Development of Uridine Diphosphate-Glucuronyltransferase Activity in Cultures of Chick-Embryo Liver

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1. The liver of the domestic fowl (*Gallus gallus*) remains capable of conjugating *o*-aminophenol with glucuronic acid after 8 days' culture. The pathway of *o*-aminophenyl glucuronide formation in cultured liver, as in fresh tissue, includes the enzyme UDP-glucuronyltransferase. 2. UDP-glucuronyltransferase activity in chick-embryo liver increases on culture from very low to adult values within 6–8 days. 3. The development of UDP-glucuronyltransferase activity in cultured chick-embryo liver requires certain serum factors in the medium. The requirements change with embryo age. Liver from embryos younger than 15 days develops enzyme activity equally well in media containing either foetal or adult serum; liver from embryos older than 16 days develops activity only with adult serum. The development of enzyme activity in liver from the older embryos appears to be stimulated by diffusible factors in adult serum and inhibited by diffusible factors in foetal serum. It is suggested that the stimulation and inhibition of enzyme formation by small, diffusible molecules may be part of the mechanism regulating UDP-glucuronyltransferase activity *in vivo*. 4. Liver from 19-day-old chick embryos cultured with foetal serum begins to develop UDP-glucuronyltransferase activity if transferred to an adult-serum medium. Its capacity to develop UDP-glucuronyltransferase activity in adult serum survives in a foetal-serum medium for at least 5 days, the longest period tested. 5. The activity of UDP-glucuronyltransferase reached in 19-day chick-embryo liver after 1 or 2 days with adult serum is maintained without further increase after transfer to a foetal-serum medium. After 3 days with adult serum UDP-glucuronyltransferase activity continues to increase when the tissue is transferred to a foetal-serum medium. Thus liver from 19-day-old embryos requires 3 days with adult serum before development of enzyme activity becomes independent of a continuous adult-serum environment.

Liver UDP-glucuronyltransferase (EC 2.4.1.17) activity is absent from early foetal mammals and appears gradually during development, reaching adult values at or after birth, the time depending on species and substrate (Dutton & Grieg, 1957; Brown & Zuelzer, 1958; Dutton, 1959, 1966). Many other enzymes such as glucose 6-phosphatase, tryptophan pyrrolase and tyrosine transaminase are also absent in the foetus during most of gestation and appear around the time of birth (Nemeth, 1954; Nemeth & Nachmias, 1958; Kretchmer, 1959).

Previous studies have described the inhibitory effect of the uterine environment on the development of tryptophan pyrrolase and glucose 6-phos-

phatase activity in the mammalian foetus (Nemeth, 1959, 1961; Nemeth & de la Haba, 1962; Dawkins, 1961, 1966). It seemed desirable to study the development of enzyme in isolated tissue where the immediate environment of the cells could be defined and controlled. Organ cultures of mammalian liver, however, have proved hard to maintain (Trowell, 1959; MacDougall, 1964). Chick-embryo liver was therefore tried.

Earlier work had demonstrated that, in fresh chick-embryo liver, UDP-glucuronyltransferase activity towards *o*-aminophenol is present, but at a low value which falls virtually to zero at the end of embryonic life and increases to high values during and after hatching (Dutton, 1963; Dutton

& Ko, 1966). This enzyme, because of its rapid rise in activity at hatching, was chosen as a model to study regulatory mechanisms.

The present paper describes the development of UDP-glucuronyltransferase activity in cultures of chick-embryo liver and identifies a number of the conditions and factors necessary for the induction of enzyme activity. Preliminary reports have already appeared (Ko & Nemeth, 1966a,b).

MATERIALS

Animals. Medium-sized fertile eggs of White Leghorn hens between 7 and 10 days of incubation were obtained from the George Shaw Farms (Westchester, Pa., U.S.A.) and from the Agricultural Research Council Poultry Research Centre (Edinburgh) and kept in an egg-incubator at 38–39.5°. Embryos were removed from eggs and decapitated; young chicks were also killed by decapitation. Livers were rapidly removed and immersed in Tyrode's solution at room temperature under sterile conditions. Mice were albinos from a mixed laboratory stock.

Materials for tissue culture. Tyrode's solution was obtained from Difco Laboratories, Detroit, Mich., U.S.A. Eagle's minimal medium, horse serum, bovine serum, foetal bovine serum, bovine serum ultrafiltrate, L-glutamine and penicillin-streptomycin solution were obtained from Microbiological Associates Inc., Bethesda, Md., U.S.A. All serum was used within 6 months of collection. The foetal bovine serum prepared by Microbiological Associates Inc. is collected at several slaughterhouses and is pooled in batches of 80 l. Embryo extract was prepared from 11-day-old embryos (White, 1963).

