sumed messenger-ribosome initiation complex is formed, and "incubation" when aminoacyl transfer is assayed. Under these conditions, $5 \times 10^{-5}M$ ATA during preliminary incubation inhibits subsequent aminoacyl transfer by more than 96 percent. However, when ATA is added only to the incubation (that is, after preliminary incubation) it has little effect on the reaction. If the preliminary incubation is performed at 0°C, or if either ribosomes or messenger are omitted from an otherwise complete reaction mixture during preliminary incubation (and added in the incubation), addition of ATA results in full inhibition. These data indicate that the ATA-sensitive reaction in the wheat embryo system is indeed the enzymatic formation of a messenger-ribosome initiation complex.

Application of ATA inhibition to the assay and direct demonstration of initiation factors is presented in Table 2. The primary feature of the assay is that the extent of initiation can be monitored by inhibiting further initiation with ATA and then measuring aminoacyl transfer in the presence of excess supernatant (*6*). In practice, a 6-minute preliminary incubation is first carried out in the presence of soluble components to be assayed for initiating activity; $5 \times 10^{-5}M$ ATA, excess supernatant (providing a saturating quantity of transfer factors), and [^{14}C]aminoacyl tRNA are then added. After a 9-minute incubation, the radioactivity is determined in material that is insoluble in hot trichloroacetic acid. Lines 1 to 3 are basic controls demonstrating that if no supernatant components are present during the preliminary incubation, or if only one of the initiation factors (C or D) is added, no incorporation is obtained. In contrast, when both factors are present (Table 2, lines 4 and 6), the system is capable of considerable aminoacyl transfer.

The possibility that the initiation factors are simply alternate functional manifestations of the aminoacyl transfer enzymes (*7*) is examined in Table 2, lines 4–7. These experiments present a comparison of incorporation with and without the addition of supernatant during the incubation. The rationale for these experiments is that if the initiation factors are different from the transfer enzymes, purification of the initiating factors (*8*) should result in a greater requirement for transfer enzymes in the incubation. The data indicate that there is such a requirement. With the initial diethylaminoethyl-cellulose fractions (Table 2, lines 4 and 5), addition of supernatant to the incubation increases incorporation approximately twofold. With the partially purified fractions (Table 2, lines 6 and 7), the requirement for supernatant is almost absolute. It thus seems apparent that the initiation factors are indeed entities distinct from the transfer enzymes.

The details of the function of the initiation factors are as yet unknown. However, the specific requirement for adenosine triphosphate (*3*) as well as the lack of participation of formylmethionyl tRNA (*4*) suggest that the initiation process of wheat embryo differs considerably from that described for *E. coli* (*2*).

ABRAHAM MARCUS
J. DEREK BEWLEY
DONALD P. WEEKS
Institute for Cancer Research, 7701 Burholme Avenue, Fox Chase, Philadelphia, Pennsylvania 19111

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6. The assay of initiation factors requires a source of transfer enzymes free of initiation factors. In the bacterial system (*2*) this is provided by the supernatant fraction since the initiation factors are predominantly bound to the ribosomes. In wheat embryo extracts, both the initiation factors and the transfer enzymes are found only in the supernatant fraction.
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8. Fractions C and D are prepared from the 100,000*g* supernatant fraction [S100 (*3*)] by fractionation on DEAE-cellulose. Fraction C passes through DEAE-cellulose at 0.1*M* KCl, and fraction D is eluted between 0.15 and 0.30*M*. Fraction C is further purified by adsorption and elution from phosphocellulose, and fraction D is purified by precipitation at *p*H 4.7 followed by fractionation with $(NH_4)_2SO_4$ between 45 and 70 percent.
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Induction of Glutamine Synthetase in Embryonic Retina: Its Dependence on Cell Interactions

Abstract. *A relation between enzyme induction in embryonic cells and cellular organization is indicated by the finding that the levels of glutamine synthetase induced by hydrocortisone in the embryonic neural retina in vitro are dependent on the associations between the retina cells. Intact retina tissue, aggregates of dissociated cells, and cells in monolayer culture showed a decreasing response, in this order, to glutamine synthetase induction. With time of culture, the enzyme activity continued to rise in the intact retina and in cell aggregates, but activity declined in monolayer cultures even though the inducer was continuously present. Dispersed cells cultured in monolayer without the inducer showed after 24 hours a loss of inducibility which could not be reversed by reaggregating such modified cells but could be prevented by maintaining the freshly dispersed cells at a low temperature.*

