XX. THE BIOCHEMISTRY AND PHYSIOLOGY OF GLUCURONIC ACID.

V. THE SITE AND MECHANISM OF THE FORMATION OF CONJUGATED GLUCURONIC ACID.

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When a substance which is metabolised with difficulty is introduced into the animal body several detoxication mechanisms can come into play, and it is well known that glucuronic acid, glycine, glutamine [Thierfelder and Sherwin, 1915] and sulphuric acid play various rôles in this connection. A scrutiny of the work for the last half century on the metabolism of certain compounds reveals that many of these (*e.g.* certain aliphatic alcohols, phenols, aldehydes, ketones, hydrocarbons, terpenes, aromatic acids, certain amines and heterocyclic nitrogen compounds) can be detoxicated by conjugation with glucuronic acid. This process of conjugation only occurs if the toxic compound carries a hydroxyl group, a $C_6H_5.COOH$, or a $C_6H_5.\overset{|}{\underset{|}{C}}.COOH$ group, or can form such a group by oxidation (or in some cases by reduction) in the organism. Thus, terpenes are oxidised to the corresponding alcohol and the latter conjugated with glucuronic acid for excretion [Fromm and Hildebrandt, 1901]:

$$C_{10}H_{16} \rightarrow C_{10}H_{15}OH \rightarrow C_{10}H_{15}O.C_6H_9O_6 \text{ (Terpinolglucuronide).}$$

Benzene is oxidised to phenol which is excreted conjugated with glucuronic and sulphuric acids [Braunstein *et al.*, 1931]. Toluene is oxidised to benzoic acid which is excreted conjugated with glycine and glucuronic acid [Epstein and Braunstein, 1931]. Chloral is reduced to trichloroethyl alcohol which is excreted as trichloroethylglucuronic acid [*cf.* Mering, 1882].

In the second stage of the detoxication the toxic body may combine with glucose to form an intermediate glucoside which is then oxidised to the glucuronic acid, or it may combine directly with preformed glucuronic acid. The former possibility was proposed by Fischer and Piloty [1891] as the mechanism by which conjugated glucuronic acids were formed in the animal, but it had then no experimental basis. None the less it has been widely accepted as an established fact [see Armstrong, 1919; Tollens, 1914]. Hildebrandt [1905; 1909] has claimed that on subcutaneous injection of coniferin (coniferylglucoside), syringin (methoxyconiferylglucoside) and bornylglucoside into rabbits the corresponding conjugated glucuronic acids were excreted in the urine. This observation has alternative explanations, either direct oxidation of the glucoside to the conjugated glucuronic acid, or previous hydrolysis of the glucoside in the body to give a free aglucone which combines with preformed glucuronic acid.

These considerations raise two questions: (1) What organ in the body

carries out the detoxication? (2) Is glucoside formation an intermediate stage in the detoxication process? With a view to providing an answer to these two questions it was decided to perfuse, by means of a pump-lung preparation, the isolated kidney alone and in conjunction with other isolated organs, and to investigate the reaction of such perfused organs to glucuronogenic and other substances added to the circulating blood.

Methods.

Four combinations of perfused tissues were used during these investigations and they are briefly described in the order in which they were employed.

(i) *The pump-lung-kidney preparation* (*Preparation A*).

The arrangement for these experiments was essentially the same as those previously described by Hemingway [1931; 1933]. The preparation was made from two dogs. The first, which weighed 16–18 kg., was anaesthetised, and about 500 ml. of blood were taken from the carotid artery. The blood was whipped, twice filtered through fine muslin and, after measurement of the volume, introduced into the apparatus.

A cannula was tied into the pulmonary artery after opening the chest wall and perfusion of the lungs commenced after removal from the thorax.

Meanwhile the second dog was anaesthetised and about 300 ml. of blood were removed. Saline was injected intravenously to replace this blood. The abdomen was opened in the middle line, the left kidney freed from its attachments and the vessels dissected. A cannula was tied in the ureter, the kidney removed and attached to the perfusion apparatus.

(ii) *The pump-lung-kidney-liver preparation* (*Preparation B*).

