 g.). It was purified by crystallization first from CHCl_3 and then from benzene. 4:5:7-Trihydroxy-2-propylanthraquinone was thus obtained as long yellow needles appearing orange in bulk, m.p. 216.5–217°. (Found, on sample crystallized from benzene and dried in a high vacuum at 100°, loss in wt. 9.6%: C, 68.1; H, 4.7. $\text{C}_{17}\text{H}_{14}\text{O}_5$ requires C, 68.45; H, 4.7%.) It is optically inactive in CHCl_3 (c, 0.2512). It readily sublimes in a high vacuum at 170°. It gives the following colour reactions. It is insoluble in aqueous 0.5N- NaHCO_3 ; it readily dissolves in cold conc. H_2SO_4 , $\text{N-Na}_2\text{CO}_3$ and N-NaOH giving in each case a red solution with a slight bluish cast, the solution colours in $\text{N-Na}_2\text{CO}_3$ and N-NaOH being a stronger blue than that in conc. H_2SO_4 . Emodin (4:5:7-trihydroxy-2-methylanthraquinone) gives identical colours with these reagents.

A solution of 4:5:7-trihydroxy-2-propylanthraquinone (0.16 g.) in pyridine (6.5 ml.) and acetic anhydride (1.6 ml.) was held at 35° for 3 days. The golden-yellow solution was then poured into water (300 ml.) and the resulting yellow solid (0.18 g.) was crystallized from ethanol. 4:5:7-Triacetoxy-2-propylanthraquinone was thus obtained as long, thin, yellow needles, m.p. 182–183°. (Found: C, 64.9; H, 4.8; CH_3CO , 32.7. $\text{C}_{23}\text{H}_{20}\text{O}_8$ requires C, 65.1; H, 4.75; $3\text{CH}_3\text{CO}$, 30.4%.)

ISOLATION OF NALGIOLAXIN

The dried ground mycelium of *P. nalgiovensis*, after exhaustive extraction with light petroleum (b.p. 40–60°) as described on p. 568, still contains considerable amounts of other substances which can be extracted in part with ether or almost completely with CHCl_3 . These extractives consist of nalgliolaxin, further amounts of nalgiovensin, other colouring matters, colourless crystalline solids and 'fat'. The fractionation of this complex mixture of extractives presents considerable technical difficulties. The method finally found most suitable, and a typical example of which is given below, was based on the following experimentally established facts. (1) Although pure nalgliolaxin is less soluble in organic solvents than is nalgiovensin, the two colouring matters cannot be satisfactorily separated from each other by fractional crystallization of the crude extractives. (2) Nalgliolaxin and nalgiovensin cannot be separated satisfactorily by chromatography on MgCO_3 , although by such chromato-

graphy they can be separated from the other colouring matters. (3) Nalgliolaxin may be partially separated from nalgiovensin by extraction of an ether or CHCl_3 solution with $\text{N-Na}_2\text{CO}_3$. (4) Fractional sublimation in a high vacuum can be successfully employed to separate nalgliolaxin from the colourless crystalline solids and traces of nalgiovensin.

The ground mycelium (261 g.), previously extracted exhaustively with light petroleum (b.p. 40–60°), was dried and re-extracted with CHCl_3 in five Soxhlets for about 60 hr. During the extraction period a colourless solid (substance A) separated in the boiling CHCl_3 solution. At the end of the extraction the dark extract was held overnight and substance A was then separated by filtration (4.16 g.). The CHCl_3 solution was evaporated yielding a brown fatty solid (11.8 g.) from which the fat present (8.0 g.) was removed by extraction with boiling light petroleum (b.p. 40–60°) (0.5 hr., 3×500 ml.) and then by macerating with cold ether (100 ml.). There is a slight loss of pigment in the latter step, but it is essential, since otherwise considerable difficulties are experienced with emulsification in subsequent CHCl_3 extractions. The brown friable residual solid thus obtained (3.78 g.) was extracted five times with ether (800 ml. each time) by refluxing for 0.5 hr. There was thus obtained an orange-yellow ether solution (a) (4 l.), and a brown residue (b) (1.33 g.).

