Enzymic Differentiation in Mammalian Liver

Injection of fetal rats with hormones causes the premature formation of liver enzymes.

Olga Greengard

The term *enzymic differentiation* is used here to denote the process whereby, in the course of prenatal or early postnatal development, the different organs of an animal acquire their characteristic, quantitative pattern of enzymes. The fundamental problem underlying this process is that of gene expression: why does an enzyme appear at a given time in a certain tissue and at a different time, or not at all, in another tissue of the same organism? To approach this problem experimentally it seems necessary to at least identify a few physiological stimuli that specifically evoke the appearance of individual enzymes in the course of development.

In adult mammals, a large number of enzymes have been increased in amount by the administration of hormones or nutritional factors (*1, 2*). But previous attempts to evoke the premature formation of an enzyme during development by the same agent that increases its amount in the adult had been unsuccessful (*3, 4*). Such negative experimental results could not be interpreted adequately until agents were found that *are* effective prenatally. My associates and I have now been able to cause premature enzymic differentiation by a procedure operationally similar to enzyme inductions in adult animals, by a single injection of appropriate hormones to fetal rats. But the same enzyme may respond to a different hormone in the adult and in the developing organ. Thus, the explanation of the aforementioned negative experimental results lies in the evolving nature of the enzyme-regulator relationship: the predominant regulatory factor for an enzyme varies with the developmental and physiological state.

Glucagon, Epinephrine, and Thyroxine

Many descriptive data about enzyme differentiation in mammalian liver are available. During late fetal and early postnatal life this process involves the appearance of new enzymes and major gains or losses in the concentration of enzymes already present at this stage (*5*). Because *growth* is uninterrupted by fetal hypophysectomy, adrenalectomy, or thyroidectomy (*6*), there has been a tendency to overlook the possibility that some of the factors necessary for the late stages of enzymic differentiation in mammals are fetal endocrine products. But, as shown by Jost (*7*), hypophysectomy of fetal rabbits does interrupt some aspects of biochemical differentiation; the liver does not acquire the normal ability to produce glycogen. Our experiments introduce an alternative, positive approach: the administration of hormones for the purpose of evoking the appearance of specific enzymes in the liver before they would normally appear.

The normal time course of the developmental formation of liver enzymes (three of these are illustrated in Fig. 1) can provide clues about the physiological stimuli that may be involved in their regulation. We postulated that the precipitous rises that occur immediately *after* birth (for example, in the concentrations of tyrosine aminotransferase and glucose-6-phosphatase) are caused by hormones secreted in response to the well-known postnatal hypoglycemia (*8*), whereas the accumulation of enzyme [for example, of reduced nicotinamide adenine dinucleotide phosphate (NADP) dehydrogenase (E.C. 1.6.99.2) and glucose-6-phosphatase] around the 18th day of gestation may be associated with the functioning of the thyroid gland that commences just before this time (*9*). If the secretion of the postulated hormone is *the only* trigger, then the early, artificial supply of this hormone should cause the appropriate enzyme changes to occur prematurely. The hormones were administered intraperitoneally to individual fetuses within the uteri of anesthetized, laparotomized dams.

Our initial studies showed that liver tyrosine aminotransferase (and serine dehydratase), normally present in insignificant amounts before birth, can be evoked by the administration of glucagon to fetal rats (*10*); but such administration did not elevate the concentration of reduced NADP dehydrogenase. On the other hand, an injection of thyroxine enhanced the prenatal development of this enzyme (*11*). Glucose-6-phosphatase responded to both hormones (Figs. 2 and 3); this is in accord with the more complex developmental pattern of this enzyme, seen in Fig. 1. Like reduced NADP dehydrogenase, it appears before birth, and, like tyrosine aminotransferase, it also exhibits a postnatal upsurge. Glucagon may not be the only hormone that mediates the effects of the early postnatal hypoglycemia; we found that epinephrine can also evoke the prenatal appearance of tyrosine aminotransferase (Fig. 4) and can enhance the prenatal accumulation of glucose-6-phosphatase. The administration to fetal rats of glucocorticoids, insulin, or growth hormone did not enhance the formation of the liver enzymes so far studied. However, it seems likely that further physiological stimuli involved in the complex process of enzymic differentiation in fetal mammals will be detected, through extension of the approach described to additional enzyme systems and different tissues.

