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The Aldehyde Reaction with Bilirubin of Bile and of Bile Pigment in the Icteric Liver

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Summary. Golden brown bile is promptly turned green, even under oil, by addition of various aliphatic and aromatic aldehydes: formaldehyde, paraldehyde, chloral hydrate, isobutyraldehyde, glyoxal, glyoxalic acid, glutaraldehyde, benzaldehyde, *p*-dimethylaminobenzaldehyde and vanillin. In the case of formaldehyde this color change is accompanied by spectroscopic alterations and by a decrease in bilirubin assay by the Malloy-Evelyn technic. Brown yellow icteric liver tissue is similarly colored green by immersion in formaldehyde, paraldehyde, chloral hydrate, benzaldehyde and *p*-dimethylaminobenzaldehyde. This color is returned to yellow by reducing agents: sodium bisulfite or thiosulfate, ferrous salts, acetone and alcohols: methyl, ethyl and ethylene glycol. Thus reduced tissue is again colored green by reexposure to aliphatic and aromatic aldehydes. This color cycle may be repeated at least three times. Formaldehyde also oxidizes ferrous salts to ferric, as well as reducing ferric salts to ferrous, incompletely in both instances, so that ferroso-ferric mixtures result. On weighing of ferrous ferricyanide precipitates an oxidation of about 16% of a ferrous sulfate solution and a reduction of 30% of a ferric chloride solution was indicated. These two salts respectively reduced biliverdin to bilirubin and oxidized bilirubin to biliverdin in reported histochemical studies.

Introduction

That the mucosa of the gallbladder and the icteric liver and kidney assume a green color after formol fixation is common knowledge among pathologists, yet it is seldom mentioned in the current textbooks (Baker, 1961; McManus and Mowry, 1960). McManus found it difficult to understand how a reducing agent like formaldehyde could effect the oxidation of bilirubin to biliverdin. Blum (1903), summarizing reports after his (1893) introduction of formol fixation, did not mention the green coloration of icteric liver or gallbladder mucosa. Nor does Hanser (1930) mention it in the: Henke-Lubarsch, Handbuch der speziellen pathologischen Anatomie.

No record of an aldehyde reaction with bilirubin was found in Lemberg and Legge's monograph (1949), or in that of With (1968).

Material

Material utilized in this study was strongly icteric livers, fixed for histologic and histochemical studies reported elsewhere (1967, 1968, 1969a, b, 1970), derived largely from the necropsy service of the U.S. Veterans Administration Hospital. As noted in those studies neutral 10% formol fixation was used in all cases, in many one in mixtures of methanol and chloroform partly at 60° C, in others anhydrous acetone, an equal volume ethanol ether mixture, 80% ethanol and others. Fresh bile was obtained from normal gallbladders at necropsy and solutions of bilirubin were also used on occasion. Bilirubin assays were done in the Veterans Hospital laboratory by a standard Malloy-Evelyn technic. Spectra were recorded on a Beckman DB recording spectrophotometer.

Experimental and Observations

Our experience confirmed the reports of McManus and Mowry (1960) and of Baker (1961). With these multiple fixations, icteric liver blocks placed in 10% formol were generally green after 2—3 days, those fixed in acetone, in methanol+chloroform, in alcohol+ether, 80% alcohol and similar fixatives usually remained grossly golden brown. Some blocks fixed in 10% formol containing 5% sodium thiosulfate were green at 48 hours, others yellow brown. Deep green coloration of the liver *is* sometimes present in the fresh state, and a few blocks were gray green after 24 hours in acetone or methanol+chloroform.

Some biliverdin might be natively present in these green formol fixed livers, its color masked in the fresh state by the presence of red blood. The color of blood changes from red to yellow brown on formol fixation and this color change should unmask the green of biliverdin. The alternative was that in some manner formaldehyde changed bilirubin to biliverdin or some closely related substance.

To eliminate the factor of blood color, the action of formaldehyde was tested on fresh gallbladder bile from a routine autopsy not involving hepatic disease. A mixture of 1 cc bile, 8 cc distilled water and 1 cc 37% formaldehyde began to show a greenish color within an hour, and by 3 days had acquired a dark green color, without sediment. A control 10% aqueous dilution of 1 cc of the same bile sample remained golden yellow for at least a week at 25° C. Similar samples, kept under mineral oil remain golden yellow to orange for at least 10 weeks. The test tubes were about $^2/_3$ full and were closed with rubber stoppers.