The orange-yellow ether solution (a) (4 l.) was extracted with four successive portions of aqueous $\text{N-Na}_2\text{CO}_3$ (400, 400, 300, 200 ml.). The first extract was an intense wine-red colour. The other extracts were progressively lighter in colour. The Na_2CO_3 solutions were acidified with dil. HCl and the amorphous solid which separated was extracted with CHCl_3 (3×300 ml.). The CHCl_3 solution, on evaporation, yielded a brown solid (1.18 g.) which was crystallized from ethanol (300 ml.) giving crude nalgliolaxin (0.42 g.), m.p. 240–245°, contaminated with traces of nalgiovensin and other substances. It was fractionally sublimed, as described below, to give almost pure nalgliolaxin (0.33 g.), m.p. 245.5–246.5°. Further crops of crystals from the ethanol mother liquor consisted mainly of crude nalgiovensin. The carbonate extracted ether solution, on evaporation to dryness, gave a crystalline orange solid (0.62 g.), m.p. 195–197°, consisting of almost pure nalgiovensin.

The brown residue (b) (1.33 g.) was dissolved in CHCl_3 (1200 ml.). A small amount of colourless undissolved material was separated by filtration and discarded, and the clear filtrate was passed through a column (3.5 cm. diam.) of MgCO_3 (British Drug Houses Ltd., 'heavy'). A total band, 10 cm. in length, was thus formed consisting of a top purple-blue band (3.5 cm.) of an at present uncharacterized colouring matter (substance B), and a red band (6.5 cm.). These bands did not spread any further when the column was washed with CHCl_3 (400 ml.). The red band was mechanically separated, acidified with 2N- HCl in ice, and the precipitated colouring matter was extracted with CHCl_3 . On removal of the CHCl_3 there remained a brown solid (0.60 g.) which contained considerable amounts of colourless solid. It was distributed equally in five sublimation tubes and was then purified by fractional sublimation in a high vacuum as follows. The oil-bath temperature was raised slowly to 160–170° and was held there for 15 min. A small sublimate of nalgiovensin was thus obtained. The oil bath was lowered and the temperature was raised to 190–200° and held there for 45 min. The resulting orange-red sublimate (0.37 g.), m.p. 241–244°, consisted of crude nalgliolaxin. The unsublimed residue consisted of colourless solid.

Proof that nalgiolaxin is not an artifact

When it became known that nalgiolaxin contains chlorine, but is otherwise closely related to nalgiovensin, it became necessary to prove that it is not formed as an artifact during the process of isolation. This was accomplished by the following method in which no reagents containing chlorine are used. The ground mycelium of *P. nalgiovensis* (160 g.), previously extracted with light petroleum, was extracted in Soxhlets with ether for 39 hr. The orange-yellow solid which separated (0.49 g.) was successively refluxed with small portions of fresh ether (15 × 50 ml.) leaving an undissolved residue (0.41 g.). A portion of this residue (0.10 g.) was dissolved in acetone (350 ml.) at room temperature and the solution was chromatographed on a MgCO_3 column (2.5 cm. diam.). A total band of 8.5 cm. was formed consisting of the usual top purple-blue band (0.4 cm.), followed by a red band (6.8 cm.) and then by a yellow band (1.3 cm.) (substance C). The column was washed with acetone (100 ml.), and was drained free from acetone. The red band was carefully separated mechanically and was acidified with 2N- H_2SO_4 . The mixture was heated on the water bath to coagulate the precipitated solid which was separated by filtration, washed and dried. It was then fractionally sublimed in a high vacuum as described above. The orange sublimate (0.04 g.) obtained at 190–200° melted at 243–244°, raised to 248–248.5° (0.03 g.) by crystallization from ethanol (40 ml.). It did not depress the melting point of pure nalgiolaxin (m.p. 248–248.5°) obtained as described above, where CHCl_3 was used as solvent and HCl was used for acidification of the MgCO_3 red bands.

Purification of nalgiolaxin

Crude nalgiolaxin, as obtained by the above isolation procedures, was easily purified by crystallization from ethanol. It was usually only necessary to crystallize twice in order to obtain material of constant melting point. In a typical example crude nalgiolaxin (0.37 g.), m.p. 241–244°, was crystallized from ethanol (500 ml.) and gave almost pure material (0.30 g.), m.p. 247–247.5°, raised to a constant m.p. 248–248.5° (0.25 g.) on recrystallization from ethanol.