Serum was dialysed in seamless sterile bags (A. H. Thomas Co., Philadelphia, Pa., U.S.A.) made of cellulose, with an average pore diameter 4.8 μ , against 0.9% (w/v) NaCl soln.-M-NaH₂PO₄ (100:1, v/v), pH 7.4, for 48 hr. at 4°. The solution outside the bag was stirred constantly and changed twice.

Sterile disposable plastic Petri dishes (100 mm. $\times$ 20 mm. and 35 mm. $\times$ 10 mm.), made for culturing tissues, were supplied by Falcon Plastics, Los Angeles, Calif., U.S.A., and by Macfarlane Robson Ltd., Edinburgh.

Materials for enzyme assay. *o*-Aminophenol from British Drug Houses Ltd. (Poole, Dorset) and from Calbiochem (Los Angeles, Calif., U.S.A.) was resublimed twice before use. Solutions of *o*-aminophenol (6 mg. in 25 ml. of water) contained ascorbic acid (50 mg.) to minimize oxidation and were stored at -20° in small portions to be thawed at room temperature when required. Solutions were discarded if yellow coloration developed.

UDP-glucuronic acid was obtained as the ammonium salt (98–100% quoted purity) and UDP-glucose as the sodium salt from the Sigma Chemical Co., St Louis, Mo., U.S.A.

Other materials. β -Glucuronidase was prepared from female rat preputial gland by the method of Levvy, McAllan & Marsh (1958). The procedure was followed up to the precipitation of the enzyme fraction with (NH₄)₂SO₄. Glucaro-(1 $\rightarrow$ 4)-lactone was prepared in solution according to Levvy (1952). *o*-Aminophenyl glucuronide was obtained from the Aldrich Chemical Co., Milwaukee, Wis., U.S.A. A sample of calf thymus DNA was given by Dr V. Schumaker

(University of Pennsylvania) and had been prepared by the method of Kay, Simmons & Dounce (1952).

METHODS

Procedure for tissue culture. The standard culture medium consisted (by vol.) of 40% of Tyrode's solution, 40% of horse serum, 20% of embryo extract and 1% of penicillin-streptomycin solution (50 units of penicillin with 50 μ g. of streptomycin/ml.). Modifications of this medium were also used and are described in the text. Eagle's minimal medium as used contained 1% of L-glutamine.

Livers from chick embryos were rapidly removed and placed in a large Petri dish (100 mm. $\times$ 20 mm.) with Tyrode's solution at room temperature. Small segments were cut from the curved edges of the liver lobes with iridectomy scissors. The segments were approx. 3 mm. long tapering to a point at both ends. They were triangular in cross-section (approx. 1 mm. $\times$ 1 mm. $\times$ 1 mm. at the widest part); two sides were untouched peritoneal surfaces, one side was a cut surface.

The dissected tissue (20 segments per dish) was transferred to small Petri dishes (35 mm. $\times$ 10 mm.) containing 2 ml. of medium and incubated at 37.5° in CO₂+air (1:19, v/v) at atmospheric pressure and 100% humidity. The explanted tissue was transferred to fresh medium every 2–3 days.

Procedures for enzyme assay. (a) *o*-Aminophenyl glucuronide synthesis by intact liver segments. Intact segments of liver were treated as slices in the procedure described by Dutton & Storey (1962). The 20 segments from a culture dish were placed in two flasks, ten in each; one flask contained 0.54 ml. of Tyrode's solution and 0.06 ml. of *o*-aminophenol-ascorbic acid solution (see the Materials section) to give final concentrations of 0.23 mM-*o*-aminophenol and mM-ascorbic acid; the other flask contained 0.6 ml. of Tyrode's solution alone. To the latter flask 0.06 ml. of the *o*-aminophenol-ascorbic acid solution was added after incubation. The flasks were gassed for 2 min. with CO₂+O₂ (1:19, v/v) and shaken at 37.5° for 60 min. After incubation, the segments of liver were analysed for DNA. The medium in each flask was assayed for *o*-aminophenyl glucuronide.

