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The Synthesis of *o*-Aminophenyl Glucuronide in Several Tissues of the Domestic Fowl, *Gallus gallus*, during Development

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1. Synthesis of *o*-aminophenyl glucuronide has been studied in tissues of the domestic fowl, *Gallus gallus*, during development. 2. The only route of synthesis detected is glucuronyl transfer from UDP-glucuronic acid, and evidence suggests this to be the major pathway. 3. The pathway exists in liver, to a lesser extent in kidney and alimentary tract, and possibly also in skin. It is absent from spleen and adrenal gland. 4. *o*-Aminophenyl glucuronide formation, UDP-glucuronyltransferase, UDP-glucuronic acid and UDP-glucose dehydrogenase exist in the embryo liver from at least 12 days of incubation. Transferase activity falls just before hatching and rises suddenly on emergence. Overall synthesis of *o*-aminophenyl glucuronide observed in liver slices confirms this pattern, which also occurs in kidney. UDP-glucuronyltransferase appears in intestinal mucosa only after hatching, and is absent from embryonic spleen. The allantoic membrane forms no *o*-aminophenyl glucuronide. 5. These findings are related to the isolated existence of an avian embryo.

Glucuronide synthesis, occurring by glucuronyl transfer from UDP-glucuronic acid to an acceptor, does not occur in the early foetal livers of all mammals so far investigated. Its rate of appearance varies with species and substrate but is generally still low at birth. The major cause of this deficient synthesis is low activity of UDP-glucuronyltransferase (EC 2.4.1.17) in the liver, even though this enzyme occurs elsewhere in the foetus. Foetal mammals must therefore be largely dependent on the maternal enzyme for conjugation of substances, administered or endogenous, by this route (for references and discussion see Dutton, 1963).

The avian embryo is isolated in its environment and must form, and live with, its own conjugates. Both adult hen (Sperber, 1947; Baldwin, Robinson & Williams, 1959) and its egg (Wolfe & Huang, 1959) are known to form glucuronides. In pigeon (Dutton & Greig, 1957) the biosynthetic pathway for these conjugates appeared similar to that in mammals. It therefore seemed worthwhile to study the mechanism of the formation of *o*-aminophenyl glucuronide in various tissues of the domestic fowl (*Gallus gallus*) throughout development.

The present work indicates that glucuronides are formed in *Gallus gallus* by the mammalian pathway, which is present in the embryo liver by the eighth day of incubation, and that liver UDP-glucuronyltransferase, after falling a short while before hatching, increases suddenly on emergence. The system provides an excellent model for the study of enzyme development over 'birth'. Preliminary accounts of parts of this work have already appeared (Dutton, 1961, 1963).

EXPERIMENTAL

Materials

Chemicals. *o*-Aminophenol, from British Drug Houses Ltd., was resublimed twice before use. UDP-glucuronic acid was obtained as the ammonium salt (98–100% quoted purity) from Sigma Chemical Co., St Louis, Mo., U.S.A. or as the trisodium salt (A grade) from the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. UDP-, UTP- and UDP-glucose were also from Sigma. ATP, NAD and NADP were from Boehringer und Soehne G.m.b.H., Mannheim, Germany. Glucaro-(1→4)-lactone was prepared in solution according to Levvy (1952). β-Glucuronidase was prepared from female rat preputial gland by the method of Levvy, McAllan & Marsh (1958), or the bacterial enzyme was obtained from Sigma.

Animals. White medium-sized eggs of Brown Leghorn hens were obtained from the A.R.C. Poultry Research Centre, West Mains Road, Edinburgh, and incubated in a standard egg-incubator at 38–39·5°. Chicks were of the same strain and source, and of either sex. Mice were mixed laboratory strain, of either sex. To induce fatty livers, mice were fed on standard rat-cake pellets (500g.) which had previously been soaked in a hot solution of cholesterol (20g.) in olive oil (75g.). After 12 days of this diet their livers contained 15–20% (wet wt.) of ether-extractable material; this is approximately the proportion of lipid

found in chick liver at hatching (see Entenman, Lorenz & Chaikoff, 1940).