From 572 flasks which gave 1645 g. of dried mycelium 2.91 g. of nalgiolaxin were obtained. This corresponds to 0.51 g. of nalgiolaxin/100 flasks or 0.18% of the weight of the mycelium. The average yield of nalgiovensin obtained during the isolation of nalgiolaxin was 1.61 g./100 flasks or about 55% of the total yield of nalgiovensin. The total yield of nalgiovensin obtained corresponds to about 1.0% of the weight of the mycelium.

General properties of nalgiolaxin. Derivatives

Nalgiolaxin is pleomorphic in nature and crystallizes from ethanol in small yellow plates and thin needles, m.p. 248–248.5°, without decomposition. (Found, on sample crystallized from ethanol and dried at 100° without loss in wt.: C, 59.8; H, 4.2, 4.3; N, S, nil; on sample sublimed in a high vacuum at 190–200°, CH_3O , 8.4; C-CH_3 , 4.0; Cl, 10.0. $\text{C}_{18}\text{H}_{18}\text{O}_6\text{Cl}$ requires C, 59.6; H, 4.2; $1\text{CH}_3\text{O}$, 8.55; 1C-CH_3 , 4.1; Cl, 9.8%.) It is dextrorotatory in CHCl_3 solution: $[\alpha]_{5790}^{25} + 40.3^\circ$ (c, 0.1072); the mercury-green line 5461 Å. is completely filtered out by the orange-yellow solution. It readily sublimes in a high vacuum at 190–200° without decomposition. It is less soluble in all organic solvents than is

nalgiovensin (q.v.). It does not dissolve in 0.5N- NaHCO_3 or in N- Na_2CO_3 except on standing, when a pale-red solution with a slight bluish cast is obtained. It dissolves readily in N-NaOH, giving a red solution with a slight bluish cast from which a purplish-red precipitate slowly separates. Its solution in cold conc. H_2SO_4 is red with a much bluer cast than that given by nalgiovensin. Its ethanolic solution gives an orange-brown colour with ethanolic FeCl_3 indistinguishable from that given by nalgiovensin and physcion. Its solution in glacial acetic acid is yellow in colour and does not fluoresce in daylight. Its CHCl_3 solution gives a red band when chromatographed on columns of MgCO_3 , the colour being a shade bluer than that given by nalgiovensin and physcion.

Although nalgiolaxin does not dissolve in cold aqueous N- Na_2CO_3 except on standing, it shows a characteristic reaction under the following conditions. Nalgiolaxin (about 2 mg.) was dissolved in CHCl_3 (5 ml.) and the yellow solution was vigorously shaken with aqueous N- Na_2CO_3 (5 ml.). On settling, the aqueous phase was light bluish-crimson in colour with a red precipitate at the interface. The CHCl_3 solution, which still remained yellow, was re-extracted a further four times with N- Na_2CO_3 , each time giving the same light bluish-crimson colour in the aqueous phase with little diminution of the yellow colour in the CHCl_3 solution. Under the same conditions nalgiovensin and physcion, in both of which the OH group in position 7 is methylated, gave only an extremely faint crimson colour in the aqueous phase with a yellow colour in the CHCl_3 lower layer, whereas emodin, ω -hydroxy-emodin and 4:5:7-trihydroxy-2-propylanthraquinone, in all of which the OH group in position 7 is free, gave a deep-red aqueous extract with a slight bluish cast and a colourless CHCl_3 lower layer.

Nalgiolaxin (0.05 g.) was dissolved in cold conc. H_2SO_4 (1.8 ml.) containing boric acid (0.13 g.). The intensely red solution was then slowly heated in an oil bath. The colour of the solution took on a bluer shade at 75°, was red-violet at 130° and permanganate-purple at 150–160°. It was held at this temperature for 0.5 hr. and was then cooled, poured on ice, diluted with water (100 ml.) and boiled for 10 min. After cooling, the mixture was extracted with CHCl_3 (100 ml.) yielding an orange-pink solution with a fine green fluorescence. On removal of the solvent, the resulting red solid gave the following colour reactions: with cold conc. H_2SO_4 , a red-violet solution with a slight red fluorescence; with cold aqueous N-NaOH or N- Na_2CO_3 , a blue-violet solution bleaching to colourless overnight; in glacial acetic acid, an orange-yellow solution with a fine green fluorescence. Catenarin (1:4:5:7-tetrahydroxy-2-methylanthraquinone), tested at the same time, gave colours and fluorescent solutions which were very similar to those just described, except that its solution in cold conc. H_2SO_4 was much bluer in tone.

