Precocious Development of Glucuronidating and Hydroxylating Enzymes in Chick Embryos Treated with Pituitary Grafts

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1. Initiation of precocious development of UDP-glucuronyltransferase by an endogenous factor is reported for the first time. 2. This development occurs in chick embryo liver and kidney after grafting of the cephalic lobe of chicken pars-distalis pituitary tissue on to the chorioallantoic membrane, and in liver results in a rise in the enzyme activity from virtually zero to 'adult' values. Aniline hydroxylase also precociously develops in the liver of grafted embryos, its activity rising from one-third to the full adult value. Specific activities of glucose 6-phosphatase, cytochrome *P*-450 and NADPH-cytochrome *c* reductase did not significantly change. 3. The response of the transferase does not require the presence of host pituitary gland nor, apart from 1 day's necessary initiation, the presence of the graft itself. 4. The host becomes competent to respond on the 14th day of incubation; response continues for at least 3 days after removal of the graft, and for 2 days in the isolated liver. Grafting of embryonic pars distalis younger than 17 days does not evoke a response in the host liver. 5. Secretion of the pituitary factor increases suddenly some 24-48 h before the naturally developing surge in liver UDP-glucuronyltransferase activity and may be responsible for initiating this rise *in vivo*. 6. The factor is probably not a growth or luteinizing hormone; its nature and the likelihood of a secondary hormone acting directly on the liver are discussed.

Induction of many enzymes during the natural development of an organism appears to be triggered by hormone action, especially over the perinatal period (Greengard, 1971; Greengard & Thorndike, 1974). Two important microsomal enzyme systems developing perinatally are those responsible for hydroxylation and glucuronidation. These processes 'detoxicate' a great range of endogenous and xenobiotic molecules, and their timely development is of obvious importance to the organism. Factors initiating this development have been much studied; however, although xenobiotics such as barbiturates or aryl hydrocarbons are well known to precociously induce these enzyme systems, the endogenous inducing systems have remained obscure.

The embryonated egg is free of direct maternal influence and amenable to environmental control. UDP-glucuronyltransferase (E.C. 2.4.1.17) activity rapidly increases from virtually zero to adult values in chick embryo liver and kidney on hatching at 21 days (Dutton & Ko, 1966); aniline hydroxylase (EC 1.14.14.1) activity increases throughout later embryonic development, reaching a peak at above adult values in chick liver immediately on hatching (Drummond *et al.*, 1972; Wishart & Dutton, 1975; G. J. Wishart & G. J. Dutton, unpublished work). Activity of both these enzymes in embryonic tissues *in ovo* can be increased to adult values or above by

xenobiotics such as phenobarbital (Wishart & Dutton, 1975). We have now investigated the role of endogenous factors in the development of these detoxicating enzymes.

We report here the precocious development of both enzyme systems after the grafting of pituitary glands from hatching and hatched chick on to the chorioallantoic membrane of a normal embryonated egg. Identification of the responsible factor(s) has not yet been possible, but information is presented on its source, its period of action and on the competence of the embryo to respond to it. A preliminary account of part of this work has already appeared (Wishart & Dutton, 1974).

Materials and Methods

Mammalian follicle-stimulating hormone (batch FSH-S10), prolactin (batch P-S10), luteinizing hormone (batch LH-S19) and thyroid-stimulating hormone (batch TSH-B7) were all kindly given by the Hormone Distribution Program, N.I.H., Bethesda, Md., U.S.A. Growth hormone was from Calbiochem, Hereford U.K., and adrenocorticotrophic hormone from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.; L-thyroxine, tri-iodo-L-thyronine, insulin, testosterone, corticosterone, cortisol, glucagon, adrenaline and dibutyryl cyclic AMP

were all from Sigma. Semi-purified preparations of chicken follicle-stimulating hormone, luteinizing hormone, thyroid-inducing hormone and prolactin (Scanes & Follett, 1972; Bolton *et al.*, 1974) were kindly given by Dr. C. L. Scanes, University of Leeds.