Chick embryos were removed from the egg through the air-space and decapitated; older birds and mice were killed by cervical dislocation. All tissues were rapidly removed and placed in crushed ice. Where tissues were small, those from several embryos were pooled.

Methods

Synthesis of o-aminophenyl glucuronide in sliced tissue. The method was that employed by Dutton & Storey (1962). When required, glucose was added to 20mM. A final volume of 0·6ml. was employed when only small amounts of tissue were available. Chick-embryo kidney preparations, and the smaller livers, were fragmented when slicing was impracticable. Incubation was for 1hr. at 37° under CO_2+O_2 (1:19, v/v).

Synthesis of o-aminophenyl glucuronide with homogenized tissue. The method was again that employed by Dutton & Storey (1962). Flasks contained (final concentrations): 33mM-tris buffer (pH7·4), 10mM-$MgCl_2$, 0·29mM- or 1·0mM-UDP-glucuronic acid, 0·14mM-*o*-aminophenol (with 0·76mM-ascorbic acid to prevent oxidation), in a total volume of 0·6ml. Tissue was present usually as 10mg. (liver and kidney) or 60mg. (intestine) wet wt. Incubation was for 30min. at 37° under N_2.

UDP-glucose-dehydrogenase activity. (a) This was measured directly by the method of Strominger, Maxwell, Axelrod & Kalckar (1957), wherein NAD is specifically reduced with UDP-glucose. Liver homogenates were centrifuged at 10000*g* for 30min., and 1·2ml. of the supernatant was added to 1·8ml. of 0·1M-glycine at pH8·7 containing 0·6μmole of UDP-glucose and 3μmoles of NAD. The extinction at 340mμ was read in a Unicam spectrophotometer against a control lacking UDP-glucose, each minute for the first 10min. and every 5min. for the next 20min.

(*b*) The enzyme was also demonstrated by measuring the amount of *o*-aminophenyl glucuronide formed when UDP-glucose (0·29mM) and NAD (2·4mM) replaced UDP-glucuronic acid in the homogenate mixture, the gas phase being O_2.

Protein nitrogen was estimated by the micro-Kjeldahl method as described by Wootton (1964).

UDP-glucuronate estimation. A boiled-liver extract was prepared as previously described (Dutton, 1959). The liver, rapidly excised, was cooled 1min. in ice, 0·5g. was weighed and dropped into 2ml. of boiling water and left for 2min.; the mixture was rapidly cooled, the tissue manually disrupted and the suspension centrifuged at 2000*g* for 5min. The supernatant, which could be stored at −20° for a few days, was added to a mouse-liver UDP-glucuronyltransferase system unfortified with UDP-glucuronic acid and the resulting stimulation of *o*-aminophenyl glucuronide synthesis taken as a measure of the UDP-glucuronic acid contained in the added extract. Overall recovery experiments indicated that this assay procedure is valid for the comparative purposes as used here.

Controls. Simultaneous controls were always run. For tissue slices, they were normal flasks with acceptor substrate added after incubation; for homogenates, UDP-Gucuronic acid was omitted or replaced by UDP-glucose. gllucuronide synthesis in these flasks appeared absent or negligible. Controls for continued suitability of materials and method were run when tissue appeared to lack UDP-glucuronyltransferase.

RESULTS

Mechanism of synthesis of o-*aminophenyl glucuronide in adult* Gallus gallus

Synthesis in liver slices. With *o*-aminophenol present, a diazotizable conjugate was obtained behaving like *o*-aminophenyl glucuronide in the colorimetric estimation. In case a non-diazotizable glucuronide, such as an acetylated glucuronide (see Wolfe & Huang, 1959), was also being synthesized, the reaction mixture, after incubation, was heated with N-hydrochloric acid at 100° for 1hr. No increase in diazotizable conjugate was subsequently observed. On treatment with β-glucuronidase the conjugate behaved like a similar amount of pure *o*-aminophenyl glucuronide or the conjugate formed by mouse liver. It was completely hydrolysed by either bacterial or rat-preputial gland β-glucuronidase, and this latter hydrolysis was inhibited 100% by a boiled glucurate solution (2mM final concentration) containing glucaro-(1→4)-lactone, an almost specific inhibitor of β-glucuronidase (Marsh & Levvy, 1958). The amount of glucuronide formed was of the order observed with mammalian preparations (Storey, 1950; Dutton & Storey, 1954; Dutton, 1959).