Triacetylnalgiolaxin. Nalgiolaxin (0.27 g.) was acetylated with anhydrous sodium acetate (0.54 g.) and acetic anhydride (6 ml.) in the usual manner. The crude acetate (0.36 g.) was crystallized from ethanol. *Triacetylnalgiolaxin* forms thin lemon-yellow plates (0.29 g.), m.p. 186.5–187°. The same acetate, m.p. and mixed m.p. 186.5–187°, was obtained by acetylating nalgiolaxin in pyridine and acetic anhydride. (Found: C, 58.8; H, 4.3; CH_3O , 6.4; CH_3CO , 25.7; Cl, 6.9. $\text{C}_{24}\text{H}_{21}\text{O}_9\text{Cl}$ requires C, 58.95; H, 4.3; $1\text{CH}_3\text{O}$, 6.35; $3\text{CH}_3\text{CO}$, 26.4; Cl, 7.25%.) Triacetylnalgiolaxin is laevorotatory in CHCl_3 solution: $[\alpha]_{5461}^{20} - 7.7^\circ$; $[\alpha]_{5790}^{20} - 6.1^\circ$ (c, 0.9454).

Pure triacetylnalgiolaxin (0.155 g.) was hydrolysed with aqueous 0.1N-NaOH (100 ml.) in N_2 as described for the hydrolysis of triacetylnalgiovensin (see p. 568). The recovered nalgiolaxin, crystallized from ethanol and sublimed, had m.p. and mixed m.p. 248–248.5° with a specimen prepared by crystallization from ethanol (see above).

Nalgiolaxin dimethyl ether. A mixture of nalgiolaxin (0.32 g.), anhydrous K_2CO_3 (12.4 g.), dimethyl sulphate (3.2 ml.) and anhydrous acetone (550 ml.) was refluxed for 17 hr. The crude product (0.31 g.) was worked up as described for nalgiovensin dimethyl ether (see p. 568), and was crystallized from ethanol. *Nalgiolaxin dimethyl ether* (0.22 g.) forms thin yellow needles, appearing yellow-green in bulk, m.p. 206–207°. (Found: C, 61.5, 61.4; H, 5.2, 4.6; CH_3O , 23.8; Cl, 8.7. $C_{20}H_{19}O_6Cl$ requires C, 61.5; H, 4.9; $3CH_3O$, 23.8; Cl, 9.1%). It is dextrorotatory in $CHCl_3$ solution: $[\alpha]_{D}^{25} + 27.7^\circ$; $[\alpha]_{D}^{21} + 23.5^\circ$ (c, 0.4401). It sublimes in a high vacuum at 185–195°. It is insoluble in aqueous N-NaOH, but dissolves in cold conc. H_2SO_4 to an intense red solution with a slight bluish cast.

CrO₃ oxidation of triacetylnalgiolaxin

Triacetylnalgiolaxin (0.32 g.) was dissolved in a mixture of glacial acetic acid (6.4 ml.) and acetic anhydride (6.4 ml.) at 55–60°, and oxidized with CrO_3 (0.64 g.) dissolved in water (0.5 ml.) and glacial acetic acid (6.4 ml.). The oxidation procedure and recovery of the oxidation products were similar to those described for the CrO_3 oxidation of triacetylnalgiovensin (see p. 569). The acid fraction, extracted with aqueous 0.5N- $NaHCO_3$, was obtained as a yellow solid (0.02 g.), m.p. 240–241°. The neutral fraction (0.28 g.) remaining on removal of the $CHCl_3$ was obtained as a yellow oil, partially solidifying on standing, and was oxidized a second time yielding a second acid fraction (0.02 g.) and a neutral fraction (0.22 g.).

