they started the separation by discarding the material extracted by 0 to 10 percent ethanol. After we found the three isozymes in the crude supernatant, we examined the fraction extracted by 0 to 10 percent ethanol and found it to contain most of the pi isozyme. Since the pi isozyme constitutes only about 5 to 10 percent of the total activity, it was easily overlooked. The pi isozyme seems to represent about half of the enzyme activity in fetal liver, however, and therefore may be important.

The existence of three isozymes of phenylalanine hydroxylase may be important clinically. A genetic defect that results in the absence of the enzyme causes the well-known disease phenylketonuria (PKU) (*14*). As reported by Auerbach and his associates (*15*), PKU is not a simple all-or-none phenomenon. Varying degrees and combinations of temporary or permanent symptoms have been observed. This range, from a mild condition—characterized by a slight increase in phenylalanine in the blood, no mental retardation, and no phenylpyruvic acid in the urine—to classical PKU, prompted these investigators to classify PKU as a special case of "hyperphenylalanemias." Much of this clinical variation could be explained by these isozymes. For instance, genetic defects less extreme than that which causes complete loss of the enzyme might result in the absence or delayed maturation of one or more of the isozymes. This variation in genetic defects would produce varying degrees of total enzyme activity and variable symptomatology and disease in the adult.

Moreover, the possibility that fetal isozymes exist (as indicated by the pattern of enzyme activity from human fetal liver) or that isozymes appear at different ages may explain the occurrence of mental retardation. As Winick and his associates have pointed out (*16*), brain growth has a rapid perinatal phase. If a fetal isozyme were absent or if appearance of other isozymes were delayed during any part of that period of rapid growth, brain growth and development would be impaired (*17*). Further, as the other isozymes developed, the perinatal insufficiency of phenylalanine hydroxylase could go unnoticed.

JOHN A. BARRANGER, PAUL J. GEIGER
A. HUZINO, SAMUEL P. BESSMAN
Department of Pharmacology,
University of Southern California
School of Medicine, Los Angeles 90033

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Turnover of Molecules Which Maintain the Normal Surfaces of Contact-Inhibited Cells

Abstract. *In confluent cultures normal chick embryo fibroblasts become highly agglutinable by concanavalin A within 6 hours after their synthesis of protein is inhibited by cycloheximide, pactamycin, or emetine. When growing cells are similarly treated, they fail to become more agglutinable. Apparently, molecules which maintain the normal structure of the cell surface must be replaced continually when cell growth is contact inhibited.*

A difference between the surfaces of normal and of cancerous cells has been revealed by several carbohydrate-binding plant agglutinins, including concanavalin A (Con A) (*1*). Normal cells, which respond to cell contact in culture by limiting their growth, are not agglutinated by these substances, while cancer cells, which do not exhibit contact inhibition of growth, are agglutinated. The cell surface sites which react with Con A are involved in growth control; partially degraded Con A reacts with transformed cell surfaces and restores their ability to become contact-inhibited (*2*). We found that normal, nonagglutinable fibroblasts from chick skin became agglutinable by Con A when they were treated with cycloheximide, an inhibitor of protein synthesis. The experimentally induced change, from a normal to a malignant-like cell surface, suggests a turnover or a loss of specific molecules of the cell surface. This fact could be useful in the study of the cell surface alteration

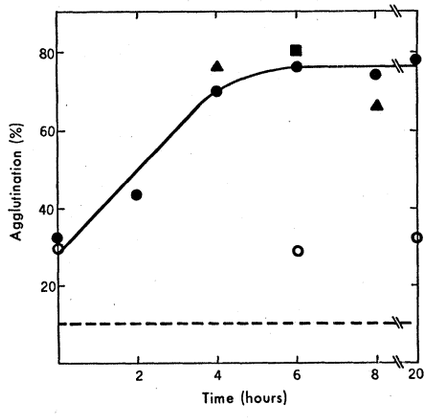

Fig. 1. The agglutinability of chick embryo fibroblasts with 200 μg of concanavalin A per milliliter of saline solution after inhibition of cellular protein synthesis. Contact-inhibited cells (closed symbols) and growing cells (open symbols) were incubated with cycloheximide (circles), emetine (triangles), or pactamycin (squares) for the time indicated. The cells were removed from plates and were shaken with concanavalin A; the percentage of cells agglutinated in clumps of two or more cells were then counted. The dashed line represents the constant background clumping of cells when concanavalin A was not present.

occurring during malignant transformation.