(b) UDP-glucuronyltransferase activity in homogenized liver segments. All segments from a culture dish were placed in 1.5 ml. of the reaction medium, complete (see below) except for UDP-glucuronic acid. The tissue was homogenized with the S-75 Sonifier (Branson Sonic Power, Danbury, Conn., U.S.A.) or the 1130 Soniprobe (Dawe Instruments Ltd., London, W.3) at 0°. A 0.5 ml. sample of the resulting mixture was used for DNA determination. Another 0.5 ml. sample was placed in a flask with 0.1 ml. of a UDP-glucuronic acid solution; the final concentrations in the reaction medium were then 33 mM-tris (pH 7.4), 10 mM-MgCl₂, 0.14 mM-*o*-aminophenol, 0.76 mM-ascorbic acid, 1.4 mM-UDP-glucuronic acid; also included in the medium was 0.1 ml. of Tyrode's solution. Another flask contained an identical mixture except that UDP-glucuronic acid was omitted. Incubation was carried out at 37.5° for 30 min. with shaking under air. The medium in each flask was then assayed for *o*-aminophenyl glucuronide.

(c) Assay of *o*-aminophenyl glucuronide. The method is that of Levvy & Storey (1949) with modifications (Dutton & Storey, 1962). Freshly prepared mixtures of 40% (w/v)

trichloroacetic acid and $2M\text{-KH}_2\text{PO}_4$ (both at pH 2.2) were used to precipitate protein. Different proportions of the two solutions were used for the assay with tissue slices (1:2, v/v) and for the assay with homogenates (1:1, v/v). For tissue slices 0.45 ml., and for homogenates 0.6 ml., of the appropriate mixture was added to the medium after incubation. Precipitated protein was removed by centrifugation and to samples of supernatant fluid (0.9 ml. for the assay with tissue slices, 0.8 ml. for the homogenate assay) 0.1 ml. of each of the following reagents was added (w/v) with shaking at 2 min. intervals: 0.1% NaNO_2 , 0.5% ammonium sulphamate and 0.1% *N*-1-naphthylethylenediamine dihydrochloride. Colour was developed in the dark at 25° for 2 hr., the solutions were centrifuged and their absorption at $560\text{ m}\mu$ was measured in the Zeiss or Unicam spectrophotometer. A calibration curve was prepared with authentic *o*-aminophenyl glucuronide put through the above procedure.

Determination of DNA. Extraction of DNA was with perchloric acid, based on the procedures of Schneider (1945) and Ogur & Rosen (1950). Ten liver segments or 0.5 ml. of homogenate were added to 1.5 ml. of 5% (v/v) perchloric acid. The segments were broken up with a glass rod. The perchloric acid-tissue mixtures were frozen and thawed and then centrifuged at $2000g$ for 30 min. The supernatant fluid was removed, 0.5 ml. of 5% perchloric acid added to the precipitate and the mixture then heated at 70° for 15 min. with occasional stirring. After cooling to room temperature the mixture was centrifuged at $2000g$ for 10 min. The supernatant fluid was analysed for hydrolysed DNA (Burton, 1956).

Histological examination. Tissues were fixed in 10% formalin for 24 hr., dehydrated with ethanol, impregnated with xylene and embedded in paraffin. Serial sections were cut at a thickness of 10μ . All the sections were affixed to glass slides and stained with haematoxylin and eosin and then examined microscopically.

RESULTS

Formation of o-aminophenyl glucuronide by chick and chick-embryo liver after culture

Formation of conjugate. Segments of chick or chick-embryo liver were cultured for 8 days in Tyrode's solution, embryo extract and horse serum; they were then removed from culture and incubated with *o*-aminophenol for 1 hr. as described for tissue slices in the Methods section; the incubation medium was then assayed for *o*-aminophenyl glucuronide. In one experiment fresh liver segments from 2-day-old chicks incubated with *o*-aminophenol for 1 hr. formed $4.5\mu\text{g.}$ of *o*-aminophenyl glucuronide/100 $\mu\text{g.}$ of DNA; when cultured for 8 days and then incubated with *o*-aminophenol they formed $4.8\mu\text{g.}$ of *o*-aminophenyl glucuronide/100 $\mu\text{g.}$ of DNA. Fresh liver segments from 11-, 15- and 19-day-old chick embryos incubated with *o*-aminophenol for 1 hr. formed 1.0, 1.5 and 0.5 $\mu\text{g.}$ of *o*-aminophenyl glucuronide/100 $\mu\text{g.}$ of DNA; when cultured for 8 days and then incubated with *o*-aminophenol they formed 1.0, 1.6 and 3.4 $\mu\text{g.}$ of

o-aminophenyl glucuronide/100 $\mu\text{g.}$ of DNA. Several additional experiments gave similar results. Thus after 8 day's culture intact liver segments can form a conjugate in the presence of *o*-aminophenol that behaves like *o*-aminophenyl glucuronide in the colorimetric estimation procedure.