It is well known that fetal liver contains a significant and gradually diminishing amount of hematopoietic cells. Thus, some of the small changes in enzyme concentration that occur in the course of normal development may merely reflect the decrease in hematopoietic cells or the increase in the number of hepatocytes. This slow change in cellular composition is not relevant to the rapid, large changes (shown in Figs. 2 and 3) that are induced by hormones within a period of hours, with-

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out change in the size or total protein content of the liver. The prenatal injection of hormones did not interfere with the viability of the fetuses; if they were not removed for assay, normal birth occurred at term.

Mechanism of Enzyme Formation

As discussed elsewhere (*2*), an increase in the rate of synthesis of specific proteins in animal tissues may or may not require concomitant synthesis of RNA. Even in cases where RNA synthesis is required, there is no evidence that the amount or rate of synthesis of messenger RNA determines the amount of protein to be made. In such studies the adult tissues were found to contain a significant basal amount of the specific protein—that is, the gene was functional before elevation of synthesis had been induced. Unfortunately, at present our only certain test of whether a given gene is or is not functioning is detection of its end product, the specific protein. Thus, when an enzyme has not yet appeared in a developing organ we cannot tell which of the many reactions necessary for its synthesis are still inoperative. Transmission of the qualitative genetic information from DNA to RNA is necessary, but not sufficient, for the occurrence of enzyme synthesis. Thus, the existence of the enzyme proves the existence of the corresponding messenger RNA, but the formation of this RNA, which has a long half-life in animal tissues (*12*), did not necessarily initiate enzyme synthesis—that is, determine the enzyme's time of appearance. The triggering nature of the formation of messenger RNA can be either entertained or excluded as a possibility, depending on whether the appearance of the enzyme does or does not depend on concomitant RNA synthesis. For example, the appearance of tryptophan oxygenase in mammalian liver was not inhibited by actinomycin (*13*); apparently the necessary RNA species was available some time before the stimulus that finally triggered the actual accumulation of this enzyme. The enzymes now being studied present a different picture: the normal postnatal accumulation of tyrosine aminotransferase (*13*) and the premature development of the enzymes in fetal liver caused by injected glucagon or thyroxine were inhibited by actinomycin (Figs. 2 and 3). The reversible, glucagon-induced appearance of tyrosine aminotransferase in fetal liver (Fig. 2) may be the most suitable experimental system now available (in animal tissue) for ascertaining whether the appearance of the qualitative message may provide the trigger necessary for synthesis to occur. But for enzyme inductions in general, a simple model according to which a single hormone specifically and directly interacts with a part of the genetic material is unsatisfactory; the observations discussed next emphasize that the same enzyme in different developmental or physiological states may respond to different hormones.

The Question of "Competence"

Requirements for developmental enzyme formation. Apparently in the intact, *late* fetus most of the necessary conditions for the synthesis of tyrosine aminotransferase have already been fulfilled, and thus a single stimulus, glucagon or epinephrine, can evoke it effectively. In the younger fetus, more than 3 days before birth, this is not the case (Fig. 4): glucagon could not evoke the enzyme. Search for an earlier step in the sequence of differentiation that renders the liver competent to synthesize tyrosine aminotransferase revealed that cyclic adenosine monophos-