Synthesis in liver homogenates. Confirmation that a glucuronide could be formed in *Gallus gallus* liver, and that the synthesis occurred by the mammalian pathway, was obtained from broken-cell studies.

Liver homogenates formed a conjugate which was identical with authentic *o*-aminophenyl glucuronide in both colorimetric estimation and behaviour with β-glucuronidase. The presence of UDP-glucuronic acid was essential; it could not be replaced by UDP-glucose (Table 1). Uridine triphosphate (3mM) alone or with glucuronate or glucuronolactone (both 0·5mM), and glucuronate alone (0·5mM or 20mM), proved even less effective than UDP-glucose; ATP (3mM) was only slightly more effective. The level of synthesis, with excess of UDP-glucuronic acid present, is similar to that found with the intact cell or with mammalian liver homogenates. On centrifuging a sucrose homogenate by the methods used previously (Stevenson & Dutton, 1962), the enzymic activity resided chiefly in the 'microsomal' fraction, again as found for mammals.

Synthesis of o-*aminophenyl glucuronide in preparations of other tissues of* Gallus gallus

Kidney and alimentary tract. Similar results were also found with slices and homogenates of adult

Table 1. *o-Aminophenyl glucuronide formation by various tissue preparations from older* Gallus gallus

Birds were aged 8 weeks or more, of either sex. Where more than two experiments were performed, the means ± S.E.M. are given, with the numbers of birds in parentheses. (a) Sliced tissue, (b) tissue homogenates fortified with UDP-glucuronic acid. Methods were as in the text.

Addition	*o*-Aminophenol conjugated (μg./mg. of protein N/30 min.) (a)	(b)
Liver		
—	3·9 ± 0·4 (8)	0·24 ± 0·01 (12)
Glucose (20 mM)	2·6, 3·0	0·1
UDP-glucuronic acid (0·29 or 1·0 mM)	—	6·9 ± 0·4 (10)
UDP-glucose (0·29 mM)	—	0·80 ± 0·14 (5)
NAD (2·4 mM)	—	0·80 ± 0·17 (8)
UDP-glucose + NAD	—	5·7 ± 0·6 (6)
Kidney		
—	0·7, 1·0	0
Glucose (20 mM)	1·6 ± 0·2 (5)	—
UDP-glucuronic acid (0·42 mM)	—	6·5 ± 0·8 (4)
UDP-glucose + NAD (2·4 mM)	—	4·6
Crop		
Glucose (20 mM)	0·2	—
Intestinal mucosa		
—	0·1	0
Glucose (20 mM)	0·45 ± 0·17 (4)	—
UDP-glucuronic acid (1·0 mM)	—	2·1 ± 0·4 (5)
UDP-glucose + NAD (3·8 mM)	—	3·0, 3·2

Gallus gallus kidney and with strips of mucosa from various lengths of the alimentary tract (Table 1). In the intact-cell preparations synthesis was increased by added glucose, as previously found (Hartiala, 1955; Stevenson & Dutton, 1962) for mammalian tissues. Homogenates of these tissues required more UDP-glucuronic acid for 'saturation' than did liver. This requirement cannot entirely be due to more rapid breakdown of UDP-glucuronic acid by these extrahepatic preparations because, while gut homogenates did destroy added UDP-glucuronic acid faster than liver homogenates, those from kidney did not.

Adrenal gland. According to Newcomer & Heninger (1960) cockerel adrenal gland possesses UDP-glucuronyltransferase activity towards certain steroids. No such activity towards *o*-aminophenol or *p*-nitrophenol was detected in homogenates of this tissue, nor did slices of adrenal gland conjugate these substrates. A similar lack of activity towards simple phenols was shown by adrenal tissue from guinea pig, rat and ox.

