CLXV. REDUCTION OF BILIVERDIN TO BILIRUBIN IN TISSUES.

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THE object of this paper is to investigate whether or not it is possible to regard biliverdin as the primary product of blood pigment breakdown in the body, and bilirubin as arising by reduction of biliverdin. By the combined action of molecular oxygen and of reducing substances like hydrazine hydrate [Warburg & Negelein, 1930] or ascorbic acid [Karrer *et al.* 1933] haemin is transformed into "green haemin". This substance has been proved by Lemberg [1935] to be a bile pigment haemochromogen, and it has been shown that it is easily convertible into biliverdin. It has been suggested that a similar mechanism is at work in the transformation of blood pigment into bile pigment in the body, leading to biliverdin. Since, however, bilirubin is the only, or the predominant, pigment in the bile of many animal species this hypothesis can be correct only if it can be shown that biliverdin is reduced to bilirubin in the body at a rate higher than the rate of blood pigment decomposition.

Little is known about reduction and oxidation of bile pigments in the tissues. When biliverdin is found in bile, its presence is sometimes due to a post-mortem oxidation of bilirubin in the bile exposed to atmospheric oxygen. Dastre & Floresco [1897] have claimed that oxidases play a role in this process. On the other hand, Haycroft & Scofield [1890] observed that the green bile pigment of ox bile became reduced to bilirubin when the bile was kept in tall cylinders. This reduction occurred in the absence of microorganisms, although it was accelerated by putrefactive bacteria. Reduction of biliverdin by liver tissue has been studied by Barry & Levine [1924] who report that at pH 7.4-7.6 biliverdin is reduced by the liver, whereas oxidation of bilirubin is favoured by pH 6.5. Glucose was found to accelerate the reduction. Although biliverdin can doubtless be formed in bile by post-mortem oxidation of bilirubin, this does not explain all findings of biliverdin in bile. It is also present in some cases as the primary bile pigment. Fresh frog's bile often contains green bile pigment and Nisimaru [1931] found that bile containing biliverdin is excreted by the liver of the bull frog when the liver is transfused with blood. Stern [1936] has obtained biliverdin as a by-product in the preparation of catalase from horse liver. The bile of the starving dog is green [Hoppe-Seyler & Thierfelder, 1924]. So is occasionally the bile of *Echidna*² and of the fowl. We have isolated small amounts of biliverdin from fresh rabbit's bile in which bilirubin predominates. In the bile of the guinea-pig we have never observed biliverdin, and for this reason we have used this animal preferably for our experiments.

Pure crystalline biliverdin has not previously been reduced to bilirubin either *in vitro* or by action of tissues. Barry & Levine used the crude mixture of oxidation products obtained by autoxidation of bilirubin in alkaline solution. Fischer *et al.* [1932] subjected mesobiliverdin ("glucobilin") to reduction with

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² We owe thanks to Dr Abbie of Sydney University, for giving us three fresh specimens of *Echidna* gall bladders, two of which contained biliverdin.

zinc dust but were unable to identify the product of reduction with mesobilirubin. For this reason we have also included a study of the reduction of biliverdin and of mesobiliverdin by chemical means.

Since bile pigment is formed to a considerable extent in the liver and is also excreted by this organ, we have particularly studied the reduction of biliverdin in guinea-pig liver, but we have also made a few experiments with other organs of the guinea-pig and with the livers of other animals.

EXPERIMENTAL.

Reduction of biliverdin and of mesobiliverdin by zinc dust.

Fischer *et al.* [1932] transformed glaucobilin (mesobiliverdin) into mesobilirubin by heating it above the melting-point, but they were unable to obtain mesobilirubin from it by reduction with zinc dust in hot methyl alcohol. This reaction gave a different compound. We have carried out the reduction at room temperature in ammoniacal solution, excluding atmospheric oxygen, since the zinc salt of mesobilirubin is readily oxidized by molecular oxygen. The reduction proceeds in a few minutes under these conditions, whereas Fischer boiled in methyl alcohol for 4 hours.

The reaction was carried out in the apparatus described by Lemberg [1935, p. 1331]. The small dropping funnel contained a solution of 20 mg. mesobiliverdin in 5 ml. 2% ammonia, the tube with the porous filter 1 g. of zinc dust and the small receiver 1 ml. of 20% HCl. During the reduction in the tube with the porous glass plate nitrogen was passing in from below the porous plate agitating the zinc dust. The end of the reduction, indicated by the change of the colour to a clear orange-yellow, was reached in 5 min. The solution was now sucked off from the zinc dust into the HCl which precipitated the pigment. By washing with water, hot glacial acetic acid, then again with water and methyl alcohol and by recrystallization from chloroform mesobilirubin was obtained in both needles and rhombic plates.

Mr Mellor has kindly compared these crystals with those of mesobilirubin prepared by catalytic hydrogenation of bilirubin, and has found them identical in shape, angles, angles of extinction and pleochroism.

Biliverdin was prepared as described by Lemberg [1932; 1934, 1]. It was reduced in the same way to bilirubin. Orange rhombic plates of the characteristic form were produced.

Reduction of biliverdin in animal tissues.

Technique. The animal was killed and bled, and the organs were removed under aseptic conditions, immediately after the death of the animal, and put into sterile Ringer solution. Slices of liver and kidney were prepared according to Warburg's method, the other tissues being finely chopped. 0.3–0.5 g. of the tissues was used for each experiment.

"Liver brei" was obtained by grinding chopped liver in a mortar with the minimum of HCl-extracted sea sand.

"Washed brei" was obtained in the following way.

The liver brei was thoroughly ground with 5 vol. of distilled water in a mortar, and the suspension was kept in the ice-chest for 1 hour. After centrifuging, the tissue was washed 5 times on the centrifuge, each time with about 5 vol. of distilled water.

The first two centrifugates, consisting of a red-brown cloudy solution, were kept as "washings".

The "liver enzymes" preparation mentioned below was obtained from the washings by precipitating the enzymes by half-saturation with ammonium sulphate, filtering the precipitate and dissolving it in 0.2 *M* phosphate buffer pH 7.6. It was then dialysed for 24 hours against 0.1 *M* phosphate buffer of the same pH in the ice-chest. In a few experiments separately mentioned, phosphate buffers were used instead of water for the extraction of the enzymes from the brei. The amount of "washed brei", "washings" and "enzyme preparation" used, always corresponded to about 0.3–0.5 g. liver.

Bacterial infection was avoided by sterilizing all vessels and solutions. Toluene was added regularly, after the first experiments had shown that it had no effect on the reduction. Very few experiments had to be discarded because of bacterial infection.

For each experiment, unless otherwise mentioned, 3 mg. of pure crystalline biliverdin dissolved in 0.1 ml. 0.2 *N* NaOH were used and the total volume of the mixture was 7 ml.

The following buffer media were used:

Ringer solution, mammalian Ringer-bicarbonate (according to Warburg [1930, p. 122]), 0.2 *M* secondary phosphate, 0.2 *M* phosphate buffer pH 7.6. In the experiment at pH 6.5 (Exp. 2, Table I) the pH was adjusted by adding 0.2 *M* phosphate buffer pH 6.0 until pH 6.5 had been reached.

Little or no difference was found in using the different media (cf. Table I).

The aerobic experiments were carried out at 37° by shaking the tissues in buffered biliverdin solutions in 50 ml. Erlenmeyer flasks with air. The qualitative anaerobic experiments were not done under strictly anaerobic conditions. The tissues were incubated at 37° with the buffered biliverdin solutions covered with a 2 in. layer of liquid paraffin. In the quantitative anaerobic experiments the reaction was carried out in vacuum tubes at a low pressure of nitrogen freed from oxygen by passage over heated copper.

Quantitative isolation of the bile pigments from the tissues is impossible. A nearly quantitative extraction followed by separation of bilirubin from biliverdin could, however, be accomplished and is described below. It identifies the yellow pigment formed as bilirubin in each case and gives a fairly accurate account of the rate of reduction, but it is rather time-consuming.

A good rough measure of the degree of reduction can be obtained in a very simple way in all experiments in which heat-coagulated proteins are not present by observing the change of colour from the blue-green biliverdin to the orange-yellow bilirubin. In the experiments with the liver enzymes, the degree of colour change of the solution corresponds satisfactorily with the degree of reduction. In experiments with slices and brei, microscopic observation is applied because the reduction is almost completely limited to the tissue. In the tables, "no" signifies blue-green colour and no reduction, + green colour and weak or doubtful reduction, + signifies a distinct reduction with change of colour from blue-green to yellowish green, ++ a greenish yellow colour corresponding to about 50% reduction or more, and +++ yellow colour and more than two-thirds to complete reduction.

A further indication of the degree of reduction, particularly in the tissues, is given by the crystallization of bilirubin. With a few exceptions bilirubin crystals were seen whenever there was a +++ reduction, in some cases even with a ++ reduction. Typical "haematoidin" crystals of both forms, rhombic plates and fine needles, were observed. In the tissue slices crystals were generally found lying within the liver cells. It was noted that the parenchymal cells of guinea-pig liver did not display a uniform behaviour to the biliverdin solutions. Some

of the cells were found to refrain from taking up bile pigment and to remain colourless, whereas other cells stored bile pigment. There were slight differences in the degree of reduction in different parts of the tissue, but less marked than the differences in bile pigment storage.

