appear that the size of the molecule has no bearing upon its antiviral activity. The variation in amino acids upon hydrolysis of samples of material from some without demonstrable cysteine to those having as high as 2% and with molecular weights from 700-30,000(7) indicates that the activity may be due to an active component so far unidentified. Analytical studies of the carbohydrate content of the active fraction indicated in most instances yields which ranged from 20-25%. In earlier experiments in which different methods of isolation were used, active antiviral fractions were obtained with one-fourth the carbohydrate content. These findings suggest that the carbohydrate is not involved in the antiviral activity of the material. Differences in carbohydrate content may reflect differences in quantitative yields of the active component in the antiviral material. The main function of the mucoprotein appears to be that of serving as a carrier for the active component. Studies are currently in progress to determine the molecular configuration of the active group.

Summary. An antiviral thermostable substance (paolin II) is isolated from oysters by extraction with acetic acid followed by fractional precipitation with ethanol. The substance shows one peak on ultracentrifugation, has a sedimentation constant of 1.6-1.8 and a molecular weight of approximately 10,000. The material yields 16 amino acids on hydrolysis with a composition of 8.6% nitrogen and 23.4% carbohydrate, indicating the mucoprotein nature of the substance.

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Identification of Isomers Differing from 9,*a*, in the Early Labelled Bilirubin of the Bile.* (31515)

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The "early labelling" of the fecal stercobilin was first demonstrated by London and associates(1). This subject has recently been reviewed with particular reference to the basic

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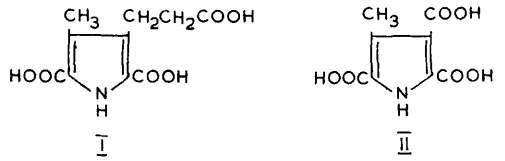

FIG. 1. Pyrrolic dicarboxylic acids I and II obtained by $KMnO_4$ oxidation of bile pigments and porphyrins.

significance of the early labelled fraction, especially the erythropoietic and non-erythropoietic components(2), and the question whether any of this material represents anabolic or "primary shunt" bile pigment in contrast to the 9,α type derived by catabolism of heme. It is conceivable that a bilirubinoid substance might be formed from an excess of the polypyrryl methane generally postulated as intermediary between porphobilinogen and uroporphyrinogen in which event an isomer other than 9,α would be anticipated.

In an earlier paper(3) it was shown that coupled oxidation of hemin *in vitro* results in random opening of the porphyrin ring at β-, γ- and δ- as well as α methene bridges, unlike the formation of bile pigment *in vivo,* in which the enzymatic oxidation of heme is highly specific for the α-bridge, and the resulting bilirubin a 9,α isomer. More recently ^{14}C-labelled bilirubin was isolated from rat bile on the third day after administration of ^{14}C-2-glycine. This bilirubin was shown to contain an isomer other than 9,α.

In these studies the ^{14}C-bilirubin was oxidized with potassium permanganate in weakly alkaline solution according to Nicolaus'(4) method. This method permits detection of isomers other than 9,α by virtue of the appearance of α,α'-dicarboxylic pyrrolic acid II which the 9,α isomer does not yield. As noted in Fig. 1, acid I alone is obtained by $KMnO_4$ oxidation of the latter, the presence of acid II clearly indicating that the sample contained another isomer.

In the present work bilirubin was obtained from a dog with a bile fistula after intravenous administration of ^{14}C-4-δ-aminolevulinic acid (^{14}C-ALA). This bilirubin was isolated in the course of a separate study(5). The samples employed in the present study were collected 1-2 hours (period 1) and 4-6 hours (period 2) after administration of the ^{14}C-ALA. The bilirubin was crystallized and recrystallized and the specific absorbance was 58,000. The specific activities of the 2 samples, respectively, were 7.7×10^5 dpm/mg and 50×10^5 dpm/mg and an amount of 0.9 mg of each was used for the present oxidation.