Spleen. Homogenates of spleen from 13-day chickens exhibited no UDP-glucuronyltransferase activity towards *o*-aminophenol.

Pathway of glucuronide synthesis in Gallus. The following evidence suggests that glucuronyl transfer from UDP-glucuronic acid is the main pathway of glucuronide formation in chicken liver, kidney and gut.

Formation of UDP-glucuronic acid from UDP-glucose and NAD (Strominger *et al.* 1957) could occur in these tissues, for these two compounds, when added together, reproduced the stimulatory effect of UDP-glucuronic acid in homogenates (Table 1); with intestinal mucosa this combination appeared even more effective than UDP-glucuronic acid itself, possibly because it was less vulnerable than the free nucleotide to gut pyrophosphatases. UDP-glucose, added alone, was virtually ineffective in all these tissues. UDP-glucuronic acid is present in *Gallus* liver, for boiled extracts of this tissue stimulated chicken- or mouse-liver UDP-glucuronyltransferase and, together with its precursor UDP-glucose, the nucleotide itself has been identified chromatographically in chick-liver extracts (Hansen, Freedland & Scott, 1956).

As in mammals, no alternative pathway was evident. Glucuronate or glucuronolactone did not stimulate glucuronide synthesis; neither did they react with UTP to form UDP-glucuronic acid.

Development of o*-aminophenyl glucuronide synthesis in liver of* Gallus gallus

Development of liver UDP-glucuronyltransferase. Liver homogenates from 8-day chick embryos possessed UDP-glucuronyltransferase activity. Difficulties in handling prevented examination at an earlier age. The activity increased (Fig. 1) to the sixteenth day of incubation, when it was about double that of the 8-day-old embryonic liver. It then fell until at 20 days' incubation the activity was as low as, or lower than, at 9 days. On hatching, at the twenty-first day, the activity rose very rapidly indeed, some tenfold increase being noted within 24 hr.

This pattern of development was apparent on wet weight, dry weight or protein-nitrogen bases of measurement, and the conjugate formed was the β-glucuronide of *o*-aminophenol by the criteria used above. The two major features, the pre-hatching fall and the post-hatching rise, were examined further.

Pre-hatching fall. This fall coincided with the condition of 'cholesterol-fatty liver' described in the chick embryo by Entenman *et al.* (1940), and which results from infiltration of lipid from the yolk sac. It is unlikely that an inhibitor of UDP-glucuronyltransferase was entering liver from the

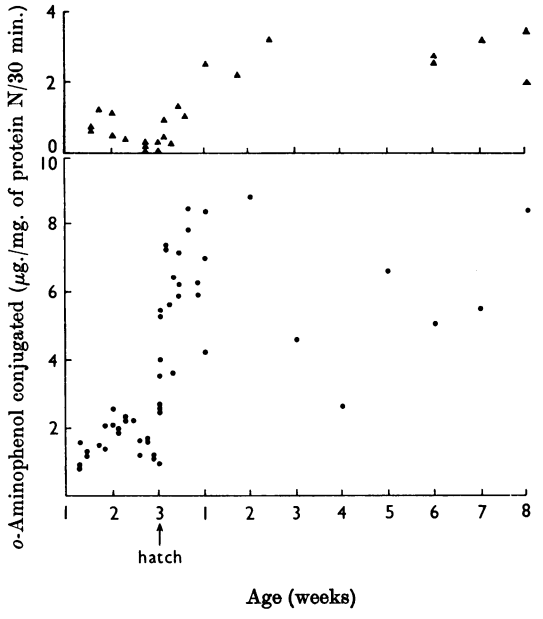

Fig. 1. Formation of *o*-aminophenyl glucuronide by developing chick liver. Methods were as described in the text. ▲, Formation with liver slices, reflecting overall synthesis of the glucuronide in the intact tissue; ●, formation with UDP-glucuronic acid-fortified liver homogenates, indicating UDP-glucuronyltransferase activity.

yolk sac at this time because homogenates from 18- to 20-days-old embryo liver, yolk or yolk sac did not inhibit activity in liver homogenate either from 16-day embryos or from mice. No increased destruction of *o*-aminophenyl glucuronide was noted in these 20-day homogenates. If mice were fed with cholesterol in olive oil until their livers acquired a comparable fat content (see under Materials), their liver UDP-glucuronyltransferase activity remained unchanged, on a protein-nitrogen basis. Fatty infiltration would therefore not seem the cause of the pre-hatching fall, especially as the lipid content of chick liver is still high (20% wet wt.), even at 2 days after hatching, when the UDP-glucuronyltransferase activity has very greatly increased.