Table I. *Aerobic reduction of biliverdin in guinea-pig liver slices.*

0.4 g. liver slices. 3 mg. biliverdin. Total volume: 8 ml.
Shaken at 37° in Erlenmeyer flasks with air.

Exp. No.	Guinea-pig	Medium	Additions	Reduction after				
				15 min.	1 hour	2 hours	4 hours	16 hours
1	21	Ringer-phosphate pH 7.4	—	—	—	+	+++, crystals	+++, crystals
2	21	Ringer-phosphate pH 6.5	—	—	—	+	+++, crystals	+++, crystals
3	21	Ringer-phosphate pH 7.4	0.1 ml. toluene	—	—	+	+++, crystals	+++, crystals
4	21	Ringer-phosphate pH 7.4	10 mg. thymol	—	—	+	++, crystals	++, crystals
5	22	Ringer	0.1 ml. toluene	—	+	—	+++, crystals	+++, crystals
6	22	Ringer-bicarbonate	„ „	—	+	—	+++, crystals	+++, crystals
7	22	Ringer-phosphate pH 7.4	„ „	—	+	—	+++, crystals	+++, crystals
8	30	Ringer-bicarbonate	„ „	—	—	—	++	+++, crystals
9	41	Ringer-bicarbonate	„ „	—	+	+	++	±
10	41	0.2 M secondary phosphate	„ „	—	+	+	++	+
11	38	Ringer-bicarbonate	„ „	+	+++, crystals	—	+++, crystals	+, a few crystals
12	38	Ringer-phosphate	„ „	+	+++, crystals	—	+++, crystals	+++, crystals
13	38	Ringer-bicarbonate	1 ml. 0.02 M KCN	±	+++, crystals	—	+++, crystals	+
14	38	Ringer-bicarbonate	0.7 g. urethane	+++	+++	—	±	No
15	39	Ringer-bicarbonate	Toluene	+	+	—	++, a few crystals	++
16	39	Ringer-bicarbonate	Toluene + 50 mg. glucose	+	++, a few crystals	—	+++, many crystals	+++, many crystals

The experiments in Table I show that there is considerable aerobic reduction of biliverdin in guinea-pig liver slices. In Exps. 1–10 the guinea-pigs were kept on the normal stock diet. Guinea-pig 38 (Exps. 11–14) had been well fed on carrots 2 days before it was killed, guinea-pig 39 (Exps. 15 and 16) had been starved over the same period. The results show a distinct variation in the speed of aerobic reduction in normally fed animals. In general almost complete reduction in the tissue with crystal formation was found after 4 hours' incubation (e.g. in animals 21 and 22). In a few other cases, however, the reduction was slow (Exp. 8) or incomplete (Exps. 9, 10). In the latter case there is even less reduction than in the starved animal liver so that it is doubtful whether the liver

of the animal 41 can be considered as normal; no visible abnormality, however, was noticed.

There is a very marked influence of the feeding of the animals on the rate of reduction as shown by a comparison between Exps. 11 and 15. Whereas the liver slices of the well fed animal reduce almost completely in 1 hour, and those of the normally fed ones in 4 hours, there is in the starved liver a ++ reduction only, with the formation of a few crystals, after 4 hours. Exp. 16 shows that this difference is due to the lack of substrate, probably glycogen, in the starved liver, since addition of glucose renders the starved liver almost as active as the well fed liver. This confirms the observation of Barry & Levine, that glucose increases the reduction of biliverdin.

Reoxidation after 16 hours, sometimes even with disappearance of bilirubin crystals, is observed occasionally (Exps. 9, 10, 11, 13, 15). A green fringe surrounding otherwise yellow tissue was often observed after 16 hours' incubation, even when the tissue had been uniformly yellow after 4 hours. It will be discussed below whether the tissue plays a role in this reoxidation.

A comparison of Exp. 2 with Exp. 1 and of Exps. 10 and 9 shows that the reduction varies little with the pH over the range 6.5–9. The finding of a strong reduction at pH 6.5 is not in accordance with the result of Barry & Levine mentioned above.

There is no appreciable influence of toluene (Exp. 3) or of thymol (Exp. 4), or of the medium; 1/400 *M* KCN does not inhibit. Less clear is the influence of urethane. It has no action of its own on bilirubin, but in presence of liver it produces almost instantaneous reduction. No bilirubin crystals are observed, and the yellow colour soon gives place again to the green colour of biliverdin so that the reduction has almost disappeared after 4 hours. A satisfactory explanation of this observation cannot be given. It might be that urethane acts by way of protein denaturation which produces a similar effect.

In order to determine the velocity of reduction of biliverdin under aerobic conditions 1 g. liver slices of the guinea-pig 22 was incubated for 4 hours at 37° with 10 ml. Ringer-bicarbonate solution containing 5 mg. biliverdin. The slices at the end of that time were completely yellow and displayed many bilirubin crystals, but no trace of green pigment. They were removed from the solution, the latter was centrifuged and its biliverdin content was determined colorimetrically against a freshly prepared biliverdin standard in Ringer-bicarbonate. 2.3 mg. of biliverdin were found in the solution, so that 2.7 mg. had been taken up by 1 g. liver tissue and had been reduced in 4 hours.

This reducing power is certainly sufficient to account for the total output of bilirubin in the guinea-pig. Provided that the activity of human liver tissue is the same the total reduction to bilirubin of the daily amount of biliverdin (about 400 mg.) can be brought about by 25 g. of liver tissue, about 1/60 of the total weight of the human liver. The reducing power of the tissue is probably much greater even, since the limiting factor of bilirubin formation in the above experiment was clearly the uptake of biliverdin by the cells from the solution, and not its reduction.

In human liver the reduction varied with the time interval between death and autopsy, so that the true rate of reduction could be properly determined. A liver excised 6 hours after death gave a strong aerobic reduction (cf. Table II) which, however, was not complete. It is impossible to recover the bile pigments sufficiently quantitatively from liver slices, and for this reason no quantitative determination of the rate of aerobic reduction could be made. In the anaerobic experiment (cf. Table V) the reduction in the tissue was almost complete after

Table II. *Aerobic reduction of biliverdin in liver slices of different animal species.*

0.4 g. liver slices. 3 mg. biliverdin. 9 ml. Ringer-bicarbonate.
Toluene. Shaken at 37° in Erlenmeyer flasks with air.

Exp. No.	Liver of	Reduction after		
		2 hours	4 hours	16 hours
1	Man	++	+*	+*
2	Horse	+	++	++
3	Cat	+	+	++
4	Rat	+	—	+++ , many crystals
5	Mouse	++	++ , crystals	+
6	<i>Echidna</i>	++	++ ‡	+
7	Fowl	+	++	++
8	Pigeon	++	++	+ †
9	Frog	±	—	±

Remarks: * ++ with lactate. † ++ crystals with lactate. ‡ +++ crystals with alcohol.

16 hours. With 1 g. of liver slices, 6 mg. of biliverdin had been incubated, of which at least 3 mg., probably 4 mg., had been reduced. This shows that certainly less than 90 g. of human liver tissue are sufficient for the reduction of the daily bile pigment output; for the reason mentioned above the real reducing power is probably greater.

Aerobic reduction of biliverdin was found in the liver slices of all animals examined, but no more than one or two experiments have been carried out with each species. Only in the livers of the mouse and the rat, and in pigeon and *Echidna* livers in presence of substrate was formation of bilirubin crystals observed.

The human liver was obtained 6 hours after death from pneumonia.

Table III. *Aerobic reduction of biliverdin in different tissues of the guinea-pig.*

Guinea-pig 38	Reduction after		
	1 hour	4 hours	20 hours
Liver	+++ , crystals	+++ , crystals	+, a few crystals
Kidney	+	+	++
Brain	+	++	No
Spleen	+	+	++
Muscle	—	—	No
Heart	—	--	No
Skin	—	—	No
Lung	—	—	No

Under aerobic conditions reduction is found in the liver and also, although less, in the kidney, in the spleen and in the grey matter of the brain. In the skin a thin layer under the epidermis showed a slight (+) and the hair sheath a marked (+++) reduction. Biliverdin might be useful for histological investigations.

Oxidation of bilirubin in tissues.

Some experiments recorded in Table I indicate that on prolonged shaking of the tissues with air bilirubin disappears again from liver slices and is reoxidized to biliverdin, the degree of this reoxidation being found to vary considerably. Reoxidation is perhaps due to exhaustion of reducing substrates, since addition of glucose to starved liver slices prevents disappearance of bilirubin (cf. Exp. 16,

Table I) which was, however, found in both well-fed and starved livers. It was therefore investigated whether liver and other tissues can accelerate the oxidation of bilirubin.

Table IV. *Oxidation of bilirubin in presence of different tissues of the guinea-pig.*

3 mg. bilirubin in 9 ml. Ringer-bicarbonate. 0.3 g. tissue slices or finely chopped tissue. Toluene. Shaking for 20 hours at 37° in Erlenmeyer flasks.

