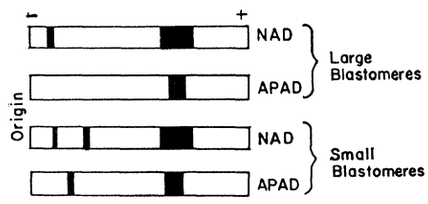

Fig. 1. Bands of activity of L-malate dehydrogenase with NAD and APAD, separated by disk electrophoresis on polyacrylamide gel and stained with nitro blue tetrazolium.

The ratio of the rates of reaction of malate dehydrogenase with acetylpyridine-adenine nucleotide (APAD) compared with NAD is 0.68 in unfertilized eggs, but rises during the early embryonic period and reaches 2.2 in 48-hour embryos (*5*). In the preparations from blastomeres separated at the 64-cell stage, the APAD/NAD ratio for malate dehydrogenase activity was 1.4 in preparations from small blastomeres and 2.3 in preparations from large blastomeres (Table 1).

Staining of the acrylamide gels revealed a slowly migrating band of NAD-malate dehydrogenase activity in small blastomeres which is not present in preparations from large blastomeres. The small cells have a total of three NAD-malate dehydrogenases which are separable by electrophoresis and the large cells have two (Fig. 1). Two of the bands in the small cells appear to be identical to the two bands seen in preparations from large blastomeres. Preparations from small blastomeres subjected to electrophoresis show two bands of APAD-malate dehydrogenase activity whereas preparations from large blastomeres show only one. The additional band of APAD-malate dehydrogenase in the small blastomeres moves slowly and remains near the anode, but it is not identical with the additional band of NAD-malate dehydrogenase. Material obtained from unfertilized eggs by homogenization or ultrasonication and subjected to disk electrophoresis on polyacrylamide gels showed five bands of malate dehydrogenase activity with NAD, and eight with APAD. Thus, as development proceeds from the egg to the 64-cell embryo, the number of malate dehydrogenases which are separable by electrophoresis decreases from five to three in small blastomeres, and to two in large blastomeres.

The method of separating small and large blastomeres in quantities large enough for chemical analysis makes possible studies of the development of biochemical differences in cells during early embryonic development. Later in development the differences in the size and density of the cells are too small to permit separation. The present experiments with the multiple forms of malate dehydrogenase provide an example of the differentiation of enzymes early in development, and indicate the feasibility of a study of the genetic and biochemical mechanisms controlling the formation of enzymes during development, analogous to the classic experiments in microorganisms (*6*).

The finding that there are as many as five forms of NAD-L-malate dehydrogenase might suggest that these are tetramers of two types of subunits, and that the five forms represent the five possible combinations of the two subunits, as suggested for mammalian lactate dehydrogenases (*7*). Vertebrate tissues have only two malate dehydrogenases which are separable by electrophoresis (*2*). The fundamental genetic control of malate dehydrogenase may be similar to that postulated for lactate dehydrogenase—that is, two genes, one for each type of subunit; but the two types of subunits may be prevented from forming hybrids in vertebrates although this is possible in the echinoderm system (*8*).

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Actinomycin: Inhibition of Cortisone-Induced Synthesis of Hepatic Gluconeogenic Enzymes

Abstract. *The cortisone-induced de novo synthesis of liver glucose-6-phosphatase, fructose-1,6-diphosphatase, aldolase, and lactic dehydrogenase was prevented by injections of Actinomycin D in the rat. In the in vitro assay systems, addition of actinomycin exerted no effect on the enzyme activities examined. The evidence favors the concept that the increased gluconeogenesis induced by corticoids entails at a certain stage an increased rate in the synthesis of gluconeogenic enzymes.*