The final products of oxidation were subjected to paper chromatography in butanol: glacial acetic:water (4:1:5) and ethanol:ammonia:water (20:1:4)(6). Active spots were detected on the strips with a Nuclear-Chicago radioactivity scanner and by autoradiography. The latter method gives much more precise locations and shapes of active spots. Control runs were made with non-radioactive synthetic pyrrolic acids I and II and the oxidation products of protoporphyrin 9 and commercial crystalline bilirubin (Nutritional Biochemicals Corp., Cleveland, Ohio) so that the R_f's of corresponding spots could be observed by means of the usual diazo reaction(4). For further confirmation ^{14}C-acids I and II were reextracted and run with the second solvent system as given above. Finally, similar oxidation and chromatography was carried out with ^{14}C protoporphyrin (sp. act. 47×10^6 dpm/mg, amount 0.12 mg) to permit observation of R_f of acids I and II by radioautography. R_f's observed by radioautography after oxidation of the ^{14}C bilirubin and in the control runs are shown in Table I.

In most cases R_f values differ slightly, due to various amounts of pigments used for oxidation, variations in temperature, etc. In those cases the general pattern of active spots must be taken into consideration. In any case, spots were reextracted and run again.

The presence of pyrrolic acids I and II (Table I) among products of ^{14}C-bilirubin oxidation with $KMnO_4$ reveals that the "early labelled" bilirubin is not exclusively the 9,α isomer but consists of a mixture of isomers.

A similar experiment has been carried out with commercial bilirubin. Since about 10-15% of natural bilirubin is of the "early labelled" type(1), *i.e.,* from sources other than destruction of mature red cells at the end of their life span, one might expect that if

TABLE I. R_f Values of Oxidation Products of Bilirubin and Controls.

Compounds subjected to $KMnO_4$ oxidation		R_f values after chromatography in 2 solvent systems: Alcohol : Ammonia : Water 20 : 1 : 4		n-Butanol : Acetic acid : Water 4 : 1 : 5	
^{14}C-bilirubin	Period 1	.06 (I—.03)*	.31 (II—.30)	.34 (II—.35)	.85 (I—.82)
	Period 2	.05 (I—.03)	.28 (II—.28)	.41 (II—.40)	.80 (I—.81)
Hemin		.03	.26	.40	.81
Bilirubin	30 mg				.74-.81†
	200 "			.09-.15	.60-.83
Hemin				.13-.26	.71-.78

* R_f values for acids I and II. † R_f values of upper and lower boundaries of spots.

isomers other than 9,α occur, they might be demonstrable if a sufficiently large amount were oxidized. Ordinarily the Nicolaus method has been applied to amounts of 5-40 mg. With such quantities of bilirubin no isomers other than 9,α were detected(7). In the present experiment 200 mg of recrystallized commercial bilirubin was used (specific absorbance 57,000). Because most of this is the 9,α isomer, the amount of acid I is extremely large in comparison with acid II, and the chromatogram has an unusually large and a much smaller spot corresponding to acids I and II, respectively (Table I). Because of this disproportion, the R_f value for acid II is smaller but the characteristic color of the spot after application of the diazo reagent confirms the presence of acid II.

Application of the $KMnO_4$ method to protoporphyrin gives two spots of almost equal size, as would be anticipated with random opening of methene bridges and correspondingly equal amounts of acids I and II.

Two possibilities may be considered to explain the presence of an isomeric bilirubin as revealed by the small amount of acid II thus demonstrated: 1) a direct synthesis or anabolic sequence from PBG bypassing uroporphyrinogen III and protoporphyrin 9 (III), as mentioned at the outset; 2) a random removal of methene bridges from protoheme 9, in small proportion, possibly occurring only in hemes unrelated to hemoglobin, as in the heme enzymes of the liver, such as catalase, tryptophane pyrrolase or cytochromes. The remarkably early appearance of the label after administration of the ^{14}C-glycine would be in accord with either possibility, as discussed by Watson(2).

Conclusions. 1. ^{14}C-bilirubin, isolated from bile of a dog within the first few hours after administration of ^{14}C-ALA, on oxidation with $KMnO_4$ yields a significant amount of α,α'-dicarboxylic pyrrolic acid I as well as II, thus revealing that this early labelled bilirubin contains a fraction of an isomer other than 9,α, the principal bilirubin of the bile. 2. Similar oxidation of large samples of commercial bilirubin (>200 mg) also produces acid I, although in very small proportion with II, thus indicating presence of an isomer other than 9,α, in a correspondingly small proportion of the total bilirubin of the bile.

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