		Oxidation after 20 hours
Liver	Tissue yellow, bilirubin crystals, solution greenish yellow	Less than control
Kidney	Tissue slightly greenish, solution olive-brown	= Control
Spleen	Tissue partly green	Slightly more than control
Lung	Tissue slightly greenish	Slightly more than control
Muscle	Fibres greenish, edges of fibres and connective tissue yellow	In the whole = control
Heart	Green on surface of tissue	More than control
Skin	Green spots in tissue, edges brown	= Control
Blood	No biliverdin found	No oxidation
Control	Solution olive-brown	—

Table IV shows that only heart accelerates the oxidation of bilirubin distinctly, and spleen and lung to a minor degree. Kidney, muscle and skin show very slight, if any, acceleration whereas liver tissue retards the oxidation. Even washed liver brei still showed this retardation. If there is any oxidation of bilirubin in the liver, it is very slow. Hence, the biliverdin-bilirubin system cannot play a catalytic role in the respiratory mechanism of the liver cell.

No oxidation of bilirubin was found in sheep's blood. After the erythrocytes had been removed by centrifuging the serum proteins were precipitated with 2 vol. of alcohol. The solution was yellow. The pigments were extracted with ether. 1% HCl failed to remove any green pigment from the yellow ethereal solution, which proves the absence of biliverdin.

Anaerobic experiments with liver slices.

The slowness of reoxidation of bilirubin in the liver of the guinea-pig is also indicated by the rather small difference between the rates of biliverdin reduction under aerobic and anaerobic conditions. This will be seen from a comparison of Exp. 1, Table V with Exp. 9, Table I, or of Exp. 4, Table V with Exp. 1, Table I. The somewhat greater difference between anaerobic and aerobic reductions in the livers of other animal species (cf. Table V with Table II) might either indicate a more rapid reoxidation of bilirubin in these livers or, more probably, inhibition of the reduction of biliverdin by oxygen competing with biliverdin as hydrogen acceptor in those livers which contain a small store of hydrogen donors. Like the aerobic, the anaerobic reduction of biliverdin is not inhibited by cyanide even in high concentration (Exps. 6 and 7), and is accelerated by glucose (Exp. 2).

Table VI shows that almost all tissues of the guinea-pig are capable of reducing biliverdin under anaerobic conditions, skin being an exception. In the skin reduction was observed only in those places mentioned above.

Biliverdin was not reduced by incubation with defibrinated sheep's blood at 37° for 24 hours. The blood was worked up in the following way. Erythrocytes were removed by centrifuging. From the green serum the proteins were precipitated with twice the volume of alcohol, and the centrifugate was distributed

between ether and water in the manner described below. Biliverdin was completely extracted from the ether with HCl.

Table V. *Anaerobic reduction of biliverdin in liver slices.*

3 mg. biliverdin in 9 ml. Ringer-bicarbonate. 0.4 g. tissue. Toluene. Under liquid paraffin.

Exp. No.	Liver of	Medium	Reduction after			
			1 hour	2 hours	4 hours	16 hours
1	Guinea pig 41	—	+	++	++	+++
2	„ 41	0.03 M glucose	+++	+++, a few crystals	—	+++, many crystals
3	„ 78	—	—	+	++, crystals	++, crystals
4	„ 21	—	+	++	+++, crystals	+++, crystals
5	„ 339	—	—	—	—	+++, crystals
6	„ 339	0.01 M KCN	—	—	—	+++, crystals
7	„ 339	0.1 M KCN	—	—	—	+++, crystals
8	Man	—	+	—	—	+++, crystals
9	Horse	—	—	++	+++	+++
10	Cat	—	—	++	++	++
11	Rabbit	—	No	—	—	+++, crystals
12	Rat	—	—	++	—	+++, crystals
13	Mouse	—	++	+++	+++, crystals	+++, crystals
14	<i>Echidna</i>	—	—	+++	—	+++, many crystals
15	Fowl	—	—	++	+++	+++, crystals
16	Pigeon	—	—	+	++	+++, many crystals
17	Frog	—	+	—	—	++, a few crystals

Table VI. *Anaerobic reduction of biliverdin in different tissues of the guinea-pig.*

3 mg. biliverdin in 9 ml. Ringer-bicarbonate. 0.4 g. tissue. Toluene. Under nitrogen at 37°.

	Reduction after	
	2 hours	20 hours
Liver	++	+++ , crystals
Kidney	++ , a few crystals	+++ , crystals
Brain	++ , a few crystals	+++ , crystals
(grey matter)		
Spleen	No	++ , crystals
Muscle	No	++ , crystals
Heart	No	++ , crystals
Lung*	No	++ , crystals
Skin	No	No

* Lung gives a reduction only if air is carefully removed from the tissue by repeated evacuation and filling with nitrogen.

Experiments with liver brei.

Liver brei reduces biliverdin as readily as liver slices (Exp. 2, Table VII); this reduction differs from that observed with slices in being extended to the solution. Cyanide (1/400 *M*) does not inhibit the reduction (Exp. 3). Careful washing of the brei with water renders it almost inactive (Exp. 4), and addition of the washings restores its activity (Exp. 5). This restored system now causes a stronger reduction in the solution than in the tissue. Exps. 6 and 7 show that washing with distilled water removes not only substrates but to a large extent also the enzymes responsible for the dehydrogenation of biliverdin. That the reduction in Exp. 6 is not due to heat-stable reducers in the washings, at least to any great extent, is proved by Exp. 8 with the washings of heated brei. The precipitate obtained by half-saturation of the washings with ammonium sulphate contains the enzymes; it is spoken of subsequently as "liver enzymes". It is almost without reducing power either alone (Exp. 9) or together with the washed liver brei (Exp. 10). This is due to the lack of substrates, which remain in the filtrate from the ammonium sulphate precipitate. The activity is restored by the addition of heated, filtered washings (Exp. 11) or by the addition of suitable substrates as will be seen below.

Table VII. *Anaerobic reduction of biliverdin in liver brei and influence of washing and heating.*

3 mg. biliverdin in 9 ml. Ringer-bicarbonate. In test-tubes under liquid paraffin at 37°. Toluene. Sol.=reduction in solution.

Exp. No.	Guinea-pig liver	Reduction after		
		2 hours	4 hours	18 hours
1	Slices	++	+++ , crystals	+++ , crystals
2	Brei	++ , sol. +	+++ , crystals, sol. ++	+++ , crystals, sol. +++
3	Brei + cyanide (1/400 <i>M</i>)	++	+++ , crystals	+++ , crystals
4	Washed brei	No	No	±, sol. no
5	Washed brei + washings	++ , sol. ++	+++ , sol. +++	+++ , crystals, sol. +++
6	Washings	Sol. ++	Sol. +++	Sol. +++ , many crystals
7	Washed brei + heated washings, filtered	+	+	++ , a few isolated crystals
8	Washings of heated brei	No	No	Sol. +
9	Ammonium sulphate precipitate of washings	No	No	Sol. +
10	Ammonium sulphate precipitate of washings + washed brei	No	No	++ , sol. +
11	Ammonium sulphate precipitate of washings + heated washings filtered	Sol. +	Sol. ++	Sol. +++ , crystals
12	Heated brei	+++ , sol. no	+++ , sol. no	+, sol. +
13	Washed brei heated	+++ , sol. no	—	+, sol. no
14	Heated brei washed	+++ , sol. no	+++ , sol. no	+++ , sol. no
15	Heated washings, unfiltered	+++ , sol. no	+++ , sol. no	+++ , sol. +

It was surprising to find that heated brei (Exp. 12), almost at once and much more quickly than unheated brei, assumes an intense yellow colour with biliverdin solution. This yellow coloration was limited to the tissue, and crystals of

bilirubin were never observed. It was also obtained with heated washed brei (Exp. 13) which before the heating had not reduced biliverdin, or with heated brei extracted with boiling water (Exp. 14). It is thus undoubtedly an artificial effect caused by the heating. The same phenomenon was observed in the coagulated proteins of heated washings (Exp. 15) and a comparison with Exp. 7 shows that it is linked with the presence of the heat-coagulum, since it does not occur when this has previously been removed by filtration. This reduction is probably caused by SH-groups in the denatured proteins. In spite of the strong yellow coloration of the coagulated proteins the degree of reduction is not great. Often the yellow colour disappears again after a few hours.

It is noteworthy that a slight reduction was still observed in the solution in Exps. 8, 12, 15 where the enzymes had been destroyed by heating but where the water-extractable matter of the tissue was present. It will be seen below that this reduction is probably caused by ascorbic acid.

Reduction of biliverdin by dehydrogenase systems.

Reduction by washed liver brei with different substrates. The experiments described make it probable that the greater part of the reduction of biliverdin is due to the action of dehydrogenase systems using biliverdin as hydrogen acceptor. We have therefore investigated several dehydrogenase systems (known to be present in the liver) for their activity with biliverdin. The first experiments were carried out with washed liver brei to which substrates were added. After what has been said of the water-extractability of the liver dehydrogenases acting on biliverdin, it will not be surprising that the results vary a good deal. Prolonged washing leads to a brei which does not reduce biliverdin even with substrate added since the dehydrogenases have been washed away. On the other hand incomplete washing leaves substrates in the brei sufficient for a reduction of biliverdin without further addition. Substrates are however washed out more readily than the enzymes, and it was thus possible to obtain preparations of washed brei with which the influence of substrate addition could be investigated. These experiments were considered necessary since it was doubtful whether all the enzymes present in the liver could be extracted and, if so, whether they were still active after ammonium sulphate precipitation and dialysis in the "liver enzymes".