In slices of embryo liver (Fig. 1) the same pre-hatching fall is evident, again suggesting that it is not due to an artifact of homogenization.

Post-hatching rise. A great increase in the UDP-glucuronyltransferase activity of chick liver occurred during the hatching period (Fig. 1). The rise could begin once the beak was through the shell, or even into the air sac, and had ceased after some 48hr.; Table 2 illustrates typical development over hatching for one batch of embryos. Over the next few weeks large individual variations in 'adult' activity became apparent.

Table 2. *Effect of stages of emergence on development of UDP-glucuronyltransferase activity in livers of one batch of* Gallus gallus *embryos*

Enzyme was measured in UDP-glucuronic acid-fortified homogenates as described in the text. *A*, Unhatched embryo, beak not in air space; *B*, unhatched embryo, beak in air space or atmosphere; *C*, emergent embryo; *D*, chick emerged for 12–48hr. Each result represents a determination in duplicate on one animal.

Age	Condition	*o*-Aminophenol conjugated (μg./50mg. wet wt. of liver/30min.)
19-day embryo	*A*	1·0, 1·1, 1·2, 1·3, 1·6
20–21-day embryo	*A*	0·8, 1·1, 2·1
20–21-day embryo	*B*	1·0, 2·1, 2·4, 2·5, 4·2
See legend	*C*	5·5
12–48hr. chick	*D*	5·6, 6·1, 6·4, 6·3, 7·8

When observed in slices from chick liver, the overall synthesis of *o*-aminophenyl glucuronide also rises on hatching (Fig. 1), although this increase is not so immediate, nor as steep, as that of the transferring enzyme itself.

When the total UDP-glucuronyltransferase activity of the liver on a wet weight of chick or embryo basis was calculated at various ages, the pre-hatching fall and the post-hatching rise remained as prominent as in Fig. 1.

Development of o*-aminophenyl glucuronide synthesis in other organs of* Gallus gallus

Kidney. In chick embryo the mesonephros functions increasingly from the fifth to the eleventh days of incubation, and then falls away; the metanephros, functioning from about the eleventh day, takes over from it smoothly (Romanoff, 1960). No attempt was made in this work to separate these two types of renal tissue and they are classed together as 'kidney'.

Homogenates of 14-day chick-embryo kidney contained UDP-glucuronyltransferase activity; measurement in younger embryos was not practicable. The activity remained low until hatching, after which it rose rapidly (Fig. 2). Increasing the concentration of UDP-glucuronic acid increased UDP-glucuronyltransferase activity very little in the embryo but more so in the hatched chick. Although this might suggest greater destruction of UDP-glucuronic acid in kidney homogenates from hatched chick, it was found that added UDP-glucuronic acid suffered little breakdown in these preparations either before or after hatching.

The overall glucuronide synthesis in sliced kidney was low until hatching, after which it rose. The