A liver, perfused by a method analogous to that employed by Bauer *et al.* [1932] was added to the perfused kidney preparation. The arrangement of the apparatus is shown in Fig. 1. From a large reservoir *R*, blood was taken by two pumps, P_1 and P_2, to supply respectively the renal and hepatic arteries (*R.A.* and *H.A.*) and a constant level reservoir, *P.R.*, which fed blood to the portal vein (*P.V.*). The kidney and the liver were supported on Büchner funnels the bottoms of which were covered with fine muslin filters. The blood which issued from the cut ends of the renal and hepatic veins was eventually gathered into a common funnel, *F*, again filtered and after perfusing the pulmonary system, collected in the main reservoir. The output of the pump P_1 was adjusted to maintain a pressure of 120–130 mm. Hg in the renal and hepatic arteries, and that of P_2 to keep the portal vein reservoir overflowing. The level of this reservoir was 10–15 cm. above the portal vein.

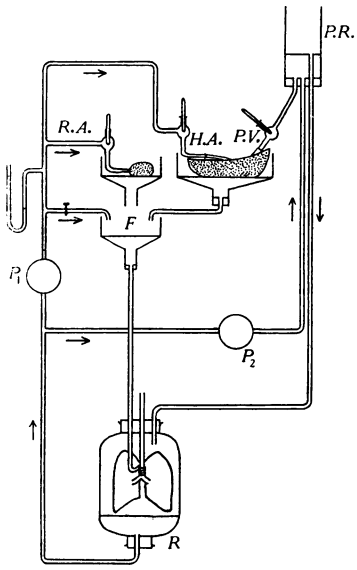

Fig. 1. Arrangement of apparatus in Preparation B.

The main reservoir and the Büchner funnels supporting the organs were placed in thermostats while the tubes conveying blood to and from the main reservoir and the lungs were water-jacketed with water circulated from one of the baths by an "autopulse" pump.

The preparation was made from two dogs and the technique of isolating the organs was identical with that described in the previous section up to the point of removing the kidney. Prior to this, the portal vein was dissected and two loose ligatures placed round it after cutting the splenic and gastric tributaries between ligatures. The hepatic artery was isolated. The aorta was clamped proximal to the renal arteries, the left kidney removed and perfusion commenced.

The portal vein was then cannulated and the liver washed with about 300 ml. of warm saline. The vein and the hepatic artery were clipped and the liver removed. A cannula was tied into the hepatic artery and after filling both cannulae with blood and attaching the appropriate tubes, perfusion was commenced. The first 200 ml. of blood coming from the liver was collected, whipped and filtered before being returned to the blood reservoir.

(iii) *The pump-lung-kidney-spleen preparation (Preparation C).*

The spleen vessels were dissected and loose ligatures placed round them. The kidney was removed and perfusion commenced, after which the spleen was removed and a cannula tied in the artery. The organ was then perfused.

(iv) *The pump-lung-kidney-limb preparation (Preparation D).*

The hind limb was selected for perfusion. The femoral vessels were dissected and loose ligatures placed round them and round the common iliac artery. The perfusion of the limb was commenced before removing the kidney, so minimising circulatory disturbances in the limb. A cannula was inserted into the femoral artery after tying the common iliac artery. The limb was washed through with defibrinated blood and, after removal by cutting through tissues and bone just below the head of the femur, perfusion was commenced. The blood issuing from the cut end of the femoral vein was collected in a funnel and led to *F*.

In all the experiments, with the exceptions mentioned below, the required amount of the substance whose conjugation was to be tested was dissolved in a small volume of saline and slowly added to the blood passing to the lungs, through funnel *F*.

Experimental.

(a) *The site of conjugation of glucuronogenic substances.*

The first organ to be considered was the kidney, since detoxication by conjugation with glycine to form hippuric acids is performed by this organ [Quick, 1932]. It seemed therefore possible that the kidney might perform a like function in regard to glucuronic acid.

As a preliminary it was necessary to demonstrate that the isolated perfused kidney was capable of eliminating preformed conjugated glucuronide. Preparation A was used in this investigation. It should first be stated that normal urine from the dog gives a slight positive test for glucuronides with naphthoresorcinol.

Our tests were carried out as follows: 2 ml. of urine, 2 ml. conc. HCl and 0·5 ml. of naphthoresorcinol (1 % in alcohol) were heated in a large test-tube for 2 mins. in boiling water. The tube was then removed from the water-bath and allowed to stand for 4 mins., and then cooled for 4 mins. in running water. The pigment was extracted with 10 ml. ether. Our experience is that this colour test for glucuronic acid, originally devised by Tollens [1908], although not a strictly quantitative test, is capable of detecting increments of glucuronic acid of the order of 0·25 mg. in 2 ml. urine, and that such increments can be compared colorimetrically.