Embryonated eggs and chickens were of White Leghorn strain obtained from Eastwood Hatcheries, Kinglassie, Fife, U.K. and incubated from day 0 at 39°C and 55% relative humidity; as breeding colonies were changed at regular intervals and slight physiological differences (e.g. in incubation period and viability) were characteristic for specific colonies, note was taken of change of colony. Organ culture and injection *in ovo* were as described by Burchell *et al.* (1972) and Wishart & Dutton (1975) respectively. Enzyme assays were as follows: for UDP-glucuronyltransferase, with *o*-aminophenol (Ko *et al.*, 1967; Skea & Nemeth, 1969) or *p*-nitrophenol (Isselbacher *et al.*, 1962) as substrates; aniline hydroxylase (Hilton & Sartorelli, 1970); glucose 6-phosphatase (Zakim & Vessey, 1973); cytochrome *P*-450 (Omura & Sato, 1964); NADH-cytochrome *c* reductase (Dallner *et al.*, 1966). Measurement of glucuronidation of *o*-aminophenol in liver slices was as described by Dutton & Storey (1962). Results quoted are means of assay duplicates differing less than $\pm 5\%$. Reaction rates were linear in all assays. Protein was estimated by the method of Lowry *et al.* (1951) and bovine serum albumin (Sigma) was used as standard.

Results

Injection of hormones

The environment within the egg appears to repress the expression of UDP-glucuronyltransferase activity (Ko *et al.*, 1967; Skea & Nemeth, 1969; Dutton, 1973, 1975). Nevertheless the injection of certain xenobiotics can overcome this repression as early as 96 h of age (Wishart & Dutton, 1975). Single or multiple injections of insulin, dibutyl cyclic AMP, glucagon, adrenaline, thyroxine, tri-iodothyronine, corticosterone, cortisol, oestriol or testosterone were therefore made on to the exposed chorioallantoic membrane of embryos of various ages, at doses up to lethal values. Daily doses of mammalian thyroid-stimulating hormone (1 U.S.P. unit), adrenocorticotrophic hormone (22 i.u.), growth hormone (0.5 U.S.P. unit), luteinizing hormone, follicle-stimulating hormone and prolactin (1 N.I.H. unit each) were similarly injected for 2–3 days, at up to 6 h intervals. Semi-purified chicken pituitary hormones and extracts or homogenates of fresh chicken pituitary were also applied. Despite enzymic or morphological evidence of successful absorption of many of these hormones [e.g. stimulation of liver phenylalanine hydroxylase activity after cortisol treatment (M. McGee-Sudjic & G. H. Wishart, unpublished work) and thyroid hypertrophy after thyroid-stimulating-hormone treatment],

results for UDP-glucuronyltransferase and aniline hydroxylase were largely negative; occasional positive results obtained with thyroxine or corticosterone were of small extent and were not reproducible. While other methods of exposure to known compounds were being developed, we investigated by more physiological means the endocrine status necessary for control of these enzymes *in ovo*.

Hypophysectomy of embryos

Embryos were hypophysectomized at 36 h as described by Betz (1965). Morphological stigmata indicative of successful hypophysectomy were evident, but UDP-glucuronyltransferase and aniline hydroxylase remained at normal embryonic activities in liver and kidney up to day 16, when last examined. On subsequent culture these livers, like those of non-hypophysectomized controls, spontaneously developed high UDP-glucuronyltransferase activity (see Burchell *et al.*, 1972). Possession of a functioning pituitary gland between 36 h and 16 days is therefore not responsible for restricting these enzymes to low activities in the embryo, nor for permitting subsequent spontaneous rise in transferase activity in cultured liver cells.