Recalling the reactivity of *p*-dimethylaminobenzaldehyde with the active $=CH_2$ group of the indolenine tautomers of the indoles, it was thought that the central CH_2 bridge of bilirubin, situated also between unsaturated, essentially aromatic linkages might be similarly reactive according to the suggested formulation below in Fig. 1, proceeding from bilirubin (II) through a methylene bilirubin (III) to a methyl biliverdin (IV) which is again reducible to a methyl bilirubin (V).

To check further on this possibility ten aldehydes were reacted with diluted bile from a normal gallbladder. Bile was added in 1 cc quantities to 9 cc distilled water dilutions of 1 cc 37% formaldehyde[1] (400 mg HCHO or 1.33 *M* concentration), 2.2 g and 220 mg chloral hydrate (1.33 *M* and 133 *mM*), 2 cc paraldehyde U.S.P. (about saturated, 1.988 g/10 cc, 1.54 *M*), 5 cc 25% glutaraldehyde[2] (1.25 g/10 cc=1.25 *M*), 1 cc benzaldehyde[2] +2 cc ethanol (about 0.95 *M*), 149 mg (1 mmol) *p*-dimethylaminobenzaldehyde[2] with 0.1 cc 12 *N* HCl and 1 cc ethanol (0.1 *M*), 152 mg vanillin[2] with 2 cc ethanol (0.1 *M*), 1 cc isobutyraldehyde[2] (*ca.* 600 mg/10 cc = 1.03 *M*), 2 cc 40% glyoxalic acid[2] (ca. 1 *M*), 2 cc 30% glyoxal (*ca.* 600 mg/10 cc = 1.03 *M*), and control distilled water, all to final 10 cc volumes.

Immediate moderate greenish reactions, as compared with the control, appeared with all but one of the aldehydes. *p*-Dimethylaminobenzaldehyde formed a bulky brick red curdy precipitate filling the fluid. By 24 hours this separated into a clear red supernatant fluid and a dark green precipitate. The formaldehyde, chloral hydrate and vanillin mixtures formed no 24 hour precipitates, remaining deep green, deep green and yellowish green respectively. The isobutyraldehyde, the paraldehyde, the glyoxal and the glyoxalic acid mixtures also

1 Baker analyzed with 11.8% methanol

2 Eastman Distillation Products Co.

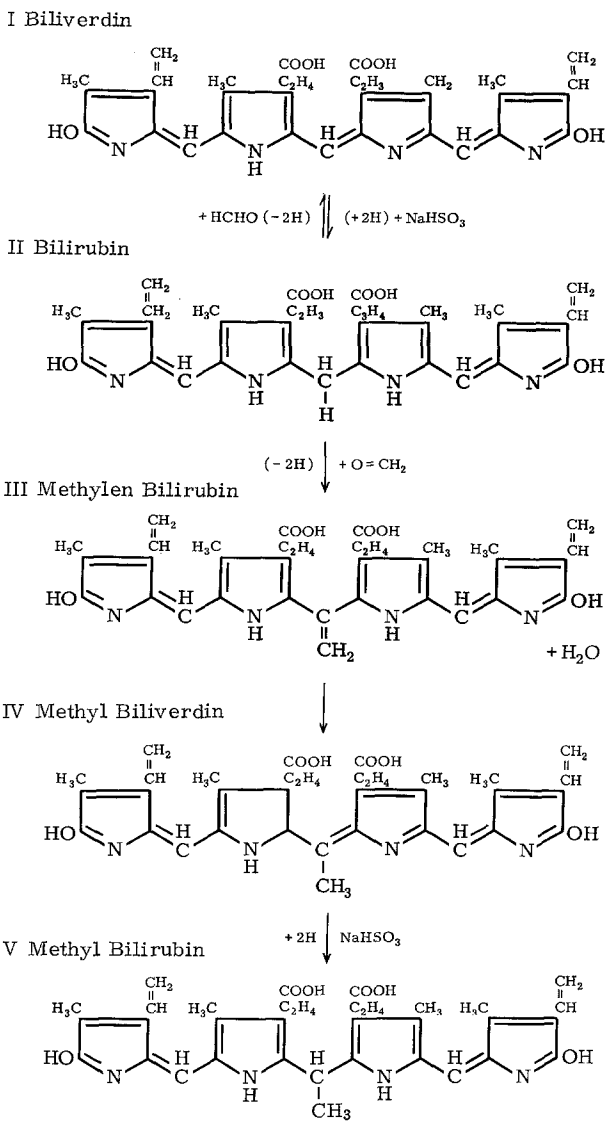

Fig. 1. Formulation of proposed formaldehyde bilirubin condensation reaction to form a hypothetical methyl biliverdin and reduction to a methyl bilirubin