Identity of conjugate. A preparation of rat preputial-gland β -glucuronidase hydrolysed both the conjugate and authentic *o*-aminophenyl glucuronide to approximately the same extent. When the specific β -glucuronidase inhibitors in a glucarolactone solution were added, hydrolysis of both conjugate and authentic *o*-aminophenyl glucuronide was prevented. The conjugate formed from *o*-aminophenol therefore seems to be *o*-aminophenyl glucuronide.

Presence of UDP-glucuronyltransferase activity. Segments of chick or chick-embryo liver were cultured for 8 days in Tyrode's solution, embryo extract and horse serum. Homogenates of these cultured liver segments formed a conjugate only if UDP-glucuronic acid was also present with the *o*-aminophenol. Addition of glucose or UDP-glucose had no effect. The conjugate behaved like authentic *o*-aminophenyl glucuronide in the estimation procedure, in hydrolysis with β -glucuronidase and in inhibition of hydrolysis by glucarolactone solution. The pathway of *o*-aminophenyl glucuronide formation by intact segments of chick or chick-embryo liver after culture therefore includes the enzyme, UDP-glucuronyltransferase.

Cellular and subcellular location of UDP-glucuronyltransferase activity. Segments of chick or chick-embryo liver (cut as described in the Methods section) were cultured in Tyrode's solution, embryo extract and horse serum. After 1-2 days in culture the segments began to shorten and curl, closing to form a sphere by 4-5 days. After 8 days in culture microscopic examination of the spheres revealed three zones: an outer capsule (five-ten cells thick) of rapidly dividing, spindle-shaped cells with markedly basophilic cytoplasm; a necrotic zone approx. three-four times as thick as the capsule and without any identifiable cells; a central core with many liver cells, of a population density approximately equal to the original tissue. A discernible capsule (one-three cells thick) first appears on the third day of culture. The necrotic zone is clearly apparent on the first day of culture as a region of disintegrating cells below the peritoneal surfaces. By the third day only an occasional cell can be found in the necrotic zone. The surviving liver cells in the core are characterized by loss of cytoplasmic basophilia compared with the original liver and a clumping of nuclear chromatin. The clumping of nuclear chromatin has also been observed in organ cultures of mouse liver (MacDougall, 1964).

Fig. 1. DNA content of chick-embryo liver segments during culture. Five culture dishes were prepared from each of 11-, 15- and 19-day embryo livers. DNA content was then determined in one dish from each age group after 0, 1, 3, 6 and 8 days' culture. This experiment was carried out 12 times. No significant differences were observed between the age groups and the results were therefore pooled so that each point on the graph represents the average of 36 determinations, 12 from each age group. The vertical lines represent the s.e.m. of each point. The culture medium contained (by vol.) 40% of horse serum, 40% of Tyrode's solution and 20% of embryo extract (see the Methods section).

The DNA content of chick-embryo liver decreases most rapidly during the first 3 days of culture, more slowly thereafter (Fig. 1). This agrees well with the microscopic observations, which revealed that the cell-free necrotic zone is established within the first 3 days of culture, while a cellular capsule is formed and increases largely after this time.

The question arises whether UDP-glucuronyltransferase activity is present in the core cells or in the capsule cells; cores and capsules were dissected apart and assayed separately; all activity was recovered in the cores, none in the capsules. To approach this question in another way, segments of spleen from 19-day-old chick embryos were cultured for 8 days in Tyrode's solution, embryo extract and horse serum. These segments became encapsulated in the same manner as liver segments, but UDP-glucuronyltransferase activity was not observed in them after culture. UDP-glucuronyltransferase activity is not present in the spleen of the chick and chick embryo (Dutton & Ko, 1966).

UDP-glucuronyltransferase activity is found in the particulate fraction of homogenized chick and chick-embryo liver (Dutton & Ko, 1966). The enzyme remained in the particulate fraction after 8 days' culture.