Table VIII shows that the washed brei of guinea-pig liver 78 answered the above requirements, whereas liver 44 was evidently extracted too much and liver 92 not sufficiently. Brei 44 (Exp. 1) was completely inactive and remained so even after addition of glucose and cozymase (Exp. 2). Brei 78 showed a very small activity (Exp. 3) which was somewhat increased by the addition of glucose and cozymase. There was also a distinct increase of the rate of reduction by addition of glucose to a mixture of washed brei and boiled filtered washings. In this case we found a considerable reduction without glucose (Exp. 5), since the washings contained other substrates, but the reduction was still accelerated by addition of glucose (Exp. 6), coenzyme being present in the washings. Of other substrates lactate (Exp. 7), formate (Exp. 10) and citrate (Exp. 9) were very efficient, but succinate only slightly so. Of non-enzymic reducers ascorbic acid and glutathione were investigated. The latter showed no reducing activity, but ascorbic acid was clearly active, although not as rapidly as some dehydrogenase systems, particularly lactic dehydrogenase.

The results with brei 92 are less conclusive since the strong reduction without substrate (Exp. 13) showed that the brei was not washed free from substrates. Alcohol, lactate, ascorbic acid and citrate increased somewhat the speed of

Table VIII. *Reduction of biliverdin by washed liver brei with different substrates.*

3 mg. biliverdin in 5 ml. Ringer-bicarbonate. 0.5 g. washed liver brei. 37°, toluene. Exps. 1-19 in test-tubes under liquid paraffin. Exps. 20-23 in open 50 ml. Erlenmeyer flasks.

Exp. No.	Substrate	Reduction after	
		2 hours	16 hours
1	Guinea-pig 44	—	No
2	„ 44	0.15 M glucose + 25 units cozymase	No
3	„ 78	—	+
4	„ 78	0.15 M glucose + 25 units cozymase	++
5	„ 78	Boiled washings	++, crystals
6	„ 78	Boiled washings + 0.15 M glucose	++, crystals
7	„ 78	0.02 M lactate	++, crystals
8	„ 78	0.02 M succinate	+
9	„ 78	0.01 M citrate	++, crystals
10	„ 78	0.04 M formate	++, crystals
11	„ 78	0.06 M glutathione	±
12	„ 78	0.01 M ascorbic acid	No
13	„ 92	—	++, crystals
14	„ 92	0.02 M lactate	++, crystals
15	„ 92	0.3 M alcohol	+++ many crystals
16	„ 92	0.02 M succinate	++, crystals
17	„ 92	0.01 M citrate	++, crystals
18	„ 92	0.04 M formate	++, crystals
19	„ 92	0.01 M ascorbic acid	+++ crystals
20	Fowl	6 units cozymase	No
21	„	6 units cozymase + 0.03 M glucose	±
22	Cat	6 units cozymase	±
23	„	6 units cozymase + 0.03 M glucose	++

reduction, and the first three caused a more perfect reduction after 16 hours' incubation (Exps. 13-19).

A few aerobic experiments with washed fowl's and cat's liver brei with and without glucose and with cozymase present show that there is a slight increase of the rate of reduction by glucose in both, somewhat more marked in cat's liver than in fowl's.

From these experiments it appears that a number of dehydrogenase systems can reduce biliverdin. Lactate, alcohol, formate and citrate are the best hydrogen donors under the conditions of the experiment, less so glucose and succinate. Of non-enzymic reducers ascorbic acid is active, but slower than the dehydrogenase systems.

The "liver enzymes".

Before studying the effect of the liver enzyme mixture on the reduction of biliverdin its action on methylene blue was investigated. In this way the presence of the different dehydrogenases in the preparation was established and a comparison between the hydrogen acceptors, biliverdin and methylene blue was made possible.

As is shown in Table IX, the preparation contains a variety of different dehydrogenases. The time necessary for reduction of 1 ml. methylene blue (1 : 5000) was determined. For comparison of these experiments with the biliverdin experiments it is necessary to keep in mind that this amount of methylene blue corresponds to 0.4 mg. of biliverdin, whereas the amount actually

Table IX. *Reduction of methylene blue by dehydrogenase systems in the "liver enzyme mixture".*

1 ml. methylene blue (1 : 5000), 3 ml. enzyme. 0.2 *M* phosphate buffer, pH 7.6. Total volume 7 ml. In vacuum tubes at a low pressure of nitrogen. In experiments with cozymase 12 units of cozymase. Reduction time in min.

Without substrate	No (>480)	Washings	88
Cozymase without enzyme	No (>480)	Distillate of washings	195
Cozymase	19	Succinate 0.01 <i>M</i>	24
Glucose 0.01 <i>M</i>	165	Citrate 0.007 <i>M</i>	64
Glucose 0.01 <i>M</i> + cozymase	23	Formaldehyde 0.02 <i>M</i>	28
Lactate 0.01 <i>M</i>	90	Acetaldehyde 0.02 <i>M</i>	1
Lactate 0.01 <i>M</i> + cozymase	13	Salicylaldehyde 0.002 <i>M</i>	1½
Alcohol 0.01 <i>M</i>	135	Xanthine 0.0003 <i>M</i>	7
Alcohol 0.01 <i>M</i> + cozymase	16		

used in the biliverdin experiments was 3 mg., i.e. 7.5 times as much. A smaller amount of biliverdin would not have allowed quantitative determinations. From this it can be understood why the reduction times in all the biliverdin experiments described below are much longer than the decoloration times of methylene blue. By some experiments with an amount of methylene blue corresponding to 3 mg. biliverdin (1.5 ml. methylene blue 1 : 1000) we verified that the reduction time was roughly 7.5 times as long as in experiments with 1 ml. 1 : 5000 methylene blue.

The experiments were carried out in vacuum tubes at a low pressure of nitrogen. The concentration of the substrate was in general 0.01 *M*, with the exception of citrate, and of aldehydes and xanthine of which optimum concentrations were employed. Since the substrate concentration is not optimum in all cases, an exact comparison of the effects of the different enzyme systems is not possible nor was this intended. The data suffice to establish the presence of different dehydrogenases and to demonstrate roughly their strengths in the preparation.

Some dehydrogenase systems require the presence of cozymase; this was prepared from bottom yeast (supplied by Tooth and Co. Ltd., Kent Brewery, Sydney), according to Myrbäck [1931], the purification being carried as far as the recovery from the mercury salt by H_2S . Cozymase when added to the "liver enzymes" produces a reduction of methylene blue without addition of substrates, which somewhat disturbs the study of those enzyme systems for which cozymase is necessary. Neither cozymase alone nor the "liver enzymes" alone reduced methylene blue. Since the velocity of the reaction in the presence of cozymase varies with the "liver enzyme" used, it is probably due to the presence of small amounts of a substrate in the enzyme preparation which requires activation by cozymase. The most likely explanation is that this is desmo-glycogen although its presence could not be demonstrated by other means. Urban [1936] has recently observed that liver protein from cat's liver, prepared in a way similar to that yielding the enzyme preparation, contains small amounts of an unidentified carbohydrate.

Glucose without cozymase causes very slow reduction and the reduction in presence of cozymase is not accelerated by glucose. Lactate however which, without cozymase, causes a reduction in about half the time of glucose, accelerates the reduction in presence of cozymase (although the lactate concentration was much less than optimum), and so does alcohol. If the reduction time due to the endogenous substrate-cozymase system is subtracted, a theoretical reduction time of 43 min. for lactate in presence of cozymase is obtained instead of 90 min. without cozymase. Acetaldehyde and also salicylaldehyde reduce very

quickly and, since heating of the "liver enzyme" destroys this effect, it is due to the action of an enzyme. Xanthine reduces also, but distinctly less. The "liver enzyme" preparation also contains succinic dehydrogenase and (somewhat less) citric dehydrogenase (in the latter case the substrate concentration was nearly optimum). It reduces methylene blue not only with the filtrate from the ammonium sulphate precipitation of the enzymes, but also with a distillate of this filtrate which gives slight aldehyde reactions. The ratio of the velocities with filtrate and with the distillate is however no correct indication of the role which the oxidation of volatile aldehydes plays in the liver since the distillate was obtained from many times the amount of filtrate.

*Reduction of biliverdin by dehydrogenase systems in the
"liver enzyme" preparation.*

The presence of a number of dehydrogenases in the "liver enzyme" preparation having thus been established, we proceeded to examine their effects on biliverdin reduction and the results are shown in Table X.

Cozymase along with enzyme but without substrate has little effect on the reduction of biliverdin, and no difference was found in experiments with different substrates with or without cozymase, which seems rather remarkable.

Glucose, in the presence of the enzyme preparation, reduced biliverdin very little (Exps. 3 and 4, Table X).

Table X. *Reduction of biliverdin by dehydrogenase systems in the
"liver enzyme" preparation.*

3 mg. biliverdin in 0.1 ml. 0.2 *N* alkali. 3 ml. enzyme in phosphate buffer *pH* 7.6. Total volume 7 ml. In test-tubes under liquid paraffin. Toluene. In experiments with cozymase 12 units of cozymase were added.