Hepatic glucose-6-phosphatase activity which represents the final common path of gluconeogenesis and glycogenolysis increases after administration of corticoid (*1*), and the findings have been confirmed (*2*). Subsequently it was demonstrated that the activity of other enzymes involved in gluconeogenesis, such as fructose-1,6-diphosphatase, phosphohexose isomerase, and lactic dehydrogenase, also increases after cortisone is injected into normal, adrenalectomized, and hypophysectomized rats (*3*). Injection of ethionine (*4*) or puromycin (*5*) also inhibits these increases, suggesting that corticoid injection induces *de novo* synthesis of the gluconeogenic enzymes. Recently it was demonstrated that injection of puromycin and actinomycin prevents deposition of liver glycogen in starved rats injected with cortisone (*6*). Since actinomycin inhibits selectively deoxyribonucleic acid-directed synthesis of RNA in both microorganisms and mammalian cells (*7*), we tested the effect of this compound on cortisone-induced increases in liver gluconeogenic enzymes. Actinomycin completely inhibits cortisone-induced enzyme synthesis, nitrogen increase, and glycogen deposition in liver.

Male Wistar rats, weighing 90 to 100 g and maintained on Purina laboratory chow and water ad libitum, were divided into three experimental groups: (i) normal rats, (ii) rats treated with cortisone, and (iii) rats treated with cortisone and actinomycin. Normal rats were used for controls because preliminary experiments showed that physiological saline injections in volumes and regimens identical with those used for the treated groups had no effect on the enzymes examined or on the nitrogen or glycogen content. In the second

group enzyme induction was achieved by injecting cortisone acetate (intramuscularly, 25 mg/100 g rat) daily for 5 days. The rats were killed on the 6th day, in accord with the experimental arrangements of previous studies (*1*, *3*, *5*). In the third group, for prevention of enzyme synthesis, animals were injected with cortisone and actinomycin (intraperitoneally, 0.008 mg/100 g rat) daily for 5 days. The actinomycin was administered ½ hour earlier than the cortisone. The animals were killed on the 6th day. From the decapitated and exsanguinated rats, livers were rapidly excised and 10 percent homogenates were prepared in isotonic KCl. The supernatant fluid was obtained by centrifuging tissue homogenates at 100,000 *g* for 30 minutes at 0°C in a refrigerated Spinco model L centrifuge. The cellularity of the liver was determined by nuclear counts (*8*).

Nitrogen content was determined by the micro-Kjeldahl technique, and glycogen was measured by the anthrone procedure (*9*). The assays for glucose-6-phosphatase (*10*), fructose-1,6-diphosphatase, and lactic dehydrogenase (*11*) followed methods described previously. Aldolase was assayed by a spectrophotometric method (*12*). Glucose-6-phosphatase was measured in the homogenate, whereas the other enzymes were measured in the supernatant fluid. Enzymatic activities were expressed in micromoles of substrate metabolized per hour per average cell at 37°C.

The behavior of gluconeogenic enzyme systems and liver nitrogen and glycogen content in normal rats after cortisone induction and after roughly concurrent administration of cortisone and actinomycin is summarized in Fig. 1. Cortisone administration increased hepatic glucose-6-phosphatase activity to 265 percent of normal, fructose-1, 6-diphosphatase to 295 percent, aldolase to 290 percent, and lactic dehydrogenase to 185 percent. Liver homogenate and supernatant nitrogen levels rose to 154 and 153 percent of normal, respectively, whereas hepatic glycogen content rose to 262 percent of normal. All increases induced by cortisone are statistically significant. In the group in which actinomycin was administered concurrently with cortisone, the synthesis of the gluconeogenic enzymes studied, as well as the rise in nitrogen and glycogen levels, was prevented. Results similar to those described here were also obtained for malic dehydrogenase activity as assayed in the direction of gluconeogenesis by measuring the conversion of malate to oxalacetate (*13*).

