related to the ease of formation of multiple intramolecular hydrogen bonds in the αi-somer (see, e.g., Knell et al., 1975), causing efficient shielding of the polar character of the carboxyl groups and of the NH-CO groups of the 'outer' pyrrolenone rings. Such bonds cannot be formed for the non-α-isomers. The introduction of a polar group in bilirubin-IXα would thus be required to open the hydrogen-bonded structure and to acquire the amphiphilic character appropriate for biliary excretion.


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**The Fate of Bilirubin-IXα Glucuronides in Cholestatic Bile:**

**Sequential Migration of the 1-Acylaglycone to the 2-, 3- and 4-Positions of Glucuronic Acid**

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Major pathways for excretion of bilirubin-IXα in mammals involve esterification of one or two propionic acid side chains with UDP-glucuronic acid to form 1-acyl-glucuronides. Diazonium cleavage at the central methylene bridge of the tetrapyrrole moiety produces a pair of dipyrrolic azopigments, but does not affect the ester linkage. Further azopigment derivatives can be prepared by reactions of the glucuronic acid moiety, i.e. methyl ester formation with diazomethane and further acetylation or silylation of the free hydroxyl groups.

When prepared from fresh normal bile, the above-mentioned azo derivatives are homogeneous on t.l.c., except for the existence of two isomers with interchanged methyl and vinyl groups on the azodipyrrrole moiety. The two isomers can be separated only after the final acetylation step.

By contrast, conjugated azopigments and their methyl esters, when prepared from cholestatic bile of man and from obstructive rat bile, separate into four main bands. Significantly, when fresh normal bile is kept at 37°C under an N2 atmosphere for 1–6h, diazonium cleavage reveals the same four azopigments formed in sequential order.

At this stage we advanced the hypothesis of sequential migrations of the 1-acyl-aglycone to other positions of glucuronic acid. Migration of acyl groups in carbohydrates via intramolecular rearrangement has been observed frequently (Bonner, 1959) and most readily occurs at slightly alkaline pH values (pH 7.9). Structures for the methyl esters are shown in Scheme 1. The azopigment moving as band 3 derives from the original bilirubin 1-acylglyco.pyranuronoside. The first transformation involves 1→2 acyl migration, yielding a 2-acylglucuronide (band 1). Further sequential 2→3 and 3→4 migrations give rise to bands 2 and 4, which are 3-acyl- and 4-acyl-glucuronides.

The conversions discussed here occur not only in bile (pH about 7.8), but also in buffered media (pH 7.9) for isolated bilirubin glucuronides and for azopigment glucuronides and their methyl esters.
Structures of azopigment methyl esters

R is the azodipyrrole moiety. The esters are numbered 1, 2, 3 and 4 in the order of decreasing mobility on t.l.c.

Molecular ions in the mass spectra of the four acetylated ($M^+ 778$) and silylated methyl esters ($M^+ 868$) reveal the presence of a set of isomeric compounds. In particular, the ion $[Si(CH_3)_3O-CH-CH-OSi(CH_3)_3]^+$ ($m/e$ 204, base peak), encompassing carbon atoms 2 and 3 of the carbohydrate moiety, at once reveals the 4-acyl structure for the trimethylsilyl derivative of material from band 4.

Ammonolysis of the azopigment acids and methanolysis (2M-HCl in methanol) of the methyl esters yields glucuronic acid as the only sugar identified by t.l.c. and g.l.c.-m.s. (mass spectrometry).

The pyranose structure for the acetylated azopigment methyl esters was established as follows. Treatment of the 1-acyl acetate derived from band 3 with HBr and acetic acid yielded 1-bromoglucopyranuronic acid methyl ester triacetate. The 1-bromo sugar was converted into the 1-methoxy derivative by treatment with methanol and $A_2O_3$. This procedure yielded a single carbohydrate derivative characterized by g.l.c.-m.s.

A more general approach, applied to the four acetylated methyl esters, consisted of treatment with HBr and acetic acid, followed by reduction with LiAlH$_4$ or LiAlH$_4$. In each case the 1,5-anhydroalditol of glucose was obtained and was characterized by g.l.c.-m.s. as the 2,3,4,6-tetra-acetate. This result proves the pyranose ring structure of the acetylated azopigment esters. Indeed, the same reactions performed for the reference compounds $\beta$-glucopyranuronic acid methyl ester tetra-acetate and $\alpha$-glucofuranuronic acid methyl ester tetra-acetate yield the 1,5-anhydroalditol and the 1,4-anhydroalditol of glucose respectively.