Exp. No.	Substrate	Guinea-pig 55	Guinea-pig 303			Guinea-pig 31			Rabbit
		16 hours	1 hour	2 hours	16 hours	A	B	C	16 hours
1	—	No	No	No	+	±	±	±	No
2	Cozymase	No	—	—	—	—	—	—	—
3	0.01 <i>M</i> glucose	+	—	—	—	—	—	—	—
4	0.01 <i>M</i> glucose + cozymase	+	—	—	—	—	—	—	—
5	0.02 <i>M</i> lactate	++ , crystals	—	—	—	—	—	—	—
6	0.02 <i>M</i> lactate + cozymase	++ , crystals	+	++	+++ , crystals	++ , crystals	++ , crystals	++ , crystals	+
7	0.3 <i>M</i> alcohol	++ , crystals	±	++	+++ , many crystals	—	—	—	—
8	0.3 <i>M</i> alcohol + cozymase	++ , crystals	—	—	—	++ , crystals	++ , crystals	±	+
9	0.02 <i>M</i> succinate	—	No	+	++	+	+	±	No
10	0.01 <i>M</i> citrate	—	No	+	++	+	+	±	—
11	0.04 <i>M</i> formate	—	—	—	—	+	+	+	—
12	0.005 <i>M</i> xanthine	No	—	—	—	±	±	±	—
13	0.2 <i>M</i> acetaldehyde	—	++	+++ , many crystals	+++ , many crystals	+++ , crystals	+++ , crystals	+++	+++ , crystals
14	0.005 <i>M</i> salicylaldehyde	+++ , many crystals	—	—	—	—	—	—	—

A. Extract with water
 B. Extract with secondary phosphate
 C. Extract with primary phosphate } Reduction time 16 hours.

Lactate and alcohol (the latter was used at rather high concentrations) are excellent hydrogen donors for biliverdin; the fastest reducers, however, are aldehydes. Neither acetaldehyde nor salicylaldehyde reacts with biliverdin alone. It was suspected that the xanthine oxidase of the liver catalysed this reaction; xanthine however had a very slight effect, if any. Succinate, citrate and formate also gave a slight reduction of biliverdin.

On the whole the results agree with those obtained with washed brei. With "liver enzyme" citrate and formate appeared to be less active than with the brei; in the case of citrate this was certainly due to the small concentration of citric dehydrogenase in the enzyme preparation. Lactate and alcohol produced a good reduction with the enzyme preparation, although the former was used in sub-optimum concentration and the enzyme preparation did not contain much alcohol dehydrogenase.

It does not make a great difference whether water, secondary phosphate or primary phosphate is used for the extraction of the enzymes. Extracts with primary phosphate contain less alcohol, succinic and citric dehydrogenases than extracts with water or secondary phosphate, to judge from the biliverdin reduction experiments.

Roughly quantitative experiments with "liver enzyme" and other dehydrogenase preparations.

Technique and estimation. 3 mg. of biliverdin were incubated with the enzyme and some toluene in 0.2 *M* phosphate buffer, pH 7.6, in vacuum tubes at low pressure nitrogen.

After incubation at 37° biliverdin and bilirubin were removed from the proteins as quantitatively as possible and separated from each other in the following way.

The mixture was made alkaline with a few ml. 0.2 *N* NaOH and 10 ml. of methyl alcohol were added. This solution was poured into a separating funnel containing 10 ml. of ether and a slight excess of glacial acetic acid. It was shaken at once (not too vigorously!) and several times extracted with ether until no more pigment was removed. Proteins were precipitated in the aqueous layer which was filtered (filtrate C). The protein precipitate on the filter was boiled out successively with methyl alcohol and with chloroform. In most cases these extracts did not contain any appreciable amount of pigment. If they did the methyl alcoholic extracts were again distributed between ether and dilute acetic acid, and from the chloroform extracts the pigments were extracted with 0.2 *N* NaOH, and transferred to ether in the way described above. The united ethereal extracts were washed with water, and biliverdin was isolated by two extractions with a few ml. of 1% HCl followed by one extraction with 5% HCl (A). From the remaining ether solution, after washing with water, bilirubin was extracted with a few ml. of 0.2 *N* NaOH (B).

Biliverdin was determined in A by adding methyl alcohol up to 50 ml. and comparing this solution colorimetrically with a biliverdin standard in methyl alcoholic HCl.

In B bilirubin was determined by the Van den Bergh reaction after addition of alcohol and a few drops of glacial acetic acid. Biliverdin (and also meso-biliverdin) does not react with diazobenzenesulphonic acid in acid solution.

The aqueous solution C was often colourless, but sometimes contained a considerable amount of bilirubin with the characteristic properties of "direct bilirubin". The pigment was not extracted from the aqueous solution by chloroform and gave the direct Van den Bergh test, with a somewhat more

bluish violet colour which, however, turned to the typical red-violet when alcohol was added. Bilirubin in C was determined by the Van den Bergh reaction, alcohol being added after the colour had been developed with the diazo-reagent.

As standard we used cobaltous sulphate for determinations of weak solutions of the azo-dye. Stronger solutions were matched more satisfactorily against an acid methyl red solution. Both solutions were standardized with pure crystalline bilirubin. The cobaltous sulphate standard was equivalent to a bilirubin concentration of 0.5 mg. in 100 ml., the methyl red standard to a concentration of 2 mg. in 100 ml.

The sum of A, B and C was often 70 % or more of the amount of biliverdin used. In many cases however the protein precipitate after extraction still contained as much as 70–80 % of the total pigment. These precipitates were always yellow and did not contain an appreciable amount of biliverdin, which was much less adsorbed on the proteins than bilirubin. The pigment contained in the precipitate therefore was assumed to be bilirubin.

The rate of reduction is estimated with a maximum error of about ± 10 % of the original biliverdin.

Glucose and biliverdin. In a number of experiments with liver slices of the guinea-pig and the rat no accelerating action of glucose on the aerobic reduction of biliverdin was found, but in other experiments a distinct acceleration was observed like that reported above with liver slices of the starved guinea-pig (Exp. 16, Table I). Glucose has also been found to accelerate the anaerobic reduction in guinea-pig liver slices (Exp. 2, Table V), in washed brei of the fowl (Exp. 21, Table VIII), and the cat (Exp. 23, Table VIII), and somewhat with the enzyme preparation of guinea-pig's liver (Exps. 3, 4 and 5, Table X). With washed brei and with enzyme mixture the acceleration was however much less marked than in those experiments with slices where it had been observed. These observations and the fact that lactic acid is a suitable hydrogen donator for biliverdin make it probable that glucose is first glycolysed and the lactic acid thus formed acts as hydrogen donator.

The glucose dehydrogenase of the liver had also to be taken into account. This enzyme was prepared from horse liver according to the procedure of Harrison [1933]. The amounts of enzyme and coenzyme used corresponded to 0.8 g. of acetone-liver. In another experiment cozymase was added.

Table XI. *Reduction of biliverdin and of methylene blue by the glucose dehydrogenase system of horse liver.*

Exp. No. ...	1	2	3	4
Enzyme (ml.)	0.4	0.4	0.4	0.4
Coenzyme (ml.)	0.5	—	0.5	—
Cozymase (12 units) (ml.)	—	1.0	—	1.0
Glucose 2 <i>M</i> (ml.)	0.2	0.2	0.2	0.2
Methylene blue 1 : 5000 (ml.)	—	—	0.4	0.4
Biliverdin 3 mg. (ml.)	0.2	0.2	—	—
Phosphate buffer pH 7.6 (ml.)	0.7	0.2	0.7	0.2
Reduction	0.6 mg. in 50 hours	0.6 mg. in 50 hours	60 min.	15 min.

Table XI shows that the enzyme preparation was somewhat less active to methylene blue than that of Harrison. The reduction of biliverdin which is caused is so small and slow that it remains doubtful whether the enzyme acts at all with biliverdin. This action is certainly much slower than that with

methylene blue, although the amount of methylene blue used was 20 times smaller than that of biliverdin.

The amount of acetone-liver from which enzyme and coenzyme had been obtained, corresponded to at least 5 times the amount of fresh liver tissue used for the qualitative anaerobic experiments in which almost complete reduction had been obtained in 4 hours. Unless the greater part of the enzyme present in the fresh liver was destroyed by the preparation, we conclude that glucose dehydrogenase cannot play a role in biliverdin reduction in the liver.

Lactate and biliverdin. It has been shown in experiments with washed liver brei (Exps. 7 and 14, Table VIII), and with "liver enzyme" (Exps. 6 and 7, Table X), that lactate is effective as hydrogen donor for biliverdin. Table XII shows that the rate of reduction of biliverdin with lactate in presence of "liver enzyme" is much greater than that caused by glucose dehydrogenase.

The amount of "liver enzyme" was the same as used for the qualitative experiments and corresponded to about 0.4 g. liver.

Table XII. *Reduction of biliverdin and of methylene blue by lactic dehydrogenase of guinea-pig liver.*

Exp. No. ...	1	2	3	4	5	6
Enzyme (ml.)	3	—	3	3	3	3
Heated enzyme (ml.)	—	3	—	—	—	—
Cozymase, 12 units (ml.)	—	—	1	1	1	1
Lactate 1% (ml.)	0.45	0.45	0.45	—	0.45	—
Methylene blue 1 : 5000 (ml.)	—	—	—	—	1	1
Biliverdin 3 mg. (ml.)	1	1	1	1	—	—
Phosphate buffer pH 7.6 (ml.)	2.55	2.55	1.55	2	1.55	2
Reduction	1.5 mg. in 16 hours	No	1.5 mg. in 16 hours	No	30 min.	38 min.