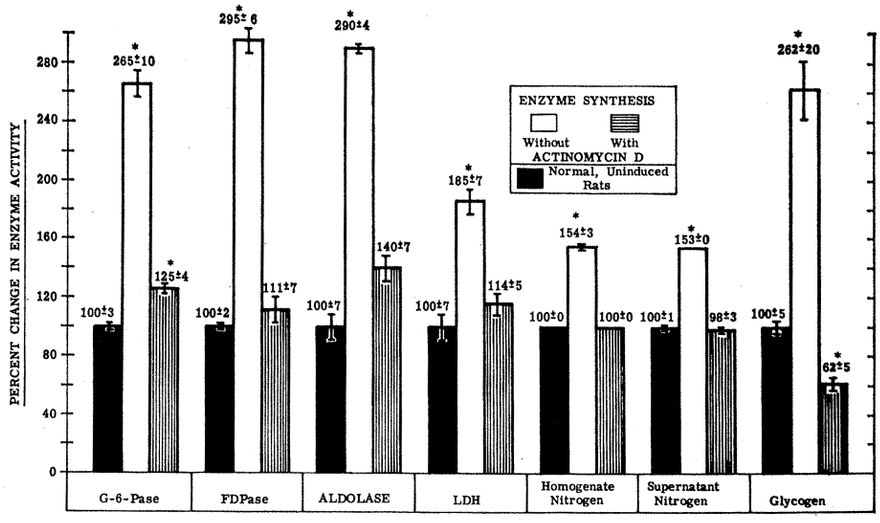

Fig. 1. Effect of actinomycin on cortisone-induced liver enzyme synthesis, nitrogen increase, and glycogen deposition. The enzyme activities and the biochemical parameters were calculated per average cell, and the data are expressed as percentages of the liver values of untreated normal rats which are taken as 100 percent. The asterisks indicate alterations statistically significantly different from values in normal rats.

In studies in vitro the addition of actinomycin (0.04 to 0.4 μg/100 mg of liver) to the assay systems for glucose-6-phosphatase, fructose-1,6-diphosphatase, aldolase, lactic dehydrogenase, or malic dehydrogenase had no effect on the enzymes. Prior incubation of the homogenate or supernatant fluid with actinomycin for 30 minutes at 37°C also failed to affect the activities of the gluconeogenic enzymes.

Cortisone administration stimulates the extent of precursor incorporation into hepatic RNA in vivo (*14*). The presently observed inhibition of the cortisone-induced rise of the levels of glucose-6-phosphatase, fructose-1,6-diphosphatase, aldolase, and lactic dehydrogenase by actinomycin is in line with the suggestion that the stimulation of the synthesis of certain RNA species may be one of the primary actions of this hormone (*7*). It is interesting that the effect of actinomycin is more specific than that of puromycin since it interferes with the cortisone-mediated induction of such enzymes as liver tyrosine transaminase and tryptophan pyrrolase but does not affect the substrate-induced elevation in the amount of the latter enzyme (*7*). In our studies the high specificity of actinomycin in inhibiting the *de novo* synthesis of hepatic gluconeogenic enzymes is indicated by the fact that the total dose capable of inhibiting enzyme synthesis was: for ethionine, 100,000 μg; for puromycin, 14,000 μg; but for actinomycin only 40 μg. On a molar basis, if the effective total concentration of the actinomycin is taken as 1, the necessary concentrations for puromycin and ethionine are approximately 2000 and 40,000, respectively.

Since the enzymes we studied include glucose-6-phosphatase and fructose-1,6-diphosphatase, which are rate-limiting in hepatic gluconeogenesis because of their slow reaction rate as well as their key position in circumventing the thermodynamic barriers in gluconeogenesis (*15*), the evidence favors the concept that the increased gluconeogenesis induced by corticoids entails at a certain stage an increased rate in the synthesis of gluconeogenic enzymes (*16*).

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Delayed Incorporation of Tritiated Thymidine into DNA

Abstract. *Delayed utilization of tritiated thymidine by regenerating mouse liver can be almost completely suppressed by a continuous infusion of nonradioactive thymidine. In addition, as shown earlier for lymphocytes, labeled granulocytes are a potential source of the tritium marker. These observations suggest that the delayed incorporation of label into DNA must be due to the transfer of labeled nucleoside, which may be derived either from the degradation of DNA or from a long-lived intracellular pool. In either case, the transferred material probably originates from all tissues that have a high rate of cell turnover.*