formed greenish black to deep green precipitates with greenish yellow, deep green, faintly greenish yellow and clear yellow supernatants in that order. Glutaraldehyde returned to brown yellow fluid and brown orange precipitate, while benzaldehyde yielded a green fluid and greenish sediment.

Except glutaraldehyde, all the aldehydes tested yielded green precipitates or green fluids, suggesting that the previously reported green coloration of bile pigment by formaldehyde is a more or less general aldehyde reaction.

Confirming the results of Tiedemann and Gmelin (1826) the untreated bile control turned green in a few days. But addition of 1% sodium thiosulfate or 0.2% sodium metabisulfite to fresh bile prevented the color change for 28 days, even with air exposure.

A parallel test was conducted on "purified bilirubin" as furnished for standardization of the Hijmans van den Bergh reaction. It was found that the untreated distilled water control also yielded a dark green precipitate only slightly less in amount than with formaldehyde. It was found that the stock 1% solution in 1 *N* $NaHCO_3$ required a 3 day treatment with an equal volume of 0.2 *M* $Na_2S_2O_3$ to change precipitate color from dark green to dark brown, with a deep yellow supernatant. Paraldehyde similarly reduced the initial green precipitate to yellow in a brown yellow supernatant. No red color was seen at any stage with *p*-dimethylaminobenzaldehyde. The glutaraldehyde precipitate remained dark green up to 8 days, in contrast to its behavior with fresh bile.

In view of the ready oxidation of bilirubin demonstrated in our tissue sections (1968), and the rather laborious preparation method involved in the isolation of bilirubin, it did not seem worthwhile to attempt the preparation of a sample of reduced, biliverdin free bilirubin for the repetition of these tests.

Total Bilirubin estimations by a standard Malloy-Evelyn technic were done on 13 samples, without and with additions of 0.5, 1 and 2 cc formol to 10 cc aliquots of 10% bile. Some samples presented a pronounced decrease of bilirubin in formol treated samples, others showed little significant alteration. Averages for the 13 samples were 6.77 mg/100 cc for the control, 4.66, 4.74 and 4.34 for the samples receiving 0.5, 1 and 2 cc formol respectively. In a total of 13 assays on samples of high total bilirubin content direct assays were also done. These averaged 70.6% of the total bilirubin.

Spectroscopically, fresh human bile yields a broad absorption band between about 380 and 500 mμ, whose maximum is a plateau at 410—430 mμ. Addition of formaldehyde occasions a relatively small decrease in absorption in the 400—450 area, a fairly pronounced decrease in the 450—500 zone with appearance of a shoulder or even a secondary peak at about 480, and a quite moderate second absorption band in the 590—600 area. The presence of absorption bands in the violet (410) and red orange (590—600) areas is usual for green dyes in solution.

Fig. 2 illustrates the effect of addition of graded doses of 40% formaldehyde in 10% fresh human bile at 24 hours reaction time. Curve *0* is that of the bile sample run at once after dilution, Curve *1* is the same solution at 24 hours to illustrate that alterations on standing are relatively slight. Curves *2*, *3* and *4* show the effect of inclusion of 0.5, 1 and 2 cc formol in the 10 cc dilute bile. During the 24 hour reaction period all specimens were protected from air by a 2—3 cm layer of mineral oil. Final dilutions of 1:250 were made for spectroscopy.

Changes on air exposure of diluted fresh bile have on occasion presented two peaks at about 400 and at about 480 mμ, with a deepening depression between as aging progresses. This again indicates passage of more blue and blue violet, so that the previous orange yellow changes to green.