Fig. 2. UDP-glucuronyltransferase activity in chick-embryo liver segments during culture. Five culture dishes were prepared from each of 11-, 15- and 19-day embryo livers. Enzyme activity was then determined in one dish from each age group after 0, 1, 3, 6 and 8 days' culture. This experiment was carried out five times. No significant differences were observed between the age groups and the results were therefore pooled so that each point in the graph represents the average of 15 determinations, five from each age group. The vertical lines represent the s.e.m. of each point. The main curve represents enzyme activity on a DNA basis, the inset graph that per culture dish (see the Methods section). The culture medium contained (by vol.) 40% of horse serum, 40% of Tyrode's solution and 20% of embryo extract.

Increase in UDP-glucuronyltransferase activity in chick-embryo liver during culture

Segments of liver from 11-, 15- and 19-day-old chick embryos were cultured for various periods (0, 1, 3, 6 and 8 days) in a medium of Tyrode's solution, embryo extract and horse serum. Livers of the three ages behaved in the same way. UDP-glucuronyltransferase activity increased from very low values at initial culture to values on the eighth day comparable, on a DNA basis, with those in the hatched chick. Development of activity was slower in the first 3 days of culture, more rapid thereafter (Fig. 2). The relatively slow development of UDP-glucuronyltransferase activity during the first 3 days of culture occurred during the period of most rapid cell death (Fig. 1).

UDP-glucuronyltransferase activity towards o-aminophenol can survive in segments of chick or chick-embryo liver cultured in horse serum, embryo extract and Tyrode's solution for at least

Table 1. *Medium requirements for development of UDP-glucuronyltransferase activity in chick-embryo liver during culture*

Segments of liver from 19-day-old chick embryos were cultured in various media. Enzyme activity was determined after 8 days and is expressed as $\mu\text{g. of } o\text{-aminophenyl glucuronide/100 } \mu\text{g. of DNA/30 min.}$

Composition of medium (% by vol.)

Medium	Tyrode's solution	Eagle's minimal medium	Embryo extract	Horse serum	Bovine serum	Foetal bovine serum	Bovine serum ultrafiltrate	Activity ($\mu\text{g. of } o\text{-aminophenyl glucuronide/100 } \mu\text{g. of DNA/30 min.}$)
Expt. 1								
A	40	—	20	40	—	—	—	28.3, 34.0
B	40	20	—	40	—	—	—	37.2, 37.8
C	60	—	—	40	—	—	—	26.9, 32.3
D	80	—	20	—	—	—	—	0, 0
E	40	—	20	—	—	—	40	0, 0
Expt. 2								
F	40	—	20	40	—	—	—	11.0, 11.5
G	40	—	20	—	40	—	—	8.5, 11.3
H	60	—	—	—	40	—	—	16.0, 9.5
I	60	—	—	—	—	40	—	0, 0

Table 2. *Differences in the development of UDP-glucuronyltransferase activity in 11- and 19-day chick-embryo liver cultured with foetal serum*

Segments of liver were cultured in a medium containing (by vol.) 40% of either horse serum or foetal bovine serum, 40% of Tyrode's solution and 20% of embryo extract. UDP-glucuronyltransferase activity was determined after 8 days' culture and is expressed as $\mu\text{g. of } o\text{-aminophenyl glucuronide/100 } \mu\text{g. of DNA/30 min.}$

Age of embryo	Serum	Activity ($\mu\text{g. of } o\text{-aminophenyl glucuronide/100 } \mu\text{g. of DNA/30 min.}$)
Expt. 1 (11 days)	Horse	26.7, 21.6
	Foetal bovine	23.4, 23.1
Expt. 2 (19 days)	Horse	18.2, 16.5
	Foetal bovine	0, 0

24 days; this was the longest period tested. Embryo extract could be omitted from the culture medium without affecting development of enzyme activity (Table 1) and without decreasing the survival of liver cells as judged by microscopic examination. Omission of serum from the medium resulted in rapid autolysis. An ultrafiltrate of serum would not serve in place of whole serum.

Segments of liver from foetal (16-day) and infant (5-day) mouse, cultured for 8 days in Tyrode's solution and horse serum, showed morphological changes similar to those observed in liver segments from the chick and chick embryo. The small amount of UDP-glucuronyltransferase activity towards *o*-aminophenol present in foetal and infant mouse liver was still evident after culture but there was no indication of increased activity. Segments from adult mouse liver after 8 days' culture had not curled up, and had lost all UDP-glucuronyltransferase activity.