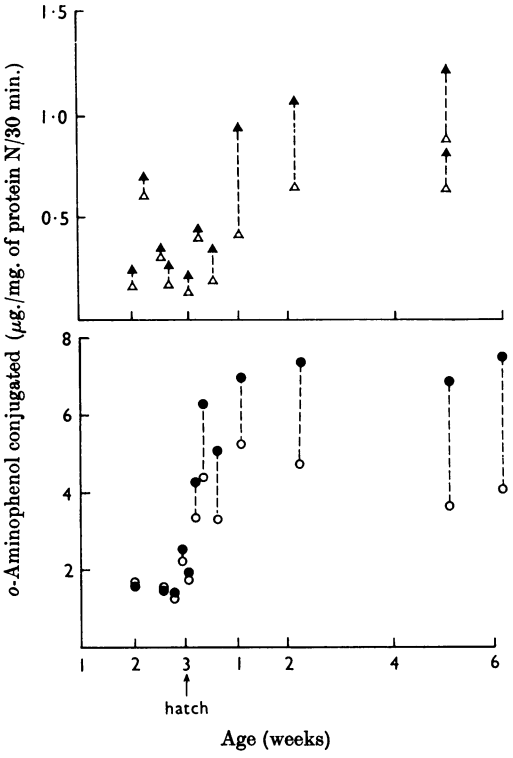

Fig. 2. Formation of *o*-aminophenyl glucuronide by developing chick kidney. Methods were as described in the text. Kidney slices ▲ with, △ without, 20mM-glucose present; ○, homogenates with 0·14mM- and ●, with 0·42mM-UDPglucuronic acid present. Broken lines join experiments employing the same tissue preparation.

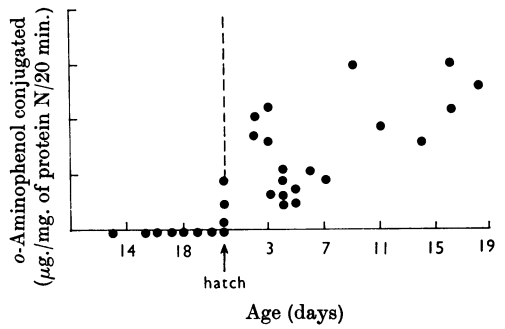

Fig. 3. UDP-glucuronyltransferase activity in gut of developing *Gallus gallus*, illustrated by formation of *o*-aminophenyl glucuronide in homogenates fortified with UDP-glucuronic acid. Methods were as described in the text. The ordinate scale is from 0 to 4 μg.

presence of 20mM-glucose stimulated this synthesis markedly (Fig. 2) in the hatched chick but not the embryo.

Intestine. *o*-Aminophenyl glucuronide was not formed in homogenates or in strips of chick-embryo gut. On hatching, UDP-glucuronyltransferase activity suddenly appeared, the time or rate of appearance not being obviously affected by the availability of food over the first 3 days. A maximum was reached in about 3 days, absolute values differing greatly with individual preparations (Fig. 3).

Other tissues. Preliminary observations suggested some synthesis of *o*-aminophenyl glucuronide by skin strips from chick and chick embryo; these appeared low both before and after hatching. No UDP-glucuronyltransferase activity could be demonstrated in homogenates from spleen of 19-day embryos. UDP-glucuronyltransferase activity was not detected in strips of intact allantoic membrane at 8 days' incubation.

Extent of glucuronide synthesis in the various organs of the chick. Before hatching it would seem that, of the chick tissues studied, liver is the only one to form appreciable amounts of *o*-aminophenyl glucuronide. After hatching, it is quantitatively the most important site, though kidney and alimentary tract also contribute largely. At 2 and 15 days, extrahepatic (gastrointestinal plus renal) UDP-glucuronyltransferase activity could be assessed on the basis of the wet weights of the whole organs at some 20–30% and 30–40% respectively of the hepatic value. Work with sliced tissue, where overall synthesis of the glucuronide is studied, suggests a similar relationship by 15 days.

UDP-glucuronic acid in developing chick liver

As embryo-liver slices formed *o*-aminophenyl glucuronide, it was not surprising to find evidence of UDP-glucuronic acid in this tissue (Table 3). As early as 11 days' incubation, the liver contained as much UDP-glucuronic acid, measured by the stimulating effect of a boiled extract when added to mouse-liver UDP-glucuronyltransferase, as that of an 8-week chick. Just before, and at hatching, stimulation fell, reaching high values again by the fourth day of emergence.

There was no evidence of an inhibitor of the mouse-liver transferase being formed over hatching and, to avoid errors from fatty-infiltration, a protein-nitrogen basis for the *Gallus* liver was used. Presumably therefore the UDP-glucuronic acid content of the extracts, and so of *Gallus* liver, is reflected in Table 3.