For the 2-, 3- and 4-acylglycuronides there remained the difficult problem of determining the site of attachment of the labile acyl group. Several reaction sequences, aimed at replacing the acyl substituent with a methyl group, were tried on the methyl esters (from bands 1, 2 and 4). One of the more successful sequences is the following.

1. Free hydroxyl groups are protected as the acetals, formed by reaction with ethyl vinyl ether and trifluoroacetic acid. (2) NH$_3$/methanol removes the acyl group and converts the CO$_2$CH$_3$ ester group into a CONH$_2$ amide function. (3) Free hydroxyl groups and the amide function are methylated with dimethyl sulphoxide anion and methyl iodide. (4) The acetal groups are hydrolysed in aqueous acid medium. (5) Alditol derivatives are prepared by reduction with NaBH$_4$ or NaB$_4$H$_4$. (6) The partially methylated alditol derivatives are acetylated.

T.l.c. of the azopigments obtained after reaction (1) and g.l.c.-m.s. of the final reaction products revealed that acetal formation of the free hydroxyl groups,
Table 1. Dimethyl derivatives of alditol acetate identified and structures assigned to methyl ester bands 1, 2 and 4

General structure of the alditol is:

\[
(\text{H})\text{H-CH(OAc)-CH(OR)-CH(OR')-CH(OR")-CH(OAc)-CON(CH}_3)_2
\]

where Ac represents an acetyl group and R, R', and R" represent either an acetyl or a methyl group.

<table>
<thead>
<tr>
<th>Azopigment methyl ester</th>
<th>Structure assigned</th>
<th>Dimethyl alditols identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Band 1</td>
<td>2-Acylglucuronide</td>
<td>2,3-Dimethyl and 2,4-dimethyl</td>
</tr>
<tr>
<td>Band 2</td>
<td>3-Acylglucuronide</td>
<td>2,3-Dimethyl and 3,4-dimethyl</td>
</tr>
<tr>
<td>Band 4</td>
<td>4-Acylglucuronide</td>
<td>2,4-Dimethyl and 3,4-dimethyl</td>
</tr>
</tbody>
</table>

other than that in 1 position, does not go to completion. For each acylglucuronide this mainly results in a mixture of two dimethyl derivatives instead of a single monomethyl derivative of alditol acetate. However, as shown in Table 1, unique assignment of structures to the acylglucuronides remains possible.


New Data on Halogenoalkane-Induced Lethality

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Effects of Illumination of Whole Barley Plants on the Protochlorophyllide-Activating System in the Isolated Plastids

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The terminal reaction of chlorophyllide a formation in higher plants is the photoreduction of protochlorophyllide. A prerequisite for this reaction to occur is that the protochlorophyllide must be present as a component of a photoactive pigment–protein complex. The latter, still retaining a high degree of photoconvertibility, and designated the protochlorophyll holochrome, has been widely isolated (Smith & Kupke, 1956; Boardman, 1962; Schopfer & Siegelman, 1968). Work from our laboratory (Griffiths, 1974, 1975b) has demonstrated the formation in *vitro* of a photoactive complex from added protochlorophyllide by isolated etioplast membranes, the process being dependent on added NADPH. This process occurs in the dark and is accompanied by a shift in the wavelength of the pigment from approx. 630 nm in the non-active state to a form absorbing maximally at 638 and 652 nm in the photoactive complex, \( \text{P638/652} \). Illumination of the latter gives rise to chlorophyllide, followed by reformation of further \( \text{P638/652} \) if a supply of protochlorophyllide and NADPH is available. Under these conditions the rate of flash-induced chlorophyllide formation provides a measure of the activity of the protochlorophyllide-activating system, an assay that we have used to study some properties of the activation process (Griffiths *et al.*, 1976). The present report describes some unexpected results that we have obtained while assaying the protochlorophyllide-activating system in plastids isolated from illuminated barley.