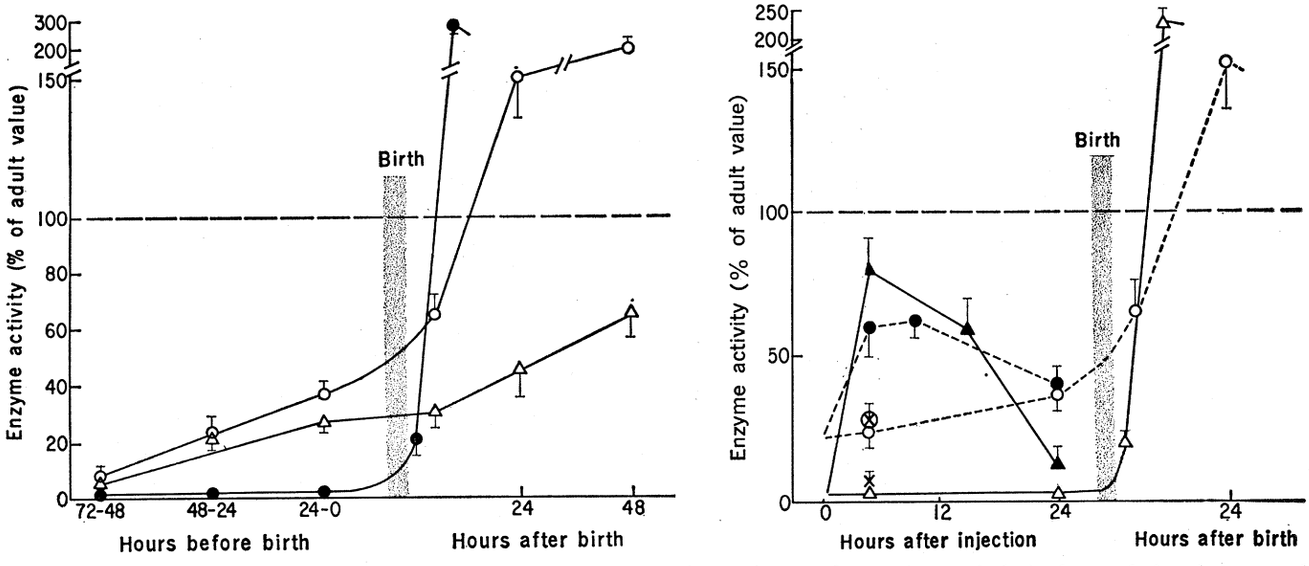


Fig. 1 (left). The developmental formation of (solid circles) tyrosine aminotransferase, (open circles) glucose-6-phosphatase, and (triangles) reduced NADP dehydrogenase in rat liver. The enzyme activities were measured, as described elsewhere (*10, 11*), in livers of 10 to 30 individual animals of the indicated ages. The mean values [brackets (line with bar) = 1 standard deviation] are expressed as percentages of those in livers of adult male rats where the activities of tyrosine aminotransferase, glucose-6-phosphatase, and reduced NADP dehydrogenase are, respectively, 43, 285, and 215 micromoles of products formed per hour per gram of fresh liver. In each fetal liver the activity of tyrosine aminotransferase, less than 2 percent of the values for adults, is considered insignificant. Fig. 2 (right). Time course of the effect of glucagon on fetal-liver enzymes. Half of the fetuses within each mother were injected with 0.05 milligram of glucagon (solid symbols) on the 20th day of gestation. The alternate, control littermates received saline (open symbols). At each of the indicated hours after injection, three to eight pregnant rats were killed and the activity of tyrosine aminotransferase (triangles) and glucose 6-phosphate (circles) was assayed in each fetal liver. In two additional pregnant rats, four of the fetuses received thyroxine plus 10 micrograms of actinomycin D (crosses). For representation of the results, see Fig. 1.

phate (AMP) has an important role in developmental enzyme formation. In late fetuses, cyclic AMP (but not AMP) and, more effectively, its dibutyryl derivative can evoke the appearance of tyrosine aminotransferase; the latter agent is also effective in younger fetuses that do not respond to glucagon (Table 1). These results suggest that glucagon and epinephrine exert their actions by way of the formation of cyclic AMP and that younger fetuses are not competent to produce sufficient cyclic AMP in response to glucagon. The concentration of glucose-6-phosphatase, synthesis of which has already been initiated at this age by thyroid hormone, is also increased by cyclic AMP more than (or as much as) by glucagon. Dibutyryl cyclic AMP did not enhance the formation of reduced NADP dehydrogenase, whose developmental formation is enhanced by thyroxine but not by glucagon. It is noteworthy that the action of cyclic AMP on enzyme systems previously described are *activations* of preformed enzymes that are demonstrable in cell-free systems, whereas the enzymes now studied are unaffected in vitro by cyclic AMP. The mechanism by which cyclic AMP directly or indirectly influences specific protein synthesis is of course unknown. It is unlikely that cyclic AMP acts by a cofactor type mechanism (*2*), since its action is inhibited by actinomycin (Table 1) and since the two enzymes whose accumulation it promotes have entirely different molecular properties, catalytic action, and cofactor requirements.