UDP-glucuronyltransferase activity in chick and chick-embryo liver after culture with foetal bovine serum

Development of UDP-glucuronyltransferase activity in liver from 11- and 19-day-old chick embryos. In a medium of horse serum, Tyrode's solution and embryo extract, segments of liver from 11- and 19-day-old chick embryos develop the same amount of UDP-glucuronyltransferase activity during culture (Fig. 2). If foetal bovine serum takes the place of horse serum in the culture medium, enzyme activity fails to develop in cultures of 19-day chick-embryo livers, but develops rapidly in liver segments from 11-day-old chick embryos (Table 2). Liver segments cultured with foetal bovine serum and with horse serum show the same morphological and histological changes. Segments curl and form spheres with equal rapidity; capsule formation and cell survival are also the same.

Fig. 3. UDP-glucuronyltransferase activity in chick and chick-embryo liver after culture with foetal bovine serum. Segments of liver from various developmental stages were cultured for 8 days in a medium containing (by vol.) 40% of foetal bovine serum and 60% of Tyrode's solution. Segments of liver from the same stages were also cultured in a medium in which the foetal bovine serum was replaced with horse serum. In the Figure, for each stage examined, enzyme activity is expressed as activity with foetal bovine serum divided by activity with horse serum; each point in the graph therefore represents a pair of culture dishes. When enzyme activity with foetal bovine serum and horse serum were within 20% of each other, the differences was considered not significant and the ratio was plotted as 100%.

Development of UDP-glucuronyltransferase activity in liver from chick embryos of various ages. Segments of liver from various embryonic stages between 8 and 20 days and from 11-day-old hatched chicks were cultivated for 8 days in horse serum and Tyrode's solution or in foetal bovine serum and Tyrode's solution. In segments from 8–14-day-old chick embryos UDP-glucuronyltransferase activity developed equally well in both media. At 15 and 16 days of embryo age, segments developed less activity in foetal serum. At 17 days or more, enzyme activity failed to develop at all in segments cultured in foetal serum; also enzyme activity in segments from hatched chicks disappeared completely in foetal serum (Fig. 3). Thus, a requirement for adult serum develops between 15 and 17 days of embryonic life.

Induction of UDP-glucuronyltransferase activity in liver from 19-day-old chick embryos by horse serum and foetal bovine serum

Liver cultured with foetal bovine serum and subsequently with horse serum. Segments of liver from 19-day-old chick embryos were cultured in foetal

Fig. 4. Development of UDP-glucuronyltransferase activity in liver from 19-day-old chick embryos cultured with foetal bovine serum, then with horse serum. Liver segments were cultured in medium containing (by vol.) 40% of foetal bovine serum, 40% of Tyrode's solution and 20% of embryo extract. The UDP-glucuronyltransferase activities in some culture dishes were estimated after 0, 3, 5 and 10 days (●). The liver segments in other culture dishes were transferred to a medium containing (by vol.) 40% of horse serum, 40% of Tyrode's solution and 20% of embryo extract after 0, 3 and 5 days, and their UDP-glucuronyltransferase activities estimated on the tenth day (○). In the Figure, enzyme activities are expressed as percentages of the activity exhibited by liver segments after culture with horse serum for the entire 10-day period. Continuous line indicates period of culture in the foetal bovine serum medium, broken line that in the horse serum medium. Each point is the average of three culture dishes; for each point the S.E.M. was less than 10% of the mean.

bovine serum, Tyrode's solution and embryo extract. As expected (see Tables 1 and 2), UDP-glucuronyltransferase activity did not appear during 8 days' culture. However, UDP-glucuronyltransferase activity began to develop if the liver segments were transferred to a culture medium containing horse serum in place of foetal bovine serum (Fig. 4). Evidently, the capacity of liver segments from 19-day-old chick embryos to develop UDP-glucuronyltransferase activity in horse serum survives undiminished in foetal bovine serum for at least 5 days, the longest period tested.

Liver cultured with horse serum and subsequently with foetal bovine serum. The UDP-glucuronyltransferase activity reached in 19-day chick-embryo liver segments after 1 or 2 days with horse

Fig. 5. Development of UDP-glucuronyltransferase activity in liver from 19-day-old chick embryos cultured with horse serum, then with foetal bovine serum. Liver segments were cultured in medium containing (by vol.) 40% of horse serum, 40% of Tyrode's solution and 20% of embryo extract. The UDP-glucuronyltransferase activities in some culture dishes were estimated after 1, 2, 3 and 8 days (○). Liver segments in other culture dishes were transferred to medium containing (by vol.) 40% of foetal bovine serum, 40% of Tyrode's solution and 20% of embryo extract after 1, 2 and 3 days and their UDP-glucuronyltransferase activities estimated on the eighth day (●). In the Figure, enzyme activities are expressed as the percentages of the activity exhibited by liver segments after culture with horse serum for the entire 8-day period. Each point is the average of three culture dishes; for each point the s.e.m. was less than 10% of the mean.