The combined acid fractions (0.04 g.) were crystallized from ethanol (5 ml.). *Diacetylchloroemodic acid 7-methyl ether* was thus obtained as yellow microplates (0.03 g.), m.p. 241–242°. (Found: C, 55.4, 55.6; H, 3.4, 3.3; Cl, 8.6. $C_{20}H_{13}O_6Cl$ requires C, 55.5; H, 3.0; Cl, 8.2%). It dissolves in 0.5N- $NaHCO_3$ to a reddish-yellow solution, whereas diacetylchloroemodic acid 7-methyl ether gives a pure yellow solution. On the other hand, their solubilities and colour reactions in N- Na_2CO_3 , N-NaOH and cold conc. H_2SO_4 are indistinguishable from each other.

The neutral fraction (0.22 g.) obtained from the second oxidation was crystallized from ethanol (125 ml.). The product (0.17 g.) was thus obtained as lemon-yellow plates and needles, m.p. 216–217°. (Found: C, 57.3, 57.3; H, 4.0, 4.1; CH_3O , 6.7. $C_{22}H_{17}O_6Cl$ requires C, 57.3; H, 3.7; $1CH_3O$, 6.7%). It differs from triacetylnalgiolaxin (m.p. 186.5–187°) in melting point and in the fact that it is optically inactive in $CHCl_3$ solution (c, 0.4337). It is insoluble in cold N-NaOH, gives no colour with ethanolic $FeCl_3$ and dissolves in cold conc. H_2SO_4 to a red solution with a very slight bluish cast.

Reduction of nalgiolaxin

A mixture of nalgiolaxin (0.18 g.), glacial acetic acid (5 ml.), red phosphorus (0.25 g.) and HI (1 ml., sp.gr. 1.7) was refluxed for 5 hr. The resulting anthranol was isolated as described in the reduction of nalgiovensin (see p. 571), and was also reduced a second time. The crude yellow anthranol

(0.135 g., m.p. 167–168°), dissolved in glacial acetic acid (150 ml.) at 60°, was oxidized to the corresponding anthraquinone with CrO_3 (0.13 g.) dissolved in water (5 ml.) and glacial acetic acid (5 ml.). The crude oxidation product (0.12 g.) was purified by crystallization from $CHCl_3$, followed by sublimation in a high vacuum at 170–175° and crystallization of the sublimate from benzene. It was thus obtained as characteristic long yellow needles (0.04 g.), m.p. 216.5–217°, not depressed on admixture with 4:5:7-trihydroxy-2-propylantraquinone (m.p. 216.5–217°) obtained by reduction of nalgiovensin. The two products also gave identical colour reactions. On acetylation in pyridine and acetic anhydride, 4:5:7-triacetoxy-2-propylantraquinone was obtained. It melted at 182–183°, not depressed on admixture with 4:5:7-triacetoxy-2-propylantraquinone (m.p. 182–183°) obtained from nalgiovensin.

Other products extracted from the mycelium of Penicillium nalgiovensin

As mentioned in previous sections the mycelium of *P. nalgiovensin* contains three other substances, A, B and C, which can be extracted with solvents. None of these substances has been characterized, but the following is a summary of what is known of them at present.

Substance A. This substance (see p. 571) separated from the boiling $CHCl_3$ solution and is present in considerable amounts, averaging about 5 g./100 flasks. It crystallizes from glacial acetic acid as fine colourless microneedles which sublime readily in a high vacuum at 250–260°, and on heating in a capillary tube sublime at about 300° without melting. It is only slightly soluble in glacial acetic acid, $CHCl_3$, acetone, benzene, ethanol and ethyl acetate. It is insoluble in water, 2N-HCl and 2N-NaOH. It gives no colour with ethanolic $FeCl_3$.

Substance B. This substance is a colouring matter which is present in the top purple-blue band during chromatography on $MgCO_3$ of the $CHCl_3$ extract (see p. 571). The crude colouring matter recovered by acidification of the separated purple-blue band gives the following colour reactions. It dissolves in cold conc. H_2SO_4 giving an intensely coloured solution which appears red-violet by transmitted light and blue-violet by reflected light. It readily dissolves in N- Na_2CO_3 and N-NaOH, giving in each case a red-violet colour. Its solution in glacial acetic acid is orange-yellow in colour and does not fluoresce in daylight. It appears to be quinonoid in nature and may be a polyhydroxyanthraquinone.