Grafting of endocrine tissues

To ensure a more physiological release of hormones and possibly of unknown factors into the embryonic circulation, various adult (6-week-old) cockerel tissues were grafted, up to lethal response, on to the chorioallantoic membrane of 12-day embryos. Skeletal muscle, brain, liver, thyroid, adrenal gland and testis were ineffective, but pituitary gland produced a consistently positive response, stimulating both UDP-glucuronyltransferase and aniline hydroxylase in the host tissues (see Wishart & Dutton, 1974). This response was time-dependent, being detectable 3 days after grafting and reaching adult values by day 4 (see Wishart & Dutton, 1974). UDP-glucuronyltransferase activity was stimulated both in liver and in kidney tissue, and towards both *o*-aminophenol and *p*-nitrophenol; the specific activity of the enzyme increased in both homogenates and microsomal fractions from liver (Table 1). The increase was observed with *o*-aminophenol concentrations of 0.1–0.6 mM and with UDP-glucuronic acid concentrations of 1.0–6.0 mM. Overall glucuronidation, as observed in liver slices, also increased to adult values (Table 1); the enzymes involved in glucuronidation, other than UDP-glucuronyltransferase, already exist at adult activity in 12-day chick embryo liver (Fyfe & Dutton, 1973, 1975). In homogenates and microsomal fractions from host livers, the specific activity of aniline hydroxylase increased some threefold to the adult values normally attained after hatching (Table 1). Cytochrome *P*-450, NADPH-cytochrome *c* reductase and glucose 6-phosphatase activities

Table 1. *Effect of grafted pituitary on enzymes in host liver*

Chick embryos were grafted on day 12 of incubation with one 6-week-old-cockerel pituitary gland. Controls were sham operated. Embryo tissues were assayed for enzyme activity 4 days later. Methods are as described in the text. Where the s.e.m. is given, the number of animals is in parentheses. —, Not done.

	UDP-glucuronyltransferase activity						Aniline hydroxylase activity (nmol of <i>p</i> -aminophenol formed per mg of protein)		
	As nmol of <i>o</i> -aminophenyl glucuronide formed/h				As nmol of <i>p</i> -nitrophenyl glucuronide formed/h per mg of protein				
	Per mg of protein			Per mg dry wt. of liver. Liver slices	Liver		Liver homogenate	Liver microsomal preparation	
	Liver homogenate	Liver microsomal preparation	Kidney homogenate		Liver homogenate	Liver microsomal preparation			
Embryo or bird									
Control embryo	0.6±0.4 (5)	0.4±0.3 (5)	7.0, 7.5	0.18, 0.14	3.6	9.0	0.9±0.2 (4)	3.2±0.5 (4)	
Grafted embryo	33±15 (7)	26±9 (5)	15.6±1.6 (3)	3.5, 2.3	15.5	62.1	3.4±0.6 (5)	9.8±2.8 (4)	
Adult cockerel (6 weeks old)	42±10 (12)	26.8±2.7 (3)	21.1±1.5 (5)	3.5	18.9	—	2.8, 3.4	—	

Table 2. *Stimulation of UDP-glucuronyltransferase development in embryo liver by grafts from different areas of cockerel pituitary*

Pituitary tissue, in the form of caudal lobe, cephalic lobe, or the whole anterior pituitary, was grafted from 6-week-old cockerels on to the chorioallantoic membrane of 12-day embryos. Then 4 days later UDP-glucuronyltransferase activity was assayed in homogenates of host liver. Where s.e.m. is given, the number of experiments is in parentheses.

Source of grafted tissue	Glucuronide formed (nmol/h per mg of host liver protein)
No grafted tissue	0–2.8 (range of 30 results)
Caudal lobe	0.2±0.1 (6)
Cephalic lobe	22.5±7.5 (4)
Entire pars distalis	32.9±15.1 (7)

were not obviously changed in host liver after the grafting. Living pituitary glands connected to the host circulation were required for these stimulatory effects; glands dead or otherwise not forming a vascularized graft were ineffective. Presumably, therefore, the gland secreted some responsible factor.