serum is maintained without further increase after transfer to a foetal-serum medium. After 3 days with horse serum, the UDP-glucuronyltransferase activity continues to increase when the tissue is transferred to a foetal-serum medium (Fig. 5). Thus liver segments from 19-day-old chick embryos require 3 days with adult serum before development of UDP-glucuronyltransferase activity becomes independent of a continuous adult-serum environment.

Induction of UDP-glucuronyltransferase activity in liver from 19-day-old chick embryos by various fractions of adult and foetal sera

Segments of liver from 19-day-old chick embryos were cultured in a medium in which the horse serum had been dialysed for 48 hr. against 0.9% sodium chloride at 4° (see the Methods section). This did not affect the usual morphological and histological changes occurring in the liver segments during culture but UDP-glucuronyltransferase activity failed to develop. When an ultrafiltrate from adult bovine serum was added to the medium, the ability of the medium to induce UDP-glucuronyltransferase activity returned (Table 3). Apparently the ultrafiltrate of adult bovine serum contains a factor or factors necessary to induce UDP-glucuronyltransferase activity in organ cultures of 19-day chick-embryo liver.

Segments of liver from 19-day-old chick embryos were cultured in a medium with dialysed foetal bovine serum in place of horse serum. The usual morphological and histological changes occurred but UDP-glucuronyltransferase activity did not develop. When an ultrafiltrate of adult bovine serum was added to the medium, UDP-glucuronyltransferase activity developed as rapidly as with

Table 3. Ability of various fractions of foetal and adult sera to support development of UDP-glucuronyltransferase activity in cultures of 19-day chick-embryo liver

Segments of liver were cultured in various media and UDP-glucuronyltransferase activity was determined after 8 days and is expressed as $\mu\text{g. of } o\text{-aminophenyl glucuronide/100 } \mu\text{g. of DNA/30 min.}$

Medium	Composition of medium (% by vol.)							Activity ($\mu\text{g. of } o\text{-aminophenyl glucuronide/100 } \mu\text{g. of DNA/30 min.}$)
	Tyrode's solution	Embryo extract	Horse serum	Horse serum (dialysed)	Adult bovine serum ultrafiltrate	Foetal bovine serum	Foetal bovine serum (dialysed)	
A	40	20	40	—	—	—	—	19.3, 17.1
B	40	20	—	40	—	—	—	0, 0
C	—	20	—	40	40	—	—	33.0
D	40	20	—	—	—	—	40	0
E	—	20	—	—	40	—	40	21.6
F	40	20	—	—	—	40	—	0
G	—	20	—	—	40	40	—	2.3, 3.4, 4.5

horse serum. Evidently the non-diffusible parts of foetal and adult serum are equally capable of supporting cell survival and, in the presence of an ultrafiltrate of adult bovine serum, equally capable of supporting development of UDP-glucuronyltransferase activity.

Addition of an ultrafiltrate of adult bovine serum to whole foetal bovine serum permits the development of only a small amount of UDP-glucuronyltransferase activity. This is in contrast with the efficacy of the ultrafiltrate of bovine serum when added to dialysed foetal bovine serum. Apparently, the diffusible part of foetal bovine serum inhibits enzyme development in cultures of 19-day chick-embryo livers.

DISCUSSION

The results indicate that chick (*Gallus gallus*) liver after 8 days' culture, like the original tissue, is able to conjugate *o*-aminophenol with glucuronic acid and this synthesis utilizes the enzyme UDP-glucuronyltransferase. Activity of this enzyme survives at low values in cultured foetal and infant mouse liver, but disappears from cultures of adult mouse liver. The only previous study of UDP-glucuronyltransferase activity in organ cultures employed adult mammalian liver under hyperbaric oxygen, where enzyme activity rapidly disappeared (MacDougall, 1964). Chick liver therefore appears to be more useful than mammalian liver for studying UDP-glucuronyltransferase activity in isolated tissues over an extended period.

The simplest conditions for maintaining UDP-glucuronyltransferase activity in isolated chick liver are in a medium containing (by vol.) 40% of horse serum and 60% of Tyrode's solution at 37° in an atmosphere of air. The medium could remain unchanged for 8 days without obvious effect. In most of our experiments embryo extract had been included in the medium and the cultures incubated under a carbon dioxide-air mixture (1:19, v/v).