It is also possible to approach the problem of competence in a negative manner, by inhibiting its development through endocrine ablations or through dietary restrictions. Such manipulation may obscure the identity of the *natural* stimulus or trigger for a developmental change in enzyme synthesis, because this trigger remains ineffective if any one of the normally nonlimiting conditions is interfered with. For example, adrenalectomy at birth inhibits, and glucocorticoid restores, the postnatal increase in tyrosine aminotransferase (*4*), even though increased glucocorticoid secretion at birth is clearly not the natural trigger. The administration of this hormone to intact fetuses cannot evoke the appearance of tyrosine aminotransferase prematurely; only glucagon or epinephrine can do this (Fig. 5). There is no reason to think that adrenalectomy prevents the occurrence of the normal postnatal trigger—that is, hypoglycemia and the consequent secretion of glucagon; it is more likely that the presence of adrenocortical secretion is one of the many conditions necessary for the action of the final trigger, the stimulus that is both necessary *and* sufficient.

Table 1. Effect of cyclic AMP on the premature formation of enzymes. Intraperitoneal injections were given to fetuses *in utero* 5 hours before assay. Within each pregnant rat some fetuses, injected with saline, served as controls; others received 0.05 milligram of glucagon; others received 0.125 milligram of cyclic AMP (cAMP) or N^6-$O^{2'}$-dibutyryl adenosine 3′-5′-cyclic phosphate (BcAMP) without or with 10 micrograms of actinomycin D (act. D). The enzyme activities (in micromoles per hour per gram of liver) are means, ± standard deviation, of results for individual livers; the numbers of individual livers are given in parentheses.

Injection	Enzyme activities	
	Tyrosine amino-transferase	Glucose-6-phosphatase
*Age: 3 or 4 days before birth**		
Saline	<2 (20)	7.5 ± 3 (5)
Glucagon	<2 (8)	12.3 ± 7 (6)
BcAMP	5.9 ± 1.4 (10)	18.7 ± 6 (7)
Age: 1 or 2 days before birth†		
Saline	<2 (30)	38 ± 10 (9)
Glucagon	30 ± 5 (15)	84 ± 4 (6)
cAMP	5.5 ± 3 (4)	79 ± 8 (4)
BcAMP	15.2 ± 6 (12)	95 ± 5 (4)
BcAMP + act. D	<2 (6)	45 ± 2 (4)

* Body length, 20 to 26 millimeters. † Body length, 37 to 41 millimeters.

In general, then, there is an essential, practical difference between the two main ways of experimentally altering the course of enzymic differentiation. The negative effects on specific enzyme formation of endocrine ablations or of metabolic inhibitors identify factors necessary for the expression of the gene, without establishing the temporal hierarchy between these factors. On the other hand, experiments designed to stimulate premature enzyme formation may also reveal the sequence in which the necessary factors acted in the course of differentiation.