It is generally agreed that embryonic cell differentiation is regulated through gene action which, in turn, often is mediated by external signals provided by cell interactions (*1*). Such interactions may occur over a long distance, as in the case of inductions by hormones; or, they may occur over a short distance and involve specific associations between cells (*2*). We now report that a hormonal inducer requires organized associations between the responding cells in order to elicit and maintain a specific, gene-controlled differentiation. The findings suggest that, in developing systems, responsiveness to inductive signals depends on the cells attaining and retaining the correct multicellular associations.

A sharp increase of glutamine synthetase (GS) marks the onset of functional differentiation of the neural retina in the chick embryo, and this increase can be induced precociously in the isolated retina in vitro (as well as in the embryo) by certain 11β-hydroxycorticosteroids that induce specifically retinal GS (*3, 4*). This induction process has a lag period of less than 2 hours; it involves the synthesis and accumulation

of new enzyme protein, as has been shown by radioimmunochemical tests (*5*), and is under gene control in that it requires RNA synthesis (*6*). To examine the dependence of GS induction on cell associations in the neural retina we followed earlier indications that dispersed retina cells were less inducible than the intact tissue (*7*), and we used the methodology of cell dissociation and aggregation to test the responsiveness of retina cells in three states of organization to GS induction by the steroid inducer.

Neural retina tissue isolated from 10-day chick embryos was cultured in Erlenmeyer flasks at 37°C on a gyratory shaker at 70 rev/min (*4, 6, 7*). Suspensions of dissociated cells were prepared by trypsinization (*8*). Cell aggregates were prepared by rotation of cell suspensions in Erlenmeyer flasks on a gyratory shaker at 70 rev/min (*8*), and monolayer cell cultures were prepared by plating cell suspensions in 60-mm Falcon petri dishes. Cultures of intact retina tissue consisted of one whole or one-half retina in 3 ml of medium; cell cultures were started with 40 or 80 million cells (the approximate number of cells in one-half or in one 10-day embryonic retina) per 3 ml of medium. The culture medium consisted of 20 percent (by volume) fetal calf serum in Eagle's basal medium equilibrated with a mixture of 5 percent CO_2 and air. Glutamine synthetase was induced by adding hydrocortisone (0.33 μg/ml) to the medium (*4, 6*). The specific activity of GS was determined as before (*3*).

The levels of GS induced in the cultures (Fig. 1) were directly related to the integrity of the tissue; they always were highest in the intact retina, intermediate in the cell aggregates, and lowest in the monolayers. The intact tissue retained its typical histological organization and continued to differentiate in culture (*3*). In cell aggregates, there was considerable tissue reconstruction, and many of the cells reestablished associations morphologically similar to those in intact retina (*8*). In monolayer cultures, the frequency of histogenetic associations was lowest; although there were many small cell clusters with aggregate-like structure, these cultures contained mostly cell plaques and single cells. Thus, there was a clear correspondence in these three types of cultures between cellular organization, that is, the frequency and precision of histogenetic cell groupings and levels of GS activity induced by the steroid inducer. Higher concentrations of the steroid inducer did not cause greater increases of GS in the monolayers, and higher enzyme activities were not obtained with the fluorosteroids dexamethasone or triamcinolone, which are probably not metabolized by these cells and which belong to the class of steroids that effectively induce GS in the intact retina (*9*).

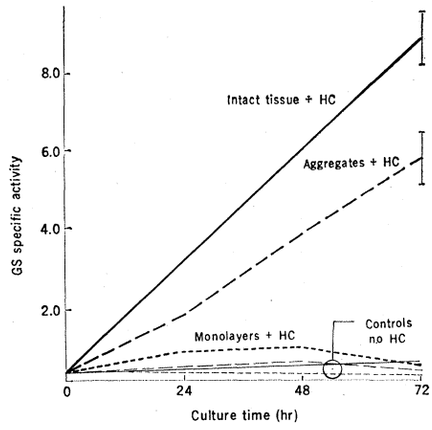

Fig. 1. Effect of tissue integrity on glutamine synthetase (GS) induction in embryonic retina in culture: differences in GS accumulation in intact tissue, cell aggregates, and cell monolayers. All cultures were induced with hydrocortisone (HC) at zero time and were stopped after 24, 48, or 72 hours of culture at 37°C. In control cultures without the inducer, GS did not increase appreciably. Each point is the average of several assays. Absolute values varied somewhat in different experiments, but the overall relationships were always as shown. All retinas were from 10-day chick embryos.