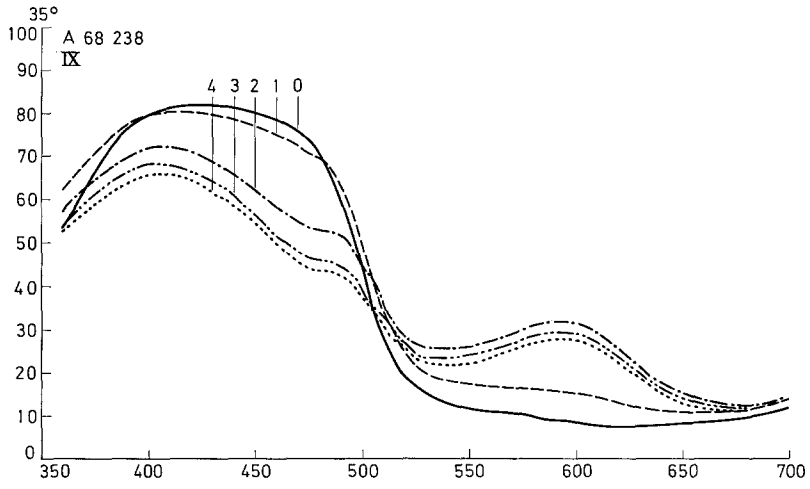

Fig. 2. —*0* Fresh bile, — — *1* No Formol, — · *2* 0.5 cc Formol, — · · *3* 1.0 cc Formol, · · · · *4* 2.0 cc Formol

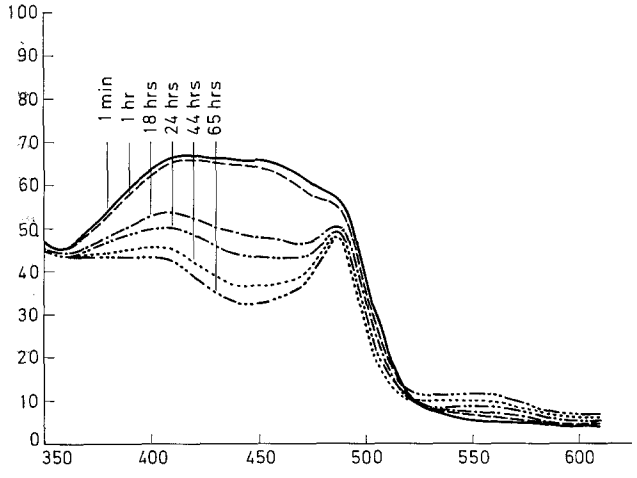

Fig. 3. Standard bilirubin solution effect, of air oxidation

The control 10 mg/100 cc hydromethanolic bilirubin solutions exposed to air 1 min, 1, 12, 24, 44, 65 and 89 hours (Fig. 3) presented an increasing depression in the 450 area so that a fairly sharp peak appeared at about 487 mμ and a broader vaguer one at about 400—410, diminishing at 65 hours almost to a mere shoulder.

Gross Color Changes in Fixed Tissue. Since it was not readily practical to study the effect of alternate oxidation and reduction in solution, and as the primary topic of investigation was the gross color changes produced in liver tissue, the bulk of the investigation was conducted on blocks of strongly icteric liver tissue fixed primarily in formol and in formol free fixatives. Here the removal

of the previous reagent, washing, and substition of a second reagent are simple and the effects on gross color are readily followed.

Tissue blocks previously fixed 24 hours in anhydrous acetone and in equal volumes of ethanol and ether were yellow to brown and both fixing solutions were light to moderate yellow in color, indicating some extraction of bile pigments. Blocks were transferred to neutral phosphate buffered 10% formol about 10 cm deep in a 15 cm test tube. In one such tube the surface of the formol was exposed to air, in a second it was covered by a 3 cm layer of mineral oil. Control blocks remained in the original fixative in a similar tube closed with a rubber stopper. The control block in acetone remained yellow brown for 3 months. The control alcohol ether block slowly assumed a greenish yellow color and the covering solvent also changed to greenish yellow. The four blocks transferred to formol, both those under oil and those exposed to air assumed a green color in about 5 hours, this deepened to deep green by the next day. No further color change occurred in 3 months and the formol solutions remained uncolored.

Histologic examination of such material did not prove profitable, colors being too faint on a histologic level for significant evaluation.

In another experiment blocks of 3 mottled dark green formol fixed livers were stored 4 months in full stoppered tubes in 10% formol (control), in 0.2 *M* $Na_2S_2O_3$, in 70% alcohol, in acetone and in 0.1 *M* ferrous sulfate in water and in 70% alcohol. The blocks stored in formol remained green, on transfer for a 24 hour period to 0.2 *M* (5%) sodium thiosulfate their color changed to deep to pale yellow. Blocks stored in $FeSO_4$ solutions, in 70% alcohol, in acetone or in thiosulfate were pale greenish yellow or yellow, and on retransfer to 10% formol for 24 hours again turned green.