The evolving complexity of regulatory systems. The nature of the mechanism that maintains the relative constancy of enzyme concentrations throughout the adult life of an animal is a major unsolved problem. This mechanism must be conditioned by the environment and by the metabolic state of the normal animal and not genetically determined in a simple way, since major reversible changes occur in the levels of numerous enzymes when an animal adapts to unusual physiological conditions. Enzymes are not eliminated from the developed organ by the lack of stimuli that promoted their formation during differentiation. For example, after the cessation of neonatal hypoglycemia, the developmental triggering stimulus for tyrosine aminotransferase, this enzyme merely decreases to approximately adult levels (Fig. 1). Similarly, thyroidectomy causes only a 20- to 30-percent decrease in the level of glucose-6-phosphatase and reduced NADP dehydrogenase in adult liver, not a reversion to prenatal values (*14*). Clearly, after the developmental formation of enzymes, additional regulators come into play that replace the original stimuli. Because of the manifold, evolving regulatory systems, differentiation is, in practice, irreversible, but, as discussed by Knox (*15*), it may well proceed by a series of individually reversible adaptive changes. This reversibility may be demonstrated when an enzyme is evoked prematurely, before the development of the additional regulatory potentialities. Tyrosine aminotransferase, which accumulates in fetal liver to maximum values in 5 hours after a single dose of glucagon, disappears again from the liver by 24 hours after administration of the glucagon (Fig. 3) if birth has not supervened. The concentration of glucose-6-phosphatase also reverts to its initial level at this time. Such reversibility could not be demonstrated for the thyroxine-induced concentrations of glucose-6-phosphatase and reduced NADP dehydrogenase (Fig. 3). A possible reason for this difference between the action of the two hormones, each given in a single dose, is that thyroxine persists longer or that the thyroid function of the fetus, already established at this time, maintains the stimulated enzyme synthesis whereas glucagon (or epinephrine) is not secreted by the fetus.

The stimuli that promote the developmental formation of enzymes may, with increasing age of the animal, become relatively unimportant, not only in maintaining the normal concentration but also in augmenting it when the stimulus is administered in excess. The elevation of levels of reduced NADP dehydrogenase by thyroxine is restricted to fetal liver; it is not seen in the normal postnatal or adult animal (*11*). For a few days after birth the liver can still respond to glucagon with a several-fold rise in tyrosine aminotransferase, but in the normal 50-day-old rat it cannot do so (Fig. 4). Epinephrine, which also evokes tyrosine aminotransferase prenatally, has very little effect on the 3-day-old rat. Conversely, gluco-

corticoids, the most important inducers of tyrosine aminotransferase and glucose-6-phosphatase in the postnatal animal, are without effect on these enzymes prenatally. There are often traces in the adult animal of the former effectiveness of developmental hormones. It is possible to modify the physiological state of the animal in such a way that this becomes apparent. After thyroidectomy in the adult rat, which decreases the level of glucose-6-phosphatase by only 25 percent, injection of thyroid hormone permits the level to rise by 200 percent (*14*). Reduced NADP dehydrogenase shows an increased response to thyroxine in hypophysectomized rats (*16*). The usual small or insignificant effect of glucagon or epinephrine on tyrosine aminotransferase levels in adult rats can be greatly enhanced by adrenalectomy plus hydrocortisone treatment (*17*).

Postnatal Consequences of Injecting Fetal Rats with Hormones

At the time of birth, mammalian liver does not yet possess some of its essential functions, such as gluconeogenesis from 3-carbon compounds and the capacity to detoxify various substances. The medical problems that arise from severe neonatal hypoglycemia, from jaundice (due to incomplete conjugation of bilirubin with glucuronate), and from the inability to metabolize drugs and steroids have been reviewed by Dawkins (*8*) and by Shelley and Neligan (*18*). The development of these liver functions involves the accumulation of new enzymes and requires days, or at least hours. Adaptation to postnatal life might be facilitated if concentrations of some of these enzymes were artificially caused to rise before birth.

We attempted to increase the levels in the newborn rat of glucose-6-phosphatase, an enzyme necessary for gluconeogenesis, and of reduced NADP dehydrogenase, a component of the microsomal drug-metabolizing system (*19*). Thyroxine, which enhances the prenatal formation of these enzymes (Fig. 3), was administered to fetal rats 1 to 2 days before term. Such rats, delivered at term, had higher levels of glucose-6-phosphatase and reduced NADP dehydrogenase than their untreated littermates (Fig. 5). A few hours after birth the levels of these enzymes in the treated rats were as high as levels normally reached 1 or 2 days later. It should be emphasized that most of the enzyme accumulations took place before birth; such accumulation requires at least 24 hours (see Fig. 4). Thus, administration of an effective agent at birth would not change the enzymic composition of