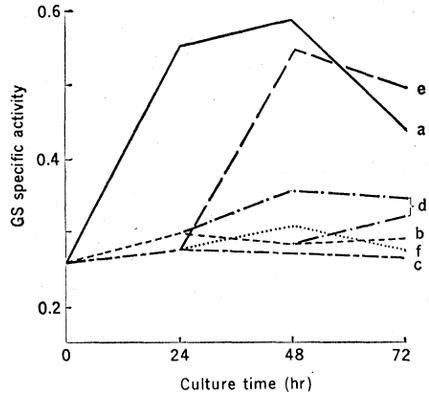

Fig. 2. Loss of inducibility of glutamine synthetase (GS) in monolayer cultures of retina cells after 24 hours at 37°C and its prevention by low temperature. Inducer (HC) was added to the monolayer cultures at the times indicated below. Other culture and assay conditions were as described in the text and in the legend for Fig. 1. (*a*) Cells cultured in medium with inducer for 72 hours at 37°C; (*b*) without inducer for 72 hours at 37°C; (*c*) with or without inducer for 72 hours at 4°C; (*d*) without inducer for 24 or 48 hours at 37°C, then inducer added; (*e*) without inducer for 24 hours at 4°C, then with inducer at 37°C; (*f*) without inducer for 24 hours at 4°C, then for 48 hours at 37°C.

The term "monolayer" cultures should in this case be used advisedly since, as was mentioned above, in the first 24 hours many of the cells formed small aggregate-like clusters under these conditions. The more clusters there were, the higher was the induced level of GS at 24 hours. Thus, the response of monolayer cultures to the inducer may have been due primarily to the presence of these aggregate-like cell clusters. This is corroborated by the findings (*10*) that even very small aggregates comprising only 50 cells are inducible. During the second day the clusters in monolayer cultures usually flattened out, and their cells tended to disperse; these changes were accompanied by a leveling or a decline in GS activity, even though the inducer was continuously present. In intact tissue and in cell aggregates cultured in medium with inducer, the enzyme continued to rise during this period. Therefore, not only the initial induction of GS but also the continued rise of the enzyme in the constant presence of the inducer depends on the multicellular organization of the retina cells.

We next determined whether the cells remained inducible if cultured without the inducer. Retina tissue and aggregates of freshly dissociated retina cells were cultured for 2 days without the inducer and were then found to respond to induction of GS by the steroid. On the other hand, if monolayer cultures were maintained for 24 to 48 hours in noninducing medium and then treated with the inducer, GS activity rose only very slightly and sometimes not at all. Higher concentrations of the inducer were equally ineffective.

To determine whether such monolayer-cultured cells which had become noninducible would regain inducibility after reaggregation into multicellular clusters, cells cultured in monolayer for 24 or 48 hours without inducer were detached from the culture dishes, dispersed into single-cell suspensions (by mild trypsinization or mechanically), and reaggregated by rotation in medium with inducer. Although aggregates formed, GS activity did not go up. Hence, the loss of GS inducibility resulting from 1-day incubation of the monolayered cells in inducer-free medium was due to changes which could not be reversed by reaggregation under the conditions examined.

The exact reasons for this rapid and durable "amnesia" of GS inducibility in monolayer-cultured retina cells are being explored; considering that synthesis of RNA and of enzyme protein are required for GS induction (*5, 6*) and that enzyme levels are subject to multiple types of regulation, changes at any of several control levels could be involved. A clue to the nature of this loss of inducibility is suggested by the finding that it can be largely prevented by suppressing metabolic activities in the dispersed cells. Freshly prepared cell suspensions in medium without the inducer were maintained at 4°C for 24 hours; the culture dishes were then transferred to 37°C, the inducer was added, and GS activity was determined after 24 hours. These cultures responded to the inducer by an increase in GS activity; controls maintained at 37°C in inducer-free medium did not respond to the subsequent addition of the inducer (Fig. 2).