Cozymase did not cause a reduction of biliverdin whether without or with enzyme (Exp. 4), and did not accelerate the rate of biliverdin reduction with lactic acid (cf. Exps. 3 and 1). The enzymic nature of the reduction follows from the fact that heating of the enzyme destroys its activity (Exp. 2). The rate of methylene blue reduction cannot be established quite clearly since cozymase produces a considerable speed of reduction of methylene blue by the "liver enzyme" without substrate. From the difference of the reduction times with and without lactate one can estimate that the lactic dehydrogenase system brings about the reduction of an amount of methylene blue equivalent to 3 mg. biliverdin in approximately 18 hours. This calculation is very inaccurate, but it allows the statement that lactic dehydrogenase does not react much more slowly with biliverdin than with methylene blue, at least if low concentrations of lactate are used. The lactic acid dehydrogenase system probably accounts for a considerable part of the biliverdin reduction in the liver.

It was considered interesting to investigate whether lactic acid dehydrogenase from other sources would be equally capable of using biliverdin as hydrogen acceptor. Table XIII shows that an enzyme prepared from zymin of bottom

Table XIII. *Reduction of biliverdin and of methylene blue by yeast lactic acid dehydrogenase.*

Enzyme ml.	Lactate 0.5 N ml.	Methylene blue 1 : 5000 ml.	Biliverdin 3 mg. ml.	Phosphate buffer pH 7.6 ml.	Reduction
3	1	1.5	—	1.5	15 min.
3	1	—	1	2	0.6 mg. in 48 hours

yeast according to Bernheim [1928] was very powerful in reducing methylene blue even without cozymase but was almost inactive towards biliverdin, although an optimum concentration of lactate was used.

Alcohol and biliverdin. Alcohol has been shown to increase the rate of biliverdin reduction by washed liver brei (Exp. 15, Table VIII), and by "liver enzyme" (Exps. 8 and 9, Table X). Table XIV shows that liver enzyme mixture with alcohol as substrate reduces biliverdin rapidly. As in the case of lactic acid dehydrogenase there is no great difference between methylene blue and biliverdin as hydrogen acceptors, but the same difficulties prevent an exact determination of the rate of reduction of methylene blue. The reduction time of an amount of methylene blue equivalent to 3 mg. biliverdin can be roughly estimated to be 18 hours.

Table XIV. *Reduction of biliverdin and of methylene blue by alcohol dehydrogenase from guinea-pig liver.*

Exp. No. ...	1	2	3	4	5
Enzyme (ml.)	3	3	3	3	—
Heated enzyme (ml.)	—	—	—	—	3
Cozymase, 12 units (ml.)	1	1	—	1	1
Alcohol 80 mg. (ml.)	0.1	—	0.1	0.1	0.1
Methylene blue 1 : 5000 (ml.)	1	1	—	—	—
Biliverdin 3 mg. (ml.)	—	—	1	1	1
Phosphate buffer pH 7.6 (ml.)	1.9	2.0	2.9	1.9	1.9
Reduction	30 min.	38 min.	2.4 mg. in 16 hours	2.4 mg. in 16 hours	No

Succinate and biliverdin. Succinate (0.02 *M*) has been shown above to accelerate biliverdin reduction in washed liver very little if at all (Exp. 16, Table VIII), somewhat more in some experiments with "liver enzyme" (Exp. 10, Table X). The experiment set up to estimate the amount of bilirubin formed by succinate and "liver enzyme" failed however to show any reduction in 18 hours, beyond the small reduction given by the enzyme without substrate.

Succinic dehydrogenase of bottom yeast was prepared from zymine according to Bernheim [1928]. Table XV shows that it caused biliverdin reduction, being a stronger succinic dehydrogenase than the "liver enzyme" (the reduction time of 7.5 times the amount of methylene blue was only twice as long). It is probable that the failure to obtain reduction with the "liver enzyme" was due to a destruction of the rather sensitive enzyme during the long period necessary for reduction of 3 mg. biliverdin. We found also that 1.5 ml. methylene blue (1 : 1000) could not be completely decolorized by "liver enzyme" in presence of succinate. Even so, the succinic dehydrogenase system reacts much more slowly with biliverdin than it does with methylene blue.

Table XV. *Reduction of biliverdin and of methylene blue by succinate in presence of "liver enzyme" and of succinic dehydrogenase from bottom yeast.*

Exp. No. ...	1	2	3	4	5	6
Liver enzyme (ml.)	3	3	3	—	—	—
Yeast enzyme (ml.)	—	—	—	3	3	3 (heated)
Succinate 1 % (ml.)	2	2	2	2	2	2
Methylene blue 1 : 5000 (ml.)	1	—	—	—	—	—
Methylene blue 1 : 1000 (ml.)	—	1.5	—	1.5	—	1.5
Biliverdin 3 mg. (ml.)	—	—	1	—	1	—
Phosphate buffer pH 7.6 (ml.)	1	0.5	1	0.5	1	0.5
Reduction	24 min.	> 24 hours	Trace in 18 hours	46 min.	1.5 mg. in 18 hours	Trace in 18 hours

Citric acid and biliverdin. Citrate has been found active in reducing biliverdin in both washed liver brei (Exps. 9 and 17, Table VIII) and "liver enzyme" (Exp. 11, Table X). The quantitative experiment (Exp. 2, Table XVI) confirms that the citric dehydrogenase system is little inferior to lactic dehydrogenase in biliverdin reduction. The "liver enzyme" citric dehydrogenase is only slightly more active to methylene blue (Exp. 1) than to biliverdin. Citric dehydrogenase was also prepared from horse acetone-liver according to Bernheim [1928] with removal of haemoglobin. Its activity was slightly less than that of the guinea-pig "liver enzyme" (Exps. 3 and 4). It did not reduce methylene blue without substrate (Exp. 5) and it was destroyed by heating (Exp. 6).

Table XVI. *Reduction of biliverdin and of methylene blue by citric dehydrogenases from guinea-pig liver enzyme and from horse acetone liver.*

Exp. No. ...	1	2	3	4	5	6
Guinea-pig liver enzyme (ml.)	3	3	—	—	—	—
Horse liver enzyme (ml.)	—	—	3	3	3	3 (heated)
Citrate 1% (ml.)	1	1	1	1	1	1
Methylene blue 1:1000 (ml.)	1.5	—	1.5	—	1.5	1.5
Biliverdin 3 mg. (ml.)	—	1	—	1	—	—
Phosphate buffer pH 7.6 (ml.)	1.5	2	1.5	2	1.5	1.5
Reduction	19 hours	1 mg. in 16 hours	27 hours	2.1 mg. in 48 hours	No	No

Aldehydes and xanthine as hydrogen donors for biliverdin. Acetaldehyde has been found to be the most efficient hydrogen donor for biliverdin in experiments with liver enzyme preparation (Exp. 14, Table X), and salicylaldehyde (Exp. 15, Table X) to be similarly active. These experiments were confirmed by roughly quantitative experiments (Exps. 1 and 5, Table XVII). Acetaldehyde reduces biliverdin rapidly in presence of "liver enzyme" preparation (Exp. 1), whereas the enzyme without substrate (Exp. 2) reduces very little. Salicylaldehyde (Exp. 5) is hardly less active; increasing its concentration above 0.004 *M* makes no difference. Neither aldehyde alone causes any reduction (cf. Exps. 4 and 8, Table XIX).

It was to be expected that the xanthine oxidase of the liver would reduce biliverdin and it has been shown above (Table IX) that the "liver enzyme" preparation from guinea-pig's liver contains xanthine oxidase. Experiments with xanthine oxidase from milk seemed to support the view that the reduction of biliverdin by aldehydes was caused by this enzyme. Two kinds of preparations were employed: the caseinogen preparation of Dixon & Thurlow [1924] and a preparation according to an unpublished method of Dixon and Lemberg, which we shall call here "globulin preparation". The latter is obtained in the following way. Milk is saturated with sodium chloride and the caseinogen-fat precipitate is removed by filtration. From the filtrate the enzyme is now precipitated with ammonium sulphate. The precipitate is dissolved in water, reprecipitated by half-saturation with ammonium sulphate and dissolved in 0.2 *M* phosphate buffer pH 7.6. The caseinogen preparation was employed as solution in the same buffer. These enzymes caused a strong reduction of biliverdin by acetaldehyde (Exps. 1 and 5, Table XVIII) and by salicylaldehyde (Exp. 9, Table XVIII). No reduction was observed with formaldehyde.

Xanthine did not however cause a biliverdin reduction either with "liver enzyme" of the guinea-pig (Exps. 13, Table X; Exp. 7, Table XVII) or with xanthine oxidase from milk (Exp. 11, Table XVIII). For complete reduction of the amount of biliverdin used for these experiments it was necessary to use a

xanthine concentration above that found optimum by Dixon & Thurlow [1924]. The possibility cannot yet be excluded that the access of biliverdin to the enzyme surface is prevented by excess xanthine more effectively than that of methylene blue, and that lower concentrations of xanthine would cause reduction of biliverdin. We have tried to verify this by using 0.0004 *M* xanthine which should still suffice to reduce 1.5 mg. of the 3 mg. of biliverdin added. If there is a reduction—in some instances the reduction with xanthine was slightly greater than in the control—it is certainly very small.