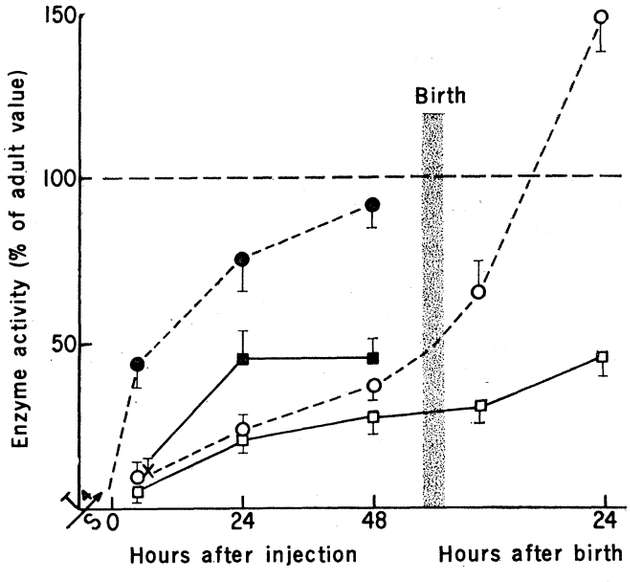
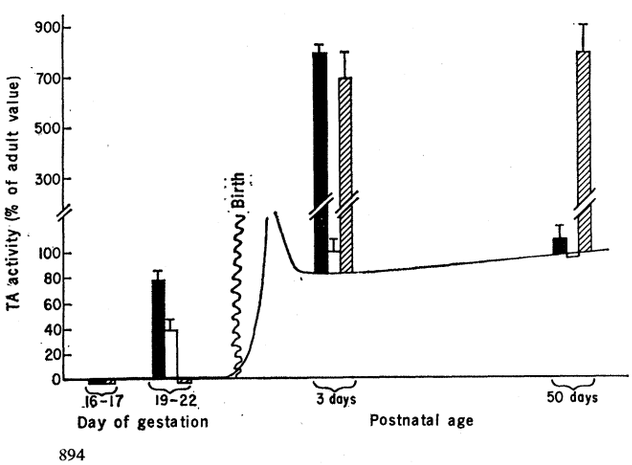
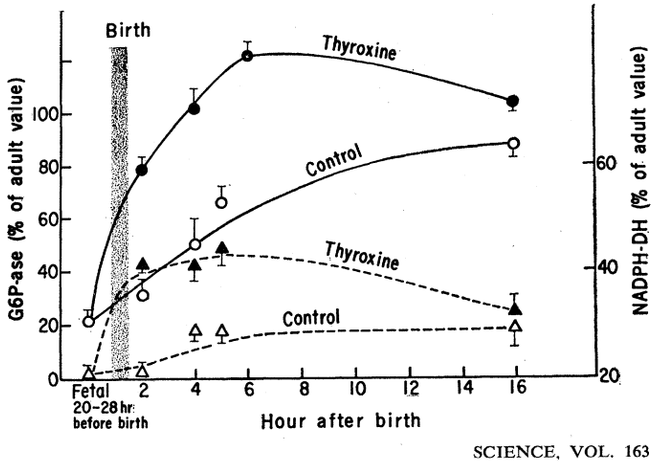