In further series, green formol fixed liver blocks (8018) were subjected to alternating changes of 0.2 *M* $Na_2S_2O_3$ and 10% formol (phosphate buffered to pH 7), allowing each to act until a definite color change appeared, leaving one block behind in each fluid change while moving the remainder on. Reduction by thiosulfate on the first round was quite slow, requiring about 7 days to reach a light still very faintly greenish yellow. The green color was restored by formol and tissues were left there 6 days over the holidays. Transfer to 2% sodium metabisulfite gave an appreciable lightening of the green color in 2 hours, and by 18 hours blocks were bright yellow. Two hours washing in 4 changes of distilled water and retransfer to 10% formol gave light green mottling on pale greenish yellow in 2 hours and in 24 hours the blocks were essentially equal in color to the original green formol stored control. Little further change was detected on longer formol storage. A third cycle of bisulfite reduction to bright yellow and formaldehyde reoxidation to green was performed, the color changes ensuing at about the same rate as on the second cycle.

In additional series on deeply icteric liver 691064 the formol fixed tissue exhibited dark green spots covering about 30% of the sectioned surface in a light green background. On reduction the spots were brownish yellow, the background light yellow. Blocks were oxidized in 10% formol (1.3 *M* HCHO), 5.9% paraldehyde (1.3 *M* CH_3CHO), 22% (1.3 *M*) and 1.65% (0.1 *M*) chloral hydrate, 10.6% (1 *M*) benzaldehyde in about 40% alcohol and *p*-dimethylaminobenzaldehyde at 0.1 *M* in 30% alcohol with and without addition of 1% concentrated

hydrochloric acid. All oxidized liver blocks to green in less than 24 hours. These blocks were reduced 48 hours with 2% $Na_2S_2O_5$ to match control bisulfite stored blocks, washed and reoxidized to complete 3 cycles of reduction and reoxidation. The weaker chloral solution and the unacidified Ehrlich reagent were somewhat slower to produce the green color than the others.

Reduction by Alcohols. Blocks of deeply icteric liver 8018, used in the cyclic oxidation reduction tests, were washed several hours in distilled water to remove free formaldehyde and placed in 60, 80 and 100% methanol. Reduction to yellow color took about 18 days in 60%, and 40 days in 80%. Further tests showed alteration from green to yellow in 50% ethanol in 3—6 days, in 70% glycol [$C_2H_4(OH)_2$] in 10 days and 10% in 17—21 days.

In trichloroethanol blocks became a darker green and a faint purplish color appeared in the solvent, which on standing some days changed to brownish yellow in the upper zone while remaining pinkish below.

The Effect of Formaldehyde on Ferric and Ferrous Salt Solutions. We (1968) have reported on a histochemical level that ferric chloride acts rather weakly to convert yellow brown reduced bile pigment to a green biliverdin phase and that ferrous sulfate has a somewhat greater reducing action to convert green bile pigment casts and granules to a yellow brown bilirubin phase. This suggested that a study of the interaction of formaldehyde with the two iron salts might yield pertinent information.

When mixtures of 1 cc 37% formaldehyde are made with 5 cc amounts of 1% $FeCl_3 \cdot 6\ H_2O$ and 1% $FeSO_4 \cdot 7\ H_2O$ and reacted after 24 hours with 1% solutions of $K_4Fe(CN)_6 \cdot 3\ H_2O$ and of $K_3Fe(CN)_6$ in comparison with control untreated $FeCl_3$ and $FeSO_4$ solutions, the controls yielded the expected dark blue precipitates of ferrous ferricyanide and ferric ferrocyanide and light green ferrous ferrocyanide and the clear orange ferric ferricyanide solution. The formaldehyde ferric chloride mixture gave a decreased, but still blue reaction with ferrocyanide and a strong dark blue reaction with ferricyanide. And the formaldehyde ferrous sulfate mixture yielded a very dark green with ferricyanide and a dark blue with ferrocyanide. Thus it appears that formaldehyde has acted to reduce Fe^{+++} and to oxidize Fe^{++}; the action on the ferric salt appearing perhaps somewhat stronger.