Primary chick skin fibroblasts, grown to confluence in F-12 medium containing serum (0.5 percent) on 60-mm tissue culture plates (Falcon) (*3*), were incubated with $2 \times 10^{-5}M$ cycloheximide, a concentration which inhibits 95 percent of the incorporation of [^{3}H]leucine into material that is precipitable by trichloroacetic acid. The cells were suspended after a 10-minute incubation at 37°C with 5 ml of 0.02 percent ethylenediaminetetraacetic acid in Puck's saline G, by gentle pipetting, and then centrifuged in the cold for 5 minutes at 1000 rev/min. The pellet of cells was suspended in saline G at a concentration of 4×10^6 cells per milliliter. A portion of the suspension (0.1 ml) was added to 0.1 ml of serial dilutions from 50 to 2000 μg of Con A per milliliter (Calbiochem) in saline G and shaken for 10 minutes at 20°C (*4*). Cells shaken by this procedure do not form excessively large clumps and, although the amount of agglutination varies, agglutination can be quantified under a microscope as the percentage of cells present in clumps of two or more cells. Agglutination increased from 30 percent in untreated cells to more than 75 percent in treated cells (Fig. 1). This increase occurred linearly over the first 4 to 6 hours of cycloheximide treatment and persisted for at least 20 hours of incubation with cycloheximide. Agglutination after 6 hours was about the same as that obtained from a standard procedure of trypsin treatment (*5*). The agglutination by Con A of the untreated cells may reflect their embryonic origin (*6*) but it was similar in degree over a concentration range of 100 to 2000 μg of Con A per milliliter of solution.

The increased agglutinability resulted from inhibition of cellular protein synthesis rather than from some other effect of cycloheximide, because inhibition of more than 95 percent of protein synthesis by $10^{-6}M$ emetine (*7*) or $10^{-7}M$ pactamycin (*8*) increased the agglutinability of cells by Con A just as cycloheximide did (Fig. 1).

The increased agglutination of cells treated with cycloheximide cannot be attributed to any irreversible damage to the cells; normal (low level) agglutination reappears within 6 hours after the removal of cycloheximide from cells previously treated for 5 hours. This reversal of agglutinability follows the same time course as did the original increase in agglutinability brought about by inhibiting protein synthesis.

The results were obtained with confluent, contact-inhibited cell cultures. Since there is evidence that the glycoproteins of the cell surface turn over rapidly in contact-inhibited cells but not in growing cells (*9*), we examined the Con A agglutination of growing cells treated with cycloheximide. Growing cells did not have an increase in agglutinability even after a 20-hour incubation in cycloheximide (Fig. 1), suggesting that the components of the cell surface are more stable in growing cells.

The increase in Con A agglutination of contact-inhibited cells that were exposed to cycloheximide probably indicates the loss of cell surface molecules containing protein. This may be a result of either the turnover or the release of these molecules by contact-inhibited cells.

There are a number of observations which relate the increase in Con A agglutinability to the failure of cells to respond to contact inhibition (*2*, *10*). Thus, the molecules which turn over in cells that are contact inhibited may be intimately involved in the maintenance of the cell surface structure necessary for contact inhibition. The cells treated with cycloheximide may be released from contact inhibition as they become agglutinable, but it is difficult to test this possibility because of the absence of growth during inhibition of protein synthesis.

JOFFRE B. BAKER*
TOM HUMPHREYS*
Department of Biology, University of California at San Diego, La Jolla 92037

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* Present address: Pacific Biomedical Research Center, University of Hawaii, Honolulu 96822.

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Modulation of Adenylate Cyclase Activity in Liver and Fat Cell Membranes by Insulin

Abstract. *Insulin depresses both the activity of adenylate cyclase stimulated by glucagon, epinephrine, and sodium fluoride in liver cell membranes and the activity of adenylate cyclase stimulated by epinephrine and adrenocorticotropin in particulate preparations from homogenates of isolated fat cells. Significant inhibition is detected with very low concentrations (10^{-11} molar) of insulin but not with unphysiologically high ($> 10^{-9}$ molar) concentrations of the hormone. These direct effects of insulin on an enzymatic system in broken-cell preparations suggest a fundamental role of adenylate cyclase activity and of cyclic adenosine monophosphate in the mechanism of action of insulin.*

It has not been possible to explain, by a single common mechanism, the effects of insulin on such diverse activities as membrane transport, lipolysis, glycogen synthesis, and protein metabolism. In that most of these can be classified as anabolic processes and are thus the reverse of processes mediated by adenosine 3′,5′-monophosphate (cyclic AMP), a fundamental effect of insulin may be to modulate the intracellular concentration of this nucleotide.

Under certain conditions insulin can decrease the concentration of cyclic AMP in adipose tissue cells (*1*) and in liver cells (*2*), and these changes appear to correlate with at least some of the biological effects of insulin in these tissues (*1–3*). The manner by which insulin lowers cyclic AMP concentrations, however, is unknown. Senft *et al.* (*4*) have reported a decrease in activity of cyclic AMP phosphodiesterase which can be reversed by insulin administration in rats made diabetic by injection of alloxan, but others (*5*) have failed to detect effects on this enzyme by administration in vivo or in vitro of insulin. Furthermore, the inability to demonstrate effects of insulin on adenyl-