It can be shown indeed that the enzyme preparations activate aldehydes for the reaction with biliverdin in another way. A large part of the catalytic action of the "enzyme mixture" and of milk xanthine oxidase on acetaldehyde is not due to an enzymic action at all, since an enzyme solution boiled for 3 min. proved no less active (Exp. 3, Table XVII; Exps. 3 and 7, Table XVIII). Heated liver enzyme alone (Exp. 4, Table XVII) reduces biliverdin very little, heated milk enzymes (Exps. 4 and 8, Table XVIII) slightly more. Salicylaldehyde differs from acetaldehyde in being much less activated by "heated enzymes" (Exp. 6, Table XVII; Exp. 10, Table XVIII).

Table XVII. *Reduction of biliverdin by "liver enzyme" of the guinea-pig with aldehydes and xanthine.*

Exp. No. ...	1	2	3	4	5	6	7
Enzyme (ml.)	3	3	3	3	3	3	3
			heated	heated		heated	
Acetaldehyde 5% (ml.)	1	—	1	—	—	—	—
Salicylaldehyde* (ml.)	—	—	—	—	1	1	—
Xanthine 4 mg. (ml.)	—	—	—	—	—	—	1
Biliverdin 3 mg. (ml.)	1	1	1	1	1	1	1
Phosphate buffer pH 7.6 (ml.)	2	3	2	3	2	2	2
Reduction in 16 hours	2.7 mg.	<0.2 mg.	2.1 mg.	<0.2 mg.	2.7 mg.	0.6 mg.	<0.2 mg.

* 4 and 50 mg. of salicylaldehyde gave the same result.

Table XVIII. *Reduction of biliverdin by aldehydes and xanthine in presence of xanthine oxidase from milk.*

Exp. No. ...	1	2	3	4	5	6	7	8	9	10	11
Caseinogen prep. 200 mg. (ml.)	3	3	—	—	—	—	—	—	3	—	3
Caseinogen heated (ml.)	—	—	3	3	—	—	—	—	—	3	—
Globulin prep. (ml.)	—	—	—	—	3	3	—	—	—	—	—
Globulin prep. heated (ml.)	—	—	—	—	—	—	3	3	—	—	—
Acetaldehyde 5% (ml.)	1	—	1	—	1	—	1	—	—	—	—
Salicylaldehyde 4 mg. (ml.)	—	—	—	—	—	—	—	—	1	1	—
Xanthine 1 mg. (ml.)	—	—	—	—	—	—	—	—	—	—	1
Biliverdin 3 mg. (ml.)	1	1	1	1	1	1	1	1	1	1	1
Phosphate buffer pH 7.6 (ml.)	2	3	2	3	2	3	2	3	2	2	2
Reduction in 16 hours	3	<0.2	3	0.3	1.5	<0.2	1.5	0.3	2.1	0.6	<0.2
	mg.	mg.	mg.*	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.

* In 5 hours less biliverdin was reduced in Exp. 3 than in Exp. 1.

With caseinogen preparation the reduction by the unheated preparation (Exp. 1) was faster (after 5 hours) than that by the heated preparation (Exp. 3); after 17 hours, reduction was complete in both cases. With the globulin preparation no difference was observed between heated and unheated enzymes (Exps. 5 and 7). Without aldehyde the heated preparations caused a slight reduction, somewhat more than heated liver enzyme.

These results prove the presence of a heat-stable catalyst in the preparations from liver and milk activating the reduction of biliverdin with acetaldehyde and

to a minor degree with salicylaldehyde. The experiments of Table XIX show that this catalyst is nonspecific.

Table XIX. *Reduction of biliverdin by aldehydes in presence of amino-compounds.*

Exp. No. ...	1	2	3	4	5	6	7	8	9	10
Egg albumin 200 mg. (ml.)	3	—	—	—	3	—	—	—	3	—
Egg albumin 200 mg. heated (ml.)	—	3	—	—	—	3	—	—	—	3
Glycine 200 mg. (ml.)	—	—	3	—	—	—	3	—	—	—
Acetaldehyde 5% (ml.)	1	1	1	1	—	—	—	—	—	—
Salicylaldehyde 4 mg. (ml.)	—	—	—	—	1	1	1	1	—	—
Biliverdin 3 mg. (ml.)	1	1	1	1	1	1	1	1	1	1
Phosphate buffer pH 7.6 (ml.)	2	2	2	5	2	2	2	5	3	3
Reduction in 18 hours	2.1	3.0	3.0	No	No	No	0.9	No	No	<0.2
	mg.	mg.	mg.				mg.			mg.

Egg albumin (Exp. 1), heat-coagulated egg albumin (Exp. 2) and glycine (Exp. 3) are able to activate acetaldehyde. Salicylaldehyde (Exps. 5-7) is also activated by glycine, but distinctly less so, and not by egg albumin. Exps. 9 and 10 are controls with egg albumin and heat-coagulated egg albumin showing that there is no reduction, or only a very slight one, without substrate. Xanthine, as would be expected, is not activated by amino-compounds. Exps. 4 and 8 show that the aldehydes need activation to react with biliverdin.

That amino-compounds activate aldehydes also towards methylene blue has been found by Haehn [1926]. This system has been studied in detail by Lieben & Getreuer [1932; 1934]; it probably involves the formation of Schiff's bases. The reduction of methylene blue by aldehydes in presence of amino-compounds however requires a higher temperature (75°) than that of biliverdin and at 37° its velocity is negligible.

These observations explain the rapid reduction of biliverdin by acetaldehyde in presence of liver enzyme or of milk xanthine oxidase, but not sufficiently the reduction by salicylaldehyde. This aldehyde is not much activated by the heat-stable system, and the observations on biliverdin reduction by xanthine do not make it probable that the strong reducing action of salicylaldehyde on biliverdin can be explained by the action of xanthine oxidase.

Since however biliverdin reduction is complicated by the action of the heat-stable system and its rate cannot be determined very accurately, experiments with methylene blue and guinea-pig liver enzyme were carried out. We have compared the velocities of methylene blue decoloration by aldehydes and xanthine in presence of liver and milk enzyme preparations, and have, in contrast to Booth [1935], arrived at the conclusion that the activation of aldehydes in the liver—at least as far as guinea-pig's and rabbit's liver is concerned—cannot be satisfactorily explained by the assumption that aldehydes are activated by the one enzyme, xanthine oxidase, alone. These experiments will be described in a second paper. They make it probable that the activation of salicylaldehyde towards biliverdin is due to the action of an aldehyde dehydrogenase different from xanthine oxidase.

This aldehyde dehydrogenase works with acetaldehyde also. Against this one might object that no considerable difference has been found between the actions of unheated and heated "liver enzymes" with acetaldehyde (cf. Exps. 1 and 3, Table XVII). A comparison of the experiments with unheated and heated egg albumin (Exps. 1 and 2, Table XIX), however, shows that heat-coagulation increases the efficacy of the proteins so that the activation due to the heat-labile enzyme system is greater than appears from the experiments of Table XVII.

Reduction of biliverdin by non-enzymic reducers. Ascorbic acid when added to washed liver brei has been shown above (Exps. 12 and 19, Table VIII) to cause reduction of biliverdin. The roughly quantitative experiment shows, however, that the reduction by vitamin C is not rapid.

3 mg. of biliverdin (dissolved in 0.1 ml. 0.2 *N* NaOH) were incubated at 37° under nitrogen with a solution of 10 mg. pure crystalline ascorbic acid (B.D.H.) in 5 ml. phosphate buffer *pH* 7.6. After 24 hours the solution was green-brown and 0.9 mg. of biliverdin had been reduced.

Crystalline glutathione did not cause a reduction of biliverdin in washed liver brei (Exp. 11, Table VIII), nor was any reduction observed when biliverdin was incubated for 3 days at 37° under nitrogen with a solution of 10 mg. crystalline glutathione in phosphate buffer *pH* 7.6. With impure ("deliquescent") glutathione a slow and partial reduction was obtained.

Reduction of mesobiliverdin in the liver. Like biliverdin, mesobiliverdin can act as hydrogen acceptor to the dehydrogenase systems of the liver. When 3 mg. of mesobiliverdin were incubated under liquid paraffin with guinea-pig liver slices these became intensely yellow after 1 hour, and after 16 hours the tissue was filled with fine needles of mesobilirubin. The heat-stable aldehyde-amino-compound system also reduces mesobiliverdin. Of 3 mg. mesobiliverdin 1.8 mg. were reduced after 16 hours' incubation with acetaldehyde and heated or unheated "liver enzyme" of the guinea-pig. The mesobilirubin crystallized from chloroform in the typical crystal forms and gave positive Gmelin and Van den Bergh reactions.

DISCUSSION.

The experimental data of this paper supply evidence that biliverdin is reduced to bilirubin in animal tissues, particularly in the liver, spleen, kidney and brain, speedily enough to allow the assumption that biliverdin is the primary product of haemoglobin breakdown, and that bilirubin results by a reduction of biliverdin in the body. These facts alone offer, of course, no positive evidence that biliverdin is the primary bile pigment, but, taken together with the *in vitro* model of haemoglobin breakdown in an earlier paper [Lemberg, 1935], and with chemical considerations, they strengthen the evidence in favour of this hypothesis.

Since the liver of the majority of species reduces biliverdin at a rate which greatly surpasses the slow rate of haemoglobin breakdown (quantitative evidence is offered for human and guinea-pig livers), one can hardly expect to find biliverdin in liver bile, even under conditions of increased haemoglobin breakdown.