Urine formed by an isolated kidney, when perfused with normal blood, also gives a positive test. It is, however, much fainter than that of normal urine, and

the intensity of the test falls to an almost zero constant value as the excretion proceeds. The urine from the isolated kidney was collected until it gave this almost zero constant value. At this point blood taken from a dog, which had been previously fed with borneol and which was known to be excreting glucuronide, was added to the circulating blood in the preparation. The result of the experiment is shown graphically in Fig. 2. It will be seen that the addition

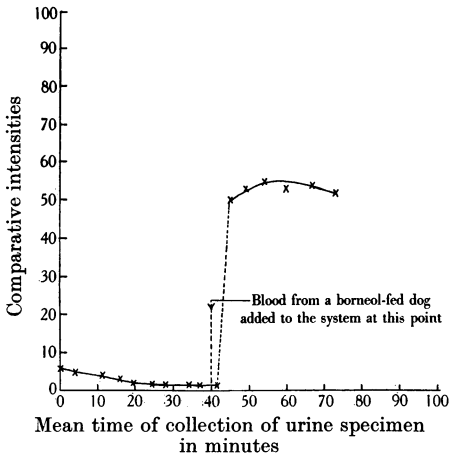

Fig. 2. Graph showing the effect of the addition of blood from a dog fed with borneol, on the intensity of the naphthoresorcinol test performed on urines excreted by an isolated perfused dog kidney. The intensity of the test performed on the bladder urine of the borneol-fed dog is taken as 100 arbitrary units of intensity.

of "borneol-blood" to the circulation of the pump-lung-kidney is followed by an immediate and marked increase of glucuronide in the urine formed by the kidney.

The next step in the investigation was to determine whether or not conjugation occurred in the isolated perfused kidney. The addition of phenol to the perfusing blood (50 mg. in 5 ml. saline to 570 ml. blood) was not followed by any increase in the glucuronide excretion, although the presence of free phenol was readily demonstrated in the urine formed within 5 mins. of the addition of the phenol to the blood. A similar experiment was performed with borneol, but in this case, owing to its low solubility, the crystalline solid was suspended in the circulating blood inside a fine muslin bag. Results as with phenol were negative.

The foregoing experiments showed that preformed conjugated glucuronic acid could readily be eliminated by the isolated perfused kidney, although the negative results in regard to conjugation left the site of formation an open question.

Embden and Glässner [1903] have shown that perfusion of the liver with blood containing phenol results in an increase of glucuronic acid in the perfused blood. A few workers have shown that there is a decreased glucuronic acid output when the liver is injured or non-functional owing to disease [Nazarjanz, 1931; Boku and Kin, 1931]. These experiments suggest that the liver is probably the site of synthesis. The results now to be described prove that this is so and also suggest that the liver alone performs this detoxication process. Preparations B, C and D were used in the series of experiments which lead to this

conclusion. The course of each experiment was similar to that already described in reference to the use of Preparation A. The following substances, all of which are known to form conjugated glucuronides in the intact animal, were added to the perfusion system: phenol, chloral hydrate, camphor and borneol.

(1) *Phenol.* This was added to the blood in two successive lots, (*a*) 30 mg. in 3 ml. normal saline, and (*b*) 70 mg. in 7 ml. normal saline. Within 4 mins. of the addition of each quantity of phenol, using the naphthoresorcinol reaction, marked increases in the glucuronide excretion were observed, (*b*) giving a considerably stronger result than (*a*). 14–15 mins. after the addition of (*b*), the glucuronide elimination by the kidney had sunk almost to zero, so that the process of conjugation and elimination was rapidly completed.

(2) *Chloral hydrate.* In the case of this substance the course of elimination of the conjugated glucuronide is given graphically in Fig. 3, which is constructed

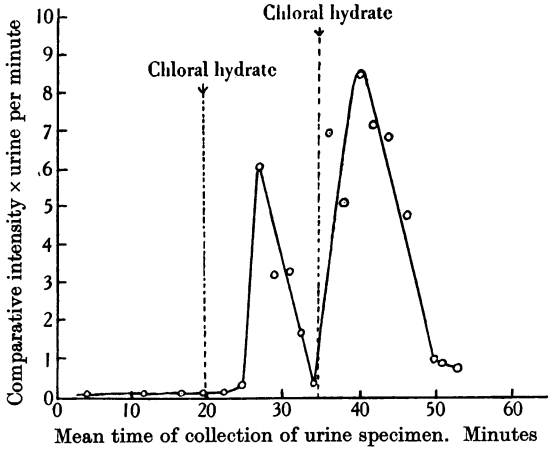

Fig. 3. Graph showing the elimination of glucuronic acid in the pump-lung-kidney-liver preparation in response to added chloral hydrate.

by multiplying the relative intensity (employing arbitrary units) of the naphthoresorcinol test by the rate of urine formation. As before the chloral hydrate was added in two lots, (*a*) 0·19 g. and (*b*) 3·03 g. each in 10 ml. normal saline (to a blood-volume of 1020 ml.) and the subsequent naphthoresorcinol reaction reached a maximum intensity in 5 to 8 mins. after each addition.