Region of pituitary gland secreting the factor

Pars-distalis samples from 6-week-old cockerels were divided down the constriction into caudal and cephalic lobes; these regions, described by Brasch & Betz (1971) at 1 day, are clearly distinguishable at 6 weeks. The lobes were then grafted on to the chorioallantoic membrane of separate groups of 12-day embryos: as seen from Table 2, only the cephalic region stimulated development of UDP-glucuronyltransferase activity. If two caudal lobes, whose wet weight exceeded that of one cephalic lobe, were grafted, there was no obvious UDP-glucuronyltransferase response; two cephalic lobes from a 6-week-old

bird usually killed the embryo. This confirms the observation of Brasch & Betz (1971) that cephalic and caudal lobes are qualitatively different, and suggests that luteinizing hormone, which occurs in the caudal lobe, and growth hormone, occurring in both lobes (Brasch & Betz, 1971), are not responsible for initiating the rise in UDP-glucuronyltransferase, whereas adrenocorticotrophic hormone, thyroid-stimulating hormone or follicle-stimulating hormone derived from the cephalic lobe, could be responsible.

Age of pituitary secreting the factor

Although gland for gland, pituitaries from 6-week-old cockerels were the most effective, younger chick glands were as good or even better sources of the stimulating factor on a wet-weight basis. The wet weight of pituitary glands changed little from 18 days of incubation to 6 days of hatched life, but at 17–18 days of incubation they were poor sources of the factor. However, in glands from embryos of 19 days a sudden rise in the ability to stimulate UDP-glucuronyltransferase activity began, which reached a peak over the hatching period (Fig. 1). The onset of this surge in stimulatory power preceded the surge of UDP-glucuronyltransferase activity (Fig. 1) and of aniline hydroxylase activity in normal hatched-chick liver, suggesting that, in untreated birds, increased secretion of the factor from their own pituitary glands could be responsible for the natural surge in UDP-glucuronyltransferase activity and for the peak in aniline hydroxylase activity. Between 19 and 20 days the concentration of the factor began to increase, but the transferase was never present. At 21 days the concentration of the factor was high in almost all birds and at this age the transferase began to appear. During free life both factor and transferase were high together, except in the very few instances where the

Fig. 1. Relation between the efficacy of pituitary grafts in stimulating liver UDP-glucuronyltransferase development in 12-day embryos and the natural development of the enzyme over hatching

○, Transferase activity in chick liver from 15 days' embryonic life to 6 days' free life. ●, Transferase activity in livers of 16-day host embryos, which at 12 days had undergone chorioallantoic grafting of a pituitary gland; this gland was donated by a bird of the age shown on the abscissa (from 15 days' embryonic life to 6 days' free life). In most cases it was the livers of these donor birds that supplied the ○ values. Transferase activity is expressed on a per mg of liver protein basis for both ○ and ●, and activity stimulated in host livers by the graft (●) was calculated as if 1 mg wet wt. of gland had been grafted; the actual wet weight of the gland grafted increased from 0.5 mg (15-day embryo) to 0.9–1.0 mg (18-day embryo–6-day chick). S.E.M. values are shown. For ● the number of birds is in parentheses; for ○ the number was 5 or above. 'Pipping' of the shell began early on day 20; hatching was complete by end of day 21.

graft appeared not to have taken and could not yield evidence of the factor.

Apart from a single result quoted earlier (Wishart & Dutton, 1974), glands from embryos of 12 to 16 days' incubation apparently did not secrete the factor when grafted (up to five in number) on to a 12-day-embryo chorioallantoic membrane; e.g. five glands from 15-day embryos (total wet wt. equivalent to two 21-day glands) evoked no increase in UDP-glucuronyltransferase activity in host tissues. From 17 days of donor age, such multiple grafting did evoke a rise in transferase activity.