The bilirubin excreted from the liver into the bile is the so-called "direct" bilirubin, insoluble in organic solvents and giving a direct Van den Bergh reaction. We have observed that reduction of biliverdin by liver enzymes yields a mixture of "direct" and "indirect" bilirubins. Further study is necessary, particularly in order to show whether the stable combination of bilirubin with liver protein, which probably explains the properties of the direct bilirubin, is linked up with the reduction mechanism, or whether it occurs independently of it.

Reduction of biliverdin was observed in all tissues although less markedly elsewhere than in liver, spleen, kidney and brain. Blood was found to be unable to reduce biliverdin, although it retarded bilirubin oxidation. The reduction of biliverdin must therefore occur before this enters the blood stream. It is nevertheless felt that the extra-hepatic bilirubin pigment formation can be explained in the same way, since the principal sites of bile pigment formation were found

to have a strong reducing action on biliverdin. One might object that in certain conditions (e.g. blood extravasations) considerable bilirubin formation is observed so that the relatively small reducing power, e.g. of muscle tissue, would not account for a sufficiently rapid reduction. The tissues however under these conditions are not comparable with normal tissue examined in slices in aerated solution, and nothing, moreover, is known about the reducing power within the phagocytic cells which are amassed in such haemorrhages and in which the haemoglobin breakdown occurs. One instance where extensive haemorrhages lead to formation of biliverdin and not of bilirubin is known, viz. the formation of uteroverdin in the dog's placenta [Lemberg & Barcroft, 1932], and biliverdin has often been observed side by side with bilirubin in the tissues.

Liver tissue was found to prevent bilirubin oxidation and for this reason the finding of biliverdin in fresh bile, particularly in liver bile, is important evidence. Biliverdin has been found in the bile mainly under conditions in which the storage of substrates (e.g. glycogen) in the liver can be considered as diminished: in the bile of starving animals, or in transfusion experiments with frog's liver. (In our experiments, frog's liver showed a smaller reducing power than mammalian or avian livers.) It has been shown in this paper that the dehydrogenase systems of the liver play an important role in biliverdin reduction. Hence, the impoverishment of the liver with regard to substrates will decrease its reducing power.

The substances capable of producing reduction of biliverdin in the liver are aldehydes, glucose, lactic acid, alcohol, citrate, ascorbic acid and to a less extent succinate. Ascorbic acid does not react rapidly with biliverdin, and some of the dehydrogenase systems reduce it more rapidly than ascorbic acid, so that the reduction of biliverdin cannot be explained by an assumption of a catalytic role of ascorbic acid. Aldehydes are activated to react with biliverdin by proteins as heat-stable catalysts and by an aldehyde dehydrogenase probably different from xanthine oxidase, whereas it is doubtful whether the last-mentioned enzyme can react with biliverdin, since xanthine could not be used as substrate.

The liver dehydrogenases capable of producing a rapid reduction of biliverdin are lactic dehydrogenase, alcohol dehydrogenase, and (somewhat less) citric dehydrogenase. It was interesting to see how the different systems would react with a hydrogen acceptor which, in contradistinction to methylene blue, is physiological, although of limited importance in this role in the body. Biliverdin, moreover, does not appear to form a reversible redox system with bilirubin. We have been unable to observe any reaction either of bilirubin with methylene blue or of biliverdin with leucomethylene blue under nitrogen. All dehydrogenase systems act more quickly with methylene blue, but in some of them (alcohol, citric and lactic dehydrogenases) the difference is less marked than in others (glucose, succinic and aldehyde dehydrogenases), and no action of biliverdin was observed with yeast lactic dehydrogenase or with xanthine oxidase. The heat-stable aldehyde-protein system is the only one found to react more rapidly with biliverdin than with methylene blue.

The study of the action of dehydrogenases on the reduction of biliverdin is, admittedly, still incomplete. Some of the more important dehydrogenases of the liver (e.g. hexosephosphate dehydrogenases, glycerophosphate dehydrogenase) have not yet been investigated. This might be the reason why the action of the established reducing systems appears not quite sufficient to account for the reduction actually observed in the tissues. Of those dehydrogenases found to reduce biliverdin rapidly, only lactic dehydrogenase is supposed to play an important role in the liver, whereas other important enzyme systems, such as

glucose dehydrogenase, succinic dehydrogenase and xanthine oxidase, were not very active or inactive with biliverdin. On the other hand, an exact comparison between the rate of reduction in the tissue and that with the isolated enzyme systems is, of course, impossible for many reasons.

Note by R. Lemberg.

The observations described above show that mesobiliverdin is reduced in the tissues to mesobilirubin, and that it is reduced by the heat-stable aldehyde system like biliverdin.

Siedel [1935] has recently observed that mesobiliverdin if injected subcutaneously disappears more rapidly than mesobilirubin and has assumed for this reason that it plays a physiological role as the "blue colour" of traumatic haemorrhages without, however, offering evidence for this assumption.

Siedel [1935] disputes the identity of the nuclear structure of mesobiliverdin with that of biliverdin, and hence the propriety of using the name "mesobiliverdin" for the substance which Fischer and his school call "glucobilin". On the former point I have adduced evidence which I consider conclusive [Lemberg, 1934, 1; 1935]. Both compounds contain two atoms of hydrogen less than the corresponding rubins and are obtained from these in the same way; both are obtained from the corresponding haemins in the same way. In the typical properties (formation of salts and ferrichlorides, solubility and basicity, light absorption of the hydrochlorides and behaviour to oxidizing agents) the two compounds show no differences. In the meantime Fischer and his school have admitted that glucobilin constitutes the green, and not the blue, oxidation stage of mesobilirubin. In his recent paper Siedel has brought experimental evidence against his earlier hypothesis that the presumable structural differences of the tetrapyrrene systems in biliverdin and mesobiliverdin were due to a "keto-enol" isomerism (better called lactim-lactam isomerism). In spite of this and of the principal objections raised against this assumption by Lemberg [1935], Siedel has not yet abandoned this hypothesis. There are slight differences in colour of the crystals in *thin* layers (both biliverdin and mesobiliverdin crystals are "steel-blue") and of the neutral solutions (those of mesobiliverdin being a purer blue than those of biliverdin), but I do not feel that they justify the assumption of a genuine structural difference. They are either caused by the difference in the side-chains, or by their influence on the fine structure which our formulae are too coarse to express. These differences disappear on salt formation. I have never observed a blue colour of mesobiliverdin hydrochloride; in the one case which Siedel perhaps had in mind in making his statement I have proved that the blueness of the colour was due to admixture of mesobiliviolin.

Against the use of the name "mesobiliverdin" Siedel refers to the fact that Fischer has thus designated the compound obtained by autooxidation of mesobilirubin in alkaline solution. He has overlooked the fact that I have isolated this compound and have identified it with "glucobilin", this being an important reason for preserving the older and well-fitting name. Since the verdins are indicator substances it does not matter to which colour, blue or green, the name applies. "Oocyan" was used for biliverdin, because in the egg shells the colour is predominantly blue, "uteroverdins" because in the tissue the colour is green. Mesobiliverdin also gives a green colour to tissue and not a blue one as Siedel assumes.

The use of inappropriate names by Fischer's school makes the understanding of bile pigment chemistry unnecessarily difficult to a wider circle of scientists. A name like "ferrobilin" for the ferrichloride of mesobiliverdin [Fischer *et al.* 1932] is unnecessary and causes mistakes when employed to designate both ferrichloride and ferribromide as has been done in this paper. The term "mesobiliviolin" is used by Siedel for a compound isomeric with mesobilirubin synthesized by him, without even mentioning the fact that the same name is applied to an oxidation product of mesobilirubin [Lemberg, 1934, 2]. Names like "copromesobiliviolin" are misleading once the prefix "copro" has been chosen to designate the kind of side-chains ("coproporphyrin", "coprobiliverdin"). Since the non-identity of urobilinogen (from both faeces and urine) with mesobilirubinogen (Watson, Lemberg) has been established, and has been confirmed by Fischer, the terms urobilinogen and urobilin should no longer be applied for the artificial products. Nor can I see any reason for replacing my term mesobiliviolin by mesobilirhodin.

Siedel's remarks on the constitution of the phycobilins will be discussed in a forthcoming paper.

SUMMARY.

Under anaerobic conditions biliverdin is reduced to bilirubin by all tissues of the guinea-pig investigated, skin being an exception, and also under aerobic conditions in liver, spleen, kidney and brain. The livers of all animals investigated were found to possess this strong reducing power for biliverdin, frog's liver alone being found distinctly less reducing than others. The significance of these findings, on the hypothesis that biliverdin is the primary bile pigment, is discussed.

Biliverdin and mesobiliverdin are reduced to the corresponding rubins by zinc dust in ammoniacal solution, and mesobiliverdin is also reduced by liver tissue.

The reduction of biliverdin in guinea-pig liver is caused by a number of dehydrogenase systems and by ascorbic acid. Aldehydes, glucose, lactate, alcohol, citrate and formate were found to act as hydrogen donors to biliverdin in the presence of liver enzymes, less so succinate. Glucose dehydrogenase is slightly active, but the greater part of the activity of glucose is due to glycolytic breakdown. Aldehydes are activated by proteins (a heat-stable system working particularly with acetaldehyde, and much faster with biliverdin than with methylene blue) and by an aldehyde dehydrogenase, probably different from xanthine oxidase. The other substrates mentioned above are activated by their specific dehydrogenases.

The bilirubin resulting from the reduction of biliverdin by liver enzymes was sometimes observed to possess the properties of "direct" bilirubin.

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