Use of this procedure to culture chick-embryo liver allowed some comparisons with enzyme development *in vivo*. As previously shown, UDP-glucuronyltransferase activity is present to a small extent in embryonic liver, falls virtually to zero just before hatching and on emergence at 21 days of incubation increases rapidly to high values (Dutton, 1963; Dutton & Ko, 1966). If liver is removed from the embryo on the eighth day of incubation and placed in culture, UDP-glucuronyltransferase activity begins to increase, reaching adult values 6–8 days afterwards. This precocious development resembles the rapid increase in tryptophan pyrrolase and glucose 6-phosphatase activity in mammalian foetuses after premature delivery (Nemeth, 1959; Dawkins, 1961, 1966).

Development of UDP-glucuronyltransferase activity in cultured chick-embryo liver requires certain serum factors in the medium. The requirements change with the age of the embryo. Liver from the early embryo (15 days of incubation or less) exhibits an increase of UDP-glucuronyltransferase activity with either foetal or adult serum in the medium, and liver from older embryos (17 days of incubation or more) only with adult serum. Whether this phenomenon reflects that changeover from an embryonic to an adult enzyme previously suggested cannot yet be decided (Dutton & Ko, 1966; Ko & Nemeth, 1966b).

The development of enzyme activity in cultures of liver from older embryos depends on diffusible factors in adult serum. Also diffusible factors in foetal serum appear to inhibit enzyme development in these same cultures. This susceptibility of the enzyme-forming system to small molecules may be part of the mechanism regulating the rapid increase of UDP-glucuronyltransferase activity at hatching.

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REFERENCES

- Brown, A. K. & Zeulzer, W. W. (1958). *J. clin. Invest.* **37**, 332.
- Burton, K. (1956). *Biochem. J.* **62**, 315.
- Dawkins, M. J. R. (1961). *Nature, Lond.*, **191**, 72.
- Dawkins, M. J. R. (1966). *Brit. med. Bull.* **22**, 27.
- Dutton, G. J. (1959). *Biochem. J.* **71**, 141.
- Dutton, G. J. (1963). *Ann. N.Y. Acad. Sci.* **111**, 259.
- Dutton, G. J. (1966). *Biochem. Pharmacol.* **15**, 947.
- Dutton, G. J. & Grieg, C. G. (1957). *Biochem. J.* **66**, 52P.
- Dutton, G. J. & Ko, V. (1966). *Biochem. J.* **99**, 550.
- Dutton, G. J. & Storey, I. D. E. (1962). In *Methods in Enzymology*, vol. 5, p. 159. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Kay, E. R. M., Simmons, N. S. & Dounce, A. L. (1952). *J. Amer. chem. Soc.* **74**, 1724.
- Ko, V. & Nemeth, A. M. (1966a). *Biochem. J.* **100**, 27P.
- Ko, V. & Nemeth, A. M. (1966b). *Biochem. J.* **100**, 27P.
- Kretchmer, N. (1959). *Pediatrics, Springfield*, **23**, 606.
- Levy, G. A. (1952). *Biochem. J.* **52**, 464.
- Levy, G. A., McAllan, A. & Marsh, C. A. (1958). *Biochem. J.* **69**, 22.
- Levy, G. A. & Storey, I. D. E. (1949). *Biochem. J.* **44**, 295.
- MacDougall, J. D. B. (1964). *Helgoländ. wiss. Meeresunters.* **9**, 312.
- Nemeth, A. M. (1954). *J. biol. Chem.* **208**, 773.
- Nemeth, A. M. (1959). *J. biol. Chem.* **234**, 2921.
- Nemeth, A. M. (1961). *Biochim. biophys. Acta*, **48**, 189.
- Nemeth, A. M. & de la Haba, G. (1962). *J. biol. Chem.* **237**, 1190.
- Nemeth, A. M. & Nachmias, V. T. (1958). *Science*, **128**, 1085.
- Ogur, M. & Rosen, G. (1950). *Arch. Biochem.* **25**, 262.
- Schneider, W. C. (1945). *J. biol. Chem.* **161**, 293.
- Trowell, O. A. (1959). *Exp. Cell Res.* **16**, 118.
- White, P. R. (1963). *The Cultivation of Animal and Plant Cells*, p. 66. New York: The Ronald Press Co.