'Threshold' effect in secretion of factor

Although single glands from embryos of 17–18 days evoked little response in host liver UDP-

glucuronyltransferase activity, a marked and far-above-double response was observed when two such glands were grafted. Presumably some opposition by the host to initiation of UDP-glucuronyltransferase development by pituitary grafting must be overcome.

Age of host responding to factor

No stimulation of UDP-glucuronyltransferase activity could be found up to 6 days afterwards when one pituitary from a 6-week-old cockerel or two from a 1-day-old chick were grafted on to the chorioallantoic membrane of embryos of 10 days or less, even though such grafting provoked morphological changes in some of these hosts, such as dwarfing, oedema and liver necrosis, conditions we had already noted after large injections of corticosteroid at this age.

Grafts performed on day 14 evoked a detectable response on day 16. If grafts were performed on day 12, a response was not detectable until day 15. To pinpoint the age of competence for response, grafts were removed from the membrane after various periods. Pituitaries were grafted on day 11 and removed on days 12, 13 or 14. UDP-glucuronyltransferase activity was assayed in host liver on day 16. Response was only evident in hosts bearing the graft for 3 days. However, if glands were grafted on day 12 and removed on days 13, 14 or 15, response was evident in the host bearing the graft for only 2 days (Table 3). Therefore, allowing 1 day for vascularization of the graft (New, 1966), it seems that embryo liver becomes

Table 3. Effect of removing grafted glands from chorioallantoic membrane after sojourn for various periods

Two 1-day chick pituitaries were grafted on to the chorioallantoic membrane of 11- or 12-day embryonated eggs. Grafts were removed 1, 2 or 3 days later. UDP-glucuronyltransferase activity was assayed in homogenates of host livers 4 days after the original grafting. Removal was by surgical excision or electrocauterization of the grafted gland and the surrounding chorioallantoic membrane to a diameter of 3–4 widths of the graft; lack of enzyme activity in controls (e.g. sojourns for 1–2 days on 11-day eggs) indicated that removal was complete. Where S.E.M. is given the number of experiments is in parentheses.

Age of host at grafting (days)	Sojourn of graft on membrane (days)	<i>o</i> -Aminophenyl glucuronide formed (nmol/h per mg of host liver protein)
11	1	1.1±0.5 (4)
	2	1.6±0.6 (5)
	3	24.2, 28.0
12	1	2.0±1.2 (4)
	2	54.6±13.1 (8)
	3	21.0±7.7 (3)

competent to respond to the pituitary factor on day 14 of incubation.

Duration of response by host liver

Hosts which had been grafted at 12 days did not usually survive beyond 4–5 days, usually because their premature attempts to retract the yolk-sac (see below) burst the sac membranes. If their liver was removed at 16 days and cultured as fragments in a chemically defined medium, its transferase activity continued to rise steeply for 1–2 days, then fell to a minimum, rising thereafter in the manner expected of cultured embryo liver (Ko *et al.*, 1967; Burchell *et al.*, 1972). For example, two 16-day host livers had reached activities *in situ* of 16 and 28 nmol of glucuronide formed/h per mg of protein. The livers were then cultured, and over 1–2 days the activities had reached a peak at 55 and 118 of these units respectively; by 3–4 days, activities had fallen to 26 and 24 units, and by 6 days' culture had risen again to 38 and 42 units, somewhat above the mean (24 units) reached by ungrafted-embryo liver after 6 days' culture. This 'carry-over' of an existing induction during the first 2 days of culture, its fall and then its succession by the 'normal' culture rise was also observed for transferase activity induced in embryo livers by exposure *in ovo* to phenobarbital. Presumably the agents responsible for increasing the transferase activities *in vivo* survive during culture similarly in both cases. If 4-day hatched chick liver is cultured as organ fragments under these conditions, a brief steep initial rise above adult activities is again noted, suggesting the presence of a similar inducing process in the cell, possibly initiated by the pituitary factor at hatching.