When 12 mmol (3.4 g) $FeCl_3 \cdot 6\ H_2O$ was treated 24 hours with equivalent 12 mmol (8.95 g) $K_3Fe(CN)_6$ in a total volume of 70 cc H_2O, no change in the deep orange solution occurred and there was no weighable precipitate. But when 1.8 cc 40% (w/v) HCHO (720 mg=24 mmol) was reacted 24 hours with the same amount of ferric chloride in 50 cc H_2O and the ferricyanide then added in 20 cc water, a dark blue precipitate resulted which on drying at 60° C to constant weight weighed 1.150 g. This is about 30.5% of what resulted from the interaction of 12 mmol $FeSO_4$ with $K_3Fe(CN)_6$.

Three equal portions (12 mmol, 3.36 g $FeSO_4 \cdot 7\ H_2O$) dissolved each in 50 cc preboiled distilled water were allowed to stand, under a 3 cm layer of mineral oil to prevent access of air, for 4 days, the one without aldehyde addition, the second with 24 mmol HCHO (1.8 cc 40% solution) and the third with 24 mmol (3.97 g) chloral hydrate. All three were then reacted with 8 mmol (2.634 g) portions of potassium ferricyanide ($K_3Fe(CN)_6$ in 20 cc H_2O. After settling overnight the overlying oil was removed by adding preboiled distilled water until the oil all overflowed into a sink, washing the surface of the water with ether and allowing ether vapor to dry off. The dark blue precipitates were transferred quantitatively to tared filters, washed with water until the wash water gave no reaction with $FeSO_4$ solution, then with 2—3 washes of absolute alcohol and of ether and dried to constant weight on a warm plate at about 50° C. The weights of ferrous ferricyanide recovered were 3.6770 g for the control, 3.0846 g for the formaldehyde treated sample, and 3.2034 g for the chloral treated. This represents a 16% conversion of Fe^{++} to Fe^{+++} with formaldehyde and a 13% with chloral.

Discussion

The demonstration that green pigments are produced in bile by a variety of aliphatic and aromatic aldehydes as well as formaldehyde suggests that the reaction is a general one of aldehydes with bilirubin (II) (see Fig. 1).

Considering at this stage that aldehydes are usually considered reducing rather than oxidizing agents the hypothesis was evolved that there was aldehyde condensation (III) with an active methylene group forming the central bridge between pyrroles 2 and 3, and that by rearrangement an alkyl (or aryl) biliverdin was formed (IV). While such a compound could again be reduced to an alkyl (or aryl) bilirubin, this substance (V) would not be susceptible to a new condensation reaction with an aldehyde since the central group would now be alkyl or aryl substituted.

On the demonstration that yellow brown nonformol fixed icteric liver blocks are turned green by exposure to formaldehyde, that green formol fixed icteric liver blocks can be returned to a golden brown color by exposure to sodium thiosulfate or bisulfite solutions, or even by ferrous salt solutions, alcohol and acetone, and finally on the demonstration that thus reduced formol fixed blocks again resume their green color on reexposure to formol, the condensation hypothesis appeared untenable and we were forced to consider an oxidation by aldehyde. Carrying the green → yellow → green shift through three cycles by alternate use of HCHO and $NaHSO_3$ appears to confirm this conclusion. Repetition of this cyclic experiment with chloral hydrate, paraldehyde, benzaldehyde and *p*-dimethylaminobenzaldehyde in parallel with formaldehyde further supports the thesis that the aldehyde reaction with bilirubin in tissue is a simple, reversible oxidation.

The change from yellow to green in tissue blocks is not a simple air oxidation in the presence of formaldehyde. It occurs with equal rapidity when the surface of the fluid is covered by a 2 cm layer of mineral oil. This precaution is adequate to preserve the golden brown color of fresh human bile for at least 70 days. The formaldehyde color change appears in 1—3 hours and is maximal in 1—2 days.

The recent demonstration (1968) that the green-brown color shift of bile pigment in tissue lay in a similar redox potential range to that of the Fe^{+++}—Fe^{++} shift and the existence of differential reactions for Fe^{+++} and Fe^{++} suggested use of these reactions to assess any possible oxidation potential of formaldehyde toward bilirubin. On the histochemical level ferric chloride solutions oxidize the yellow brown (bilirubin) phase to the green (biliverdin) state and ferrous sulfate reduces the green pigment toward the yellow brown phase.