The enzyme responsible for UDP-glucuronic acid formation, UDP-glucose dehydrogenase, was detected in embryo liver from 12 days. There was little apparent change in its activity over emer-

Table 3. *Content of UDP-glucuronic acid in* Gallus *liver during development*

Boiled extract of chick or embryo liver (see Methods section) was added to a preparation of mouse-liver UDP-glucuronyltransferase. Results are expressed as μg. of *o*-aminophenol conjugated by 50mg. wet wt. of mouse-liver homogenate/30min. on addition of extract from 1mg. of protein N of *Gallus* liver (enzyme preparation was not 'saturated' with UDP-glucuronic acid: addition of pure nucleotide to 0·29mM increased the amount of *o*-aminophenol conjugated to 4–5μg.).

Liver-extract source	*o*-Aminophenol conjugated (μg.)
11-day embryo	2·3
13-day embryo	3·1
14-day embryo	2·6
16-day embryo	1·9
18-day embryo	1·2
19-day embryo	0·8
21-days, hatching embryo	0·4
1-day chick	0·9
4-day chick	2·0
7–21-day chicks	2·0
8-week chick	2·1

gence, the activities remaining lower than that in adult *Gallus* liver. The relation between the UDP-glucuronic acid present in liver and the activity of UDP-glucose dehydrogenase is obviously complex and was not pursued further in this investigation.

DISCUSSION

The above results indicate that in *Gallus gallus* synthesis of *o*-aminophenyl glucuronide and of UDP-glucuronic acid follows the pathway observed in mammals, and that no other pathways are evident.

As in mammals, the main site of glucuronide formation appears to be liver, with quantitatively significant contributions from kidney, gastro-intestinal tract and, possibly, skin. With different substrates, these contributions may differ in relative extent; for example, Wolfe & Huang (1959) found that only traces of aminobenzoate glucuronides were formed by chicken-kidney slices. Other sites may exist. Newcomer & Heninger (1960) have reported UDP-glucuronyltransferase activity towards certain steroids in cockerel adrenal gland. As neither slices nor homogenates of this tissue yield activity towards phenols, a transferase specific for steroids may exist there; however, G. J. Dutton & B. R. Skea (unpublished work) have not so far confirmed the presence of such an enzyme in cockerel adrenals.

In the developing embryo, the mechanism for glucuronide synthesis is present in the liver from an early stage, accounting for at least part of that glucuronide found by Wolfe & Huang (1959) in allantoic fluid from the incubated egg. Other tissues in the egg, such as embryonic skin and kidney, may also form some glucuronide.

The chick embryo therefore seems somewhat better equipped for 'detoxication' than the more maternally dependent mammalian foetus (see Dutton, 1963, for discussion). This accords with the observed high activity of various drug-metabolizing (Brodie & Maickel, 1962) and conjugatory (Brauer, Julian & Krebs, 1963) enzymes in chick-embryo liver. Several other hepatic functions, deficient in foetal mammals, can be adequately performed by the early chick embryo (Ballard & Oliver, 1963). Such precocity would be useful to an animal with an isolated embryo and which eats a mixed diet on emergence.

The demonstrable fall in liver UDP-glucuronyltransferase activity just before hatching is not due to inhibition and would seem to be due to a falling-off in its synthesis rather than to dilution of the enzyme protein by infiltrating fat. The subsequent rise in UDP-glucuronyltransferase activity is as sudden as that of xanthine oxidase (Morgan, 1930) and, like xanthine oxidase, the enzyme increases during emergence: as soon as the beak is through the shell, or even into the air-space. As UDP-glucuronyltransferase is not in the yolk or yolk sac, this rise cannot be due to incorporation from that source. It seems likely to be due to renewed synthesis of UDP-glucuronyltransferase activity, as with other postnatally developing enzymes (see Nemeth, 1962). Whether this involves renewal of the embryonic UDP-glucuronyltransferase protein or its replacement by an 'adult' enzyme requires investigation as do the factors responsible for the dramatic surge of UDP-glucuronyltransferase activity which coincides with access of the organism to a less limited oxygen supply.

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