Duration of secretion of factor by grafted gland

The 1-day chick pituitary gland can continue secreting the factor for at least 5 days on the chorioallantoic membrane of a host aged 12–16 days. The 1-day chick pituitaries were grafted on to the membrane of a 12-day embryo, removed on day 15 and regrafted on to another 12-day host. Liver UDP-glucuronyltransferase was assayed on day 16 of this second embryo's life. As 2 days of this second sojourn on the membrane would have been required to evoke a response (see above), the pituitaries can be presumed to secrete factor for at least 5 days on the membrane. Further, similar pituitaries cultured *in vitro* for 2 days, and then grafted on to the membrane, could evoke a response in UDP-glucuronyltransferase activity at least 2 days later (J. Leakey, unpublished work).

Role of the host hypothalamus in response to the factor

Although the chick pituitary can perform many of its roles when grafted ectopically (Brasch & Betz, 1971), the hypothalamus is required for certain functions, such as response to stress (Wise & Frye,

1973). To check on the role of the host's hypothalamus in response to the enzyme-stimulating factor, two 1-day chick pituitaries were grafted on to the chorioallantoic membrane of 13-day embryos hypophysectomized by partial decapitation at 36h; these embryos bore characteristic stigmata of hypophysectomy and possessed no pituitary gland on dissection. By 16 days, UDP-glucuronyltransferase activity had risen exactly as in similarly grafted non-hypophysectomized controls. Clearly the factor initiating transferase development can be produced by isolated pituitary tissue independently of the host's hypothalamus, and presumably is not a response by the host to stress.

Role of the host liver in response to the factor

When 12-day embryo liver fragments were cultured on the chorioallantoic membrane of a 12-day embryonic host together with a pituitary graft, they exhibited a rise in UDP-glucuronyltransferase activity similar to that evoked in the host's liver. However, if fresh 12-day embryo liver fragments were excised and cultured *in vitro* alongside pituitary gland, or exposed during culture on paper strips to effluent from pituitary cultured above them (J. Leakey & G. J. Dutton, unpublished work), they showed no significant increase above the usual spontaneously induced UDP-glucuronyltransferase activity. As the cultured glands, when subsequently grafted on to a chorioallantoic membrane, stimulated transferase in the host liver, they had remained viable during culture *in vitro* with the liver fragments, though they may not have been secreting factor in the absence of a possible embryonic response. Probably, therefore, stimulation of embryo liver UDP-glucuronyltransferase by the pituitary factor required the mediation of other embryonic tissues which, under influence of the factor, would supply the direct initiator of enzyme response to the liver, and which might also be needed to promote secretion of the factor itself by the pituitary.

Role of thyroid and adrenal glands in response to the factor

Injection of thiourea (up to 4mg) at various periods into grafted eggs somewhat inhibited the response of UDP-glucuronyltransferase in host liver, but inhibition was never more than 50%. Thiourea-treated hosts possessed hypertrophied thyroids and did not prematurely retract the yolk-sac. Dexamethasone, when injected in order to inhibit adrenocorticotrophic hormone production by grafted pituitary, never inhibited the enzyme response and indeed precociously stimulated liver UDP-glucuronyltransferase development in ungrafted control embryos, provided they were 13 days or older when injected; the significance of this finding is discussed later.

Associated effects of pituitary grafting

After 4 days' exposure to a pituitary graft from a 6-week-old cockerel on the chorioallantoic membrane, host liver increased by some 50% in wet weight and by 30% in protein content over controls, whereas weights of the host embryo itself were only some 60–80% of controls. Increase in liver transferase and hydroxylase per embryo was therefore more marked after grafting than Table 1 would suggest. After this period of exposure to a graft, the yolk-sac of a 16-day embryo was usually found at various stages of retraction into the abdomen, an event which normally occurs over days 19–20 and is complete some 14h before hatching (El-Ibiyani *et al.*, 1966). Both thyroid (Balaban & Hill, 1971) and adrenal (Moog & Richardson, 1955) hormones have been implicated in yolk-sac retraction; we found application of thyroxine, but not of corticosteroids, induced precocious retraction (J. Leakey & G. J. Dutton, unpublished work) and thiourea always inhibited the process. However, considerable stimulation of UDP-glucuronyltransferase and aniline hydroxylase activities has occurred after grafting, without the accompaniment of these phenomena, which therefore are not necessarily produced by the factor initiating development of the embryos.