Mixtures of ferric and ferrous salt solutions with ferricyanide and ferrocyanide solutions yielded the expected clear orange ferric ferricyanide solution, the dark blue precipitates of Turnbull's and Prussian blue and the greenish white ferrous ferrocyanide. Addition of formaldehyde to ferric chloride and ferrous sulfate solutions caused them both to develop deep blue precipitates with both double iron cyanides. Since formaldehyde is thus shown to oxidize Fe^{++} and to reduce Fe^{+++} it seems logical to conclude that it can also oxidize bilirubin. Ethanol and methanol apparently reduce biliverdin to bilirubin, perhaps more readily in the presence of some water.

Thus one might write a reaction as reversible $BR + HCHO \rightleftharpoons BV + CH_3OH$, or as between compounds I and II, Fig. 1, in which the color of the pigment would depend, by mass action, on the nature of the storage fluid. There is a slow reducing action by methanol and methanol water mixtures as well as by ethanol water mixtures, acetone and alcohol ether mixture. These reductions are slower and apparently weaker than the formaldehyde oxidation. Hence the above reaction goes to the green biliverdin side.

In regard to the possibility of a Cannizzaro type reaction in the production of green pigment by action of an aldehyde on bilirubin, the Cannizzaro reaction requires presence of free alkali and usually the presence of an aromatic aldehyde. While mixtures of formaldehyde and benzaldehyde in the presence of $Ca(OH)_2$ or other suitable alkali do produce benzyl alcohol and calcium formate, the bilirubin biliverdin conversion occurs readily also in acid formaldehyde solutions. Since the Cannizzaro disproportionation reaction apparently requires the alkaline conditions, it is not believed that this mechanism can be invoked in the present reaction. It is noted that deliberately acidified Ehrlich's reagent was a more rapid and active oxidant of reduced icteric liver than an unacidified portion of the same solution.

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References

Baker, R. D.: Essential pathology. Baltimore: Williams & Wilkins Co. 1961.

Blum, F.: Das Formaldehyd als Hartungsmittel. Z. wiss. Mikr. **10**, 314—315 (1893).

— Formaldehyd (Formol, Formalin). In: Encyklopädie der mikroskopischen Technik (P. Ehrlich, R. Krause, M. Mosse, H. Rosin and C. Weigert, eds.), p. 391—406. Berlin u. Wien: Urban & Schwarzenberg 1903.

Hanser, R.: 5/1 Leber.3. Atrophie, Nekrose, Ablagerungen und Speicherungen (sog. Degeneration); und 5/2:4. Gallenblase und Gallenwege. In: Handbuch der speziellen pathologischen Anatomie und Histologie (F. Henke und O. Lubarsch, eds.). Berlin: Springer 1930.

Lemberg, R., Legge, J. W.: Hematin compounds and bile pigments, their constitution, metabolism and function. New York: Interscience Publ., Inc. 1949.

Lillie, R. D., Pizzolato, P.: A stable histochemical Gmelin reaction of bile pigments with dry bromine-carbon tetrachloride solution. J. Histochem. Cytochem. **15**, 600—602 (1967).

— — Histochemical studies of oxidation and reduction reactions of the bile pigments in obstructive icterus, with some notes on hematoidin. J. Histochem. Cytochem. **16**, 17—28 (1968).

— — Histochemical metal uptake reactions of the bile pigments of obstructive icterus and of hematoidin. J. Histochem. Cytochem. **17**, 467—474 (1969).

— — Histochemical azo coupling reactions of the bile pigments of obstructive icterus and of hematoidin. I. Diazonium salts used. J. Histochem. Cytochem. **17**, 738—748 (1969).

— — Histochemical azo coupling reactions of the bile pigments of obstructive icterus and of hematoidin. II. Effect of blockade, bleaching and extraction procedures on the pigments and their azo derivatives. J. Histochem. Cytochem. **18**, 75—79 (1970).

McManus, J. F. A., Mowry, R. W.: Staining procedures, histologic and histochemical. New York-Evanston-London: Hoeber Med. Div., Harper and Row, Publ. 1960.

Tiedemann, F., Gmelin, L.: Recherches Experimentelles Physiologiques et Chimiques sur la Digestion. Translated from German by A. J. L. Jourdan. Paris: L. B. Bailliere, publ. 1826.

With, T. K.: Bile pigments, chemical, biological and clinical aspects. New York and London: Academic Press 1968.

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