It will be observed that the second dose, some 15 times the amount of the first, did not produce a proportional increase in the glucuronide elimination, which no doubt implies some time limitation of the capacity of the liver to conjugate the chloral. It is also clear that, under the conditions of the experiment, only a part of the added chloral hydrate is eliminated *via* the kidney as conjugated glucuronide.

(3) *Camphor.* Camphor was added to the perfusing blood (1070 ml.), (*a*) in 20 ml. of saline which had previously stood in contact with solid camphor for 24 hours at 37°, and (*b*) by placing solid camphor in a fine muslin bag in the circulating system (at *F*, Fig. 1) so that the blood percolated the solid. In both cases marked increases in the glucuronide excreted were observed.

(4) *Borneol.* Solid borneol was percolated by the circulating blood (1015 ml.) as in (3) above. Positive results were again obtained.

In some early experiments the dogs used were initially under chloralose

anaesthesia. In all such cases the urine first formed in the liver-kidney perfusion system gave a marked indication of the presence of conjugated glucuronide. Chloralose, a condensation product of chloral and glucose, contains free chloral which (see (2) above) forms conjugated glucuronide in Preparation B. Chloralose anaesthesia was therefore abandoned after these early experiments.

Although the foregoing experiments proved that the liver is one of the sites of conjugation of glucuronic acid, the possibility remained that other organs might perform a like function. To test this possibility Preparations C and D were devised. The technique used was the same as that employed with Preparation B using phenol as the potential glucuronogenic substance. One experiment was performed with Preparation C, two with Preparation D, and in none of these was any conjugated glucuronide formed.

(*b*) *The mechanism of conjugation of glucuronogenic substances.*

(1) *Experiments with glycosides.* If the glycoside is an intermediate compound in the detoxication process then perfusion of the liver with blood containing glycosides should result in the formation of conjugated glycuronic acids. A second series of experiments was therefore made with Preparation B. In these (1) β-phenyl-*d*-glucoside and (2) β-bornyl-*d*-glucoside were added to the perfusing blood in separate duplicate experiments. The β-isomerides were used since all conjugated glucuronides formed *in vivo* are of the β-glycosidic type. The former glucoside was synthesised by the method of Fischer and Armstrong [1901], the latter by that of Fischer and Raske [1909]. β-Phenyl-*d*-glucoside (105 mg. in 10 ml. saline) was added to the blood (1035 ml.) in the perfusion system, whilst in the case of β-bornyl-*d*-glucoside the crystalline solid (65 mg.) was percolated as before by the blood (775 ml.). At no stage in either experiment after the addition of the glucoside was any increased elimination of glucuronide observed, although the conditions employed were identical with those giving positive results with known glucuronogenic substances. It should be added that in the case of β-phenyl-*d*-glucoside, the unchanged substance was found in the urine within 5 mins. of its addition to the perfusing blood. No free phenol was detected.

Another experiment was performed in which β-bornyl-*d*-galactoside (synthesised by the method of Robertson [1929]) was added to Preparation B. No evidence of the formation of conjugated galacturonic acid was obtained.

(2) *The influence of cyanide on the conjugation process.* The process of conjugation may require the cellular integrity of the liver or, on the other hand, it may depend on the existence of a preformed enzyme. In the former event one might expect the process of conjugation to be inhibited or abolished by cyanide. A suitable experiment, performed with Preparation B in which KCN (0·003 *M*) was added to the perfusing blood, confirmed this expectation, since added phenol was eliminated in the urine unchanged and no glucuronic acid was formed.

SUMMARY.

1. A description is given of a new technique for perfusing various organs (liver, spleen, tissues of the hind limb) in combination with the kidney.

2. It is demonstrated by using such preparations that the main, if not the only, site of formation and conjugation of glucuronic acid is the liver. The process of conjugation is abolished by cyanide.

3. No support is found for the hypothesis that the production of a glucoside is an intermediate stage in glucuronide formation.

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