Discussion

The above results demonstrate for the first time an endogenous process initiating the surge of UDP-glucuronyltransferase activity in developing liver and kidney, and suggest that regulation of aniline hydroxylase is associated with it.

We conclude that secretion of a factor by the cephalic lobe of the chicken adenohypophyseal gland is initially responsible for the effects noted. This factor could also initiate the surge of the transferase activity *in vivo* on hatching. In the colony yielding the results of Fig. 1 the natural surge in transferase activity began the first few hours after hatching and had reached its irregular plateau some 3 days later. This would provide, *in vivo*, 24–48h for the factor to initiate transferase response in liver and a further 3 days to increase the enzyme activity to 'adult' values; allowing for vascularization of grafts, these periods correspond reasonably well to those obtained in the grafting experiments. Evidence that the aniline hydroxylase system is affected by the factor *in vivo* is less apparent, for the enzyme develops gradually from before 12 days of incubation, reaching 40–50% of adult activity just before hatching. However, at hatching there is a sudden marked peak in aniline hydroxylase activity and some other cytochrome *P*-450-dependent microsomal mono-oxygenases, from this value to a value 2–2.5 times above the adult value, which is reached at 4–5 days (Drummond *et al.*, 1972; G. J. Wishart & G. J. Dutton, unpublished work); this

peak could result from the surge in the secretion of the factor. Subsequently, UDP-glucuronyltransferase may be maintained by other means, though results of hypophysectomy in adult rats (Mowat & Arias, 1970) suggest that the pituitary gland assists maintenance of transferase activity towards some substrates, possibly through the maintenance of the membrane micro-environment. It is noteworthy that grafted mouse or rat pituitary gland will also induce embryo liver transferase activities (J. Leakey & G. J. Dutton, unpublished work).

The area of chicken gland evoking the effect suggests that the factor could be thyroid-stimulating hormone, follicle-stimulating hormone or adrenocorticotrophic hormone. As simple injections of various pituitary-hormone preparations did not reproduce the effect of grafting, the factor may be a known trophic hormone whose critical rate of entry into embryonic circulation is not attained in this manner, an unknown one, or one which requires unrecognized synergists.

A secondary agent stimulated by the pituitary graft and acting directly on host liver appears probable because of the failure of hormone preparations or pituitary cultures to influence transferase in isolated host liver and because the rise in transferase activities from zero to adult values occurs after removal of the pituitary graft (Table 3). Identification of the secondary agent would assist identification of the factor itself.

Thyroxine is not an obvious secondary agent because thiourea does not completely inhibit premature transferase induction, although it does inhibit premature yolk-sac retraction. Dexamethasone acts *in vivo* as a glucocorticoid with a long half-life (Bunim *et al.*, 1958). Its marked induction of the transferase and the age dependence of the host response strongly suggest a glucocorticoid as the secondary agent, and therefore adrenocorticotrophic hormone as the factor.

Hormones, especially those of adrenocortical origin, have short half-lives in birds (see Bell & Freeman, 1971), and their injection might be expected to yield the unsatisfactory results noted above if, for example, the transferase requires a higher concentration of corticosteroid for response than does tyrosine aminotransferase (Knox & Eppenberger, 1966) or phenylalanine hydroxylase. By using a new method of continuous application of hormones *in ovo* (Leakey & Dutton, 1975), it appears likely that the factor could operate through the host's corticosteroid hormones and may itself be, or possess an effect similar to, adrenocorticotrophic hormone.

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