Fig. 3 (left). Enhancement of the accumulation of liver enzymes by injecting fetal rats with thyroxine. The experiments were designed like those of Fig. 2, but, instead of glucagon, thyroxine (solid symbols) was injected (1 to 3 micrograms per fetus) and the activity of reduced NADP dehydrogenase (squares) was measured in addition to that of glucose-6-phosphatase (circles). Fig. 4 (bottom left). Variation of the enzyme-regulator relationship with age. The solid line represents the levels of tyrosine aminotransferase (TA) in normal rats, and the height of the superimposed columns reflects the increase (mean + standard deviation) measured 5 hours after intraperitoneal injection of glucagon (solid columns), epinephrine (open columns), or hydrocortisone acetate (hatched columns). The amounts, per fetus, of the injected hormones were 0.05, 0.01, and 0.25 milligram, respectively; in the newborn rat the amounts were 0.25, 0.05, and 2.5 milligrams, respectively, per 100 grams of body weight. The small bars below the solid line indicate that the hormone had no effect. The 50-day-old rats given epinephrine were adrenalectomized (to avoid induction by stress-stimulated glucocorticoid secretion); all other rats were intact. Fig. 5 (bottom right). Liver glucose-6-phosphatase (G6P-ase) (circles) and reduced NADP dehydrogenase (NADPH-DH) (triangles) in newborn rats that had received prenatal injections of thyroxine. Half of the fetuses within each of seven pregnant rats had received intraperitoneal injections of 3 micrograms of thyroxine. The next day (that is, at term) they were delivered by section and the enzyme levels in the livers of the experimental animals (solid triangles) and saline-injected control animals (open triangles) were measured at times indicated by the numbers on the abscissa. The prenatal values refer to additional measurements on untreated fetuses 1 day before birth. The values are means (brackets indicate 1 standard deviation) of results with 6 to 15 individual livers.





the liver within the critical postnatal hours. Furthermore, any treatment may adversely affect the delicate metabolic balance of the newborn, whereas fetal rats may tolerate artificial interference better, since their metabolism is buffered by that of the mother.

The foregoing results (Fig. 5) demonstrate the possibility of producing newborns with a "precocious" enzyme pattern. Injection of fetuses with a combination of appropriate hormones that may extensively enhance biochemical differentiation could be looked upon as a way to shorten the necessary period of gestation. Such enhancement by the prenatally initiated formation of enzymes necessary for important liver functions may be of particular benefit to prematurely born animals.

Summary

The course of enzymic differentiation in liver can be altered in a positive, biologically meaningful direction by the administration of glucagon, epinephrine, and thyroxine to fetal rats *in utero*. The premature accumulations of specific enzymes occur within hours after such administration, are inhibited by actinomycin, and provide a suitable system for studying the mechanism of gene expression. Glucagon and epinephrine are probably the natural stimuli for the formation of enzymes that accumulate precipitously during the hours immediately following birth. Their action may be mediated through cyclic AMP; dibutyryl cyclic AMP can evoke the appearance of tyrosine aminotransferase in fetal livers too young to respond to glucagon. Thyroxine is important in promoting aspects of enzymic differentiation that occur during late fetal life.

Rats injected prenatally with thyroxine were born with precociously elevated levels of liver enzymes. Such artificial stimulation of the course of enzyme differentiation during the fetal stage may facilitate the metabolic adjustment of newborn or prematurely born animals to extrauterine existence.

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Two Visual Systems

Brain mechanisms for localization and discrimination are dissociated by tectal and cortical lesions.

Gerald E. Schneider

The term *vision* subsumes a complex variety of processes, thus, for fruitful scientific discussion, a reference to "vision" usually requires further specification. Likewise the term *blindness* is not self-defining. An animal or patient showing what appears to be total blindness under one set of conditions may reveal considerable visual capacity in a different situation. Such phenomena have led to discrepant conclusions in the literature on the neurological bases of vision, particularly on visual defects following various types of brain damage. The discrepancies have often been resolved through careful attention to *stimulus* conditions: variations in level of illumination, movement of stimuli, and type of pattern have led to the definition of particular types of partial blindness. However, the nature of the *response* has received less attention in studies of visual processes: an anopia found in a test requiring one type of response may vanish or turn out to be an amblyopia in tests requiring a different response.

For example, after preliminary neurological testing of golden hamsters with total ablations of the superior colliculi of the midbrain, I concluded that they were essentially blind (though their pupils still reacted to light). Unlike normal animals, they could find food only by touch and olfaction. I initially assumed that an inability to localize a stimulus in visual space (that is, to make orienting movements of the head or body in the direction of a stimulus within the field of vision) implied an inability to identify shapes and patterns visually, since shapes and patterns are defined by the spatial arrangement of their parts. But subsequent experiments (*1*) which required different responses have forced me to drop this assumption, for the "blindness" appeared only when orienting movements were required. Study of other hamsters after ablations of visual areas of the cerebral cortex showed that a related assumption—the assumption that the ability to localize objects

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