The above finding suggests that the loss of GS inducibility in monolayered retina cells is due to durable metabolic changes that occur when the cells are maintained in a separated state. That the persistent separation of the cells from tissue-like association is a causal factor in initiating these changes is shown by their prevention when the cells are reaggregated immediately after their dissociation from the tissue and thereby enabled to restore histogenetic contacts. Other interpretations are conceivable at present; however, these experimental findings provide means for further analyzing the amnesia of GS inducibility in retina cells in the context of enzyme regulation in neural differentiation and specifically in relation to the role of cell contact in developmental processes (*1, 2, 8, 11*).

The requirement for multicellular organization in the hormonal induction of GS in the retina is consistent with other evidence that the increase of GS is a characteristic feature of differentiation in this tissue. This requirement distinguishes GS induction in the embryonic retina from inductions of adaptive enzymes in monolayer cultures of adult rat hepatoma cells which do not depend on histotypic organization (*12*). It should be recalled that the formation of various specialized intercellular junctions (synaptic and others) marks the development of the neural retina; it is therefore possible that, in the embryonic retina, the role of cell interactions in enzyme induction is especially prominent but that similar conditions apply in principle also in other developing systems to induction of enzymes associated with differentiation.

JOHN E. MORRIS*
A. A. MOSCONA
Department of Biology, University of Chicago, Chicago, Illiniois 60637

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* Present address: Department of Zoology, Oregon State University, Corvallis 97331.

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Pineal Gland: Dibutyryl Cyclic Adenosine Monophosphate Stimulation of Labeled Melatonin Production

Abstract. *In organ cultures of intact rat pineal glands,* $N^6O^{2'}$*-dibutyryl adenosine 3′,5′-monophosphate stimulates the conversion of tritiated trytophan to tritiated melatonin, as does* L*-norepinephrine. Potential sites of stimulation of melatonin production by dibutyryl cyclic adenosine monophosphate are discussed, based on observations that the dibutyryl analog also stimulates the conversion of serotonin labeled with carbon-14 to carbon-14-labeled melatonin without altering hydroxyindole-*O*-methyl transferase activity or intracellular accumulation of serotonin labeled with carbon-14.*

Melatonin is synthesized from serotonin by *O*-methylation of the intermediate *N*-acetylserotonin (*1*). In mammals this conversion takes place only in the pineal gland, because of the presence of the unique enzyme hydroxyindole-*O*-methyl transferase (*2*). Norepinephrine stimulates the conversion of labeled tryptophan (*3*) and serotonin (*4*) to melatonin by pineal glands in two different organ culture systems. Weiss and Costa (*5*) reported that norepinephrine also stimulated the activity of adenyl cyclase in homogenates of pineal glands, implying that this monoamine may elevate the concentration of adenosine 3′,5′-monophosphate (cyclic AMP) in the gland. The role of cyclic AMP as an intermediate or "second messenger" in many hormone-target organ systems is becoming increasingly apparent (*6*), and we investigated whether the stimulatory effects of norepinephrine on melatonin synthesis in the pineal gland would be mimicked by cyclic AMP.

We tested the hypothesis that cyclic AMP may directly stimulate melatonin production using an organ culture system (*7*). Intact pineal glands were obtained from female Osborne-Mendel rats (150 to 170 g) and incubated for 24 hours in 0.5 ml of chemically defined media (*8*) supplemented with serum albumin (fraction V, Pentex, 1 mg/ml). Labeled melatonin formed from uniformly labeled [^{3}H]tryptophan or [^{14}C]serotonin (Amersham Searle) was extracted into 8.0 ml of chloroform from 0.05 to 0.10 ml of media added to 2 ml of 0.5*M* sodium borate buffer (*p*H 10). Chloroform extracts were washed once with 2 ml of this buffer and twice with 2.0 ml of 0.1*N* HCl or 1*N* HCl in the case of [^{14}C]serotonin incubations. Ninety to ninety-five percent of added melatonin was extracted by this procedure. Samples of the chloroform extracts were evaporated to dryness, and the radioactivity was measured in a liquid scintillation spectrometer. To verify the identity of the radioactive product in the chloroform extract, portions of all sample extractions within one treatment group were evaporated in an N_2