Substance C. This substance is present in the bottom yellow band during chromatography on $MgCO_3$ of the ether extract (see p. 572), and is present in only very small amounts. The yellow band begins to change colour quickly on exposure to air passing through brown to purple. The crude material recovered by immediate acidification of the separated yellow band gives the following colour reactions. It dissolves in cold conc. H_2SO_4 giving a greenish solution which becomes an intense dark green on standing. It is insoluble in N- Na_2CO_3 but readily dissolves in N-NaOH to a yellowish solution which, on shaking, quickly changes colour giving finally a red solution with a slight bluish cast. Substance C appears to be an anthranol, probably of the emodin-anthranol type, since a specimen of 4:5:7-trihydroxy-2-propylantraquinone behaves very similarly on chromatography on $MgCO_3$ and gives almost identical colour reactions (see p. 571).

SUMMARY

1. Two hitherto undescribed colouring matters, *nalgiovensin* and *nalgiolaxin*, have been isolated from the mycelium of laboratory cultures of *Penicillium nalgiovensis* Laxa.

2. Nalgiovensin, $C_{18}H_{16}O_8$, crystallizes in orange needles or plates, m.p. 199–200°. It has been shown to be *dextro*-4:5-dihydroxy-7-methoxy-2-hydroxypropylanthraquinone in which the nature of the hydroxypropyl group, whether $-\text{CHOH} \cdot \text{CH}_2 \cdot \text{CH}_3$, $-\text{CH}_2 \cdot \text{CHOH} \cdot \text{CH}_3$ or $-\text{CH}(\text{CH}_3) \cdot \text{CH}_2\text{OH}$, is at present undetermined.

3. Nalgiolaxin, $C_{18}H_{15}O_8\text{Cl}$, crystallizes in yellow needles or plates, m.p. 248–248.5°. It has been shown to be a monochloroderivative of nalgiovensin and is believed to be *dextro*-1(or 8)-chloro-4:5-dihydroxy-7-methoxy-2-hydroxypropylanthraquinone.

4. Nalgiolaxin is believed to be the first example of a derivative of anthraquinone containing chlorine which has been recorded from any natural source.

5. Nalgiovensin and nalgiolaxin are both dextro-rotatory in chloroform solution and are of unusual interest since optically active, naturally occurring, derivatives of anthraquinone are rare except those which occur as glycosides.

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The Influence of a High-Sucrose Diet on the Calcium and Phosphorus Percentage of the Rat Femur, and a Comparison with its Effect on the Enamel and Dentine of the Rat Incisor Teeth

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The enamel and dentine of the incisors of rats maintained on a diet containing 67% sucrose contained significantly higher percentages of calcium and phosphorus than the same tissues of animals maintained on a stock diet of Lever cubes (Hartles, 1951). The calcium/phosphorus ratios on the high-sugar diet were, however, significantly lower than those on the stock diet showing that there was a relatively greater increase in phosphorus than in calcium.

The left femur of each of these animals was removed at the same time as the teeth and kept for subsequent analysis. The work reported here is concerned with the effect of the high-sugar diet on the percentage of calcium and phosphorus in these bones, and includes a comparison with the effect on the incisor teeth. The results show that in the femur there is no significant difference in the calcium and

phosphorus percentage or the calcium/phosphorus ratios on the two diets. The high-sugar diet, therefore, affected the incisor teeth but not the bone.

EXPERIMENTAL

Animals and diet. The animals and diets were those used in the tooth study (Hartles, 1951): group I, twenty-two animals maintained on a diet of Lever rat cubes; group II, twenty-seven animals weaned at 21 days on to the diet containing 67% sucrose.

The animals were killed with ether, the left femora removed at the same time as the teeth and placed in absolute ethanol for 48 hr. The atomic Ca/P ratio of the stock diet was 1.36 and of the high-sugar diet 1.35.

Treatment of bones. Each femur was stripped of adhering tissue, broken into small fragments, wrapped in a small filter-paper parcel and placed in absolute ethanol overnight.