Tritiated thymidine has been widely used in the study of DNA synthesis and cell kinetics. Interpretation of work in vivo has been based on the assumption that, once administered, thymidine is available for incorporation into DNA for only a brief period, and that there is no reutilization of label after such incorporation has taken place. Results of a number of recent studies, however, indicate that labeling of DNA may continue to occur for some time after the administration of H^3-thymidine (*1–4*). Delayed tritium labeling has usually been ascribed to the reutilization of DNA, and pathways which have been considered include the transfer of large fragments of nucleic acids from initially labeled cells, or a specific "trephocytic" function of lymphocytes, whereby whole DNA molecules or DNA fragments are passed on to dividing tissues as part of some nutritive process. This communication presents evidence that the substance transferred is thymidine itself, and that it is probably derived from all tissues in which there is a high rate of cell turnover.

In the experimental procedure, a high concentration of nonlabeled competitor was made continuously available in a system in which delayed transfer of label is known to occur. Such a system is regenerating liver following partial hepatectomy, as described by Bryant (*2*).

Using his experimental method, male CDF_1 mice were given three intraperitoneal injections of H^3-thymidine at 12-hour intervals, and 28 hours after the last injection a subtotal hepatectomy was performed with removal of an average of 68 percent of the original liver mass. DNA synthesis in regenerating mouse liver is maximal on the second day after hepatectomy (*5*) or at least 48 hours after the final administration of the thymidine tracer. Since a tracer dose of H^3-thymidine is available for immediate DNA synthesis for only 1 to 2 hours (*6*), labeling of regenerating liver which exceeds the baseline activity of the excised tissue is presumably due to reutilization of the original tritium marker.

Nine animals that were treated as described received, in addition, continuous intravenous infusions (*7*) of nonradioactive thymidine, beginning just prior to hepatectomy and continuing until the time of autopsy. A 0.9-ml volume of isotonic fluid, containing 31.5 mg of thymidine, was delivered each day into the inferior vena cava through a catheter inserted in a tail vein. A "swivel-conduit" allowed the recipient almost free movement within its cage, although the tail was necessarily maintained in a vertical position. Three other mice received infusions of saline instead of thymidine; four had no infusions; and three had sham operations, with manipulation of the liver, to provide controls for the degree of liver radioactivity at the time of autopsy.

All the animals were killed 72 hours after operation, and radioautographs were prepared from touch preparations and histologic sections of the residual right lateral and caudate lobes. Kodak N.T.B.-3 emulsion was used and the preparations were exposed for a period of 30 days.

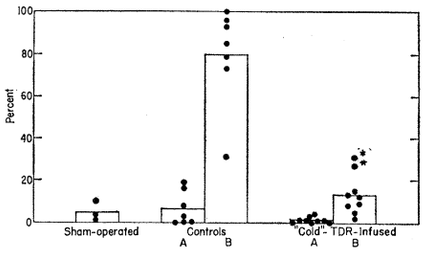

Fig. 1. Percentages of labeled liver cells of mice given tritiated thymidine and then sham operated or partially hepatectomized. *A*, specimens removed at hepatectomy; *B*, samples of regenerated liver removed 3 days after hepatectomy. Controls include three animals which received saline infusions and four which had no infusions. Note the high percentage of labeling in the regenerated liver of the controls and the suppression of labeling in animals receiving cold thymidine (TDR) after hepatectomy. Asterisks denote two animals that had unintentional interruption of continued infusion.

The mean weight of the excised livers in the experimental animals was 1.09 g. In nine controls, the mean weight of excised livers was 1.05 g; these animals were killed immediately after hepatectomy, and the weight of the residual liver was found to average 0.5 g. Regenerated liver at autopsy in the experimental animals averaged 0.96 g. The mean loss in weight of the hepatectomized mice was 4.6 g. There were no significant differences in any of these measurements in mice treated with thymidine or saline, or in those which did not receive infusions.

The results obtained from the radioautographs are summarized in Fig. 1. Less than 3 percent of parenchymal cells were labeled in livers removed at hepatectomy, or at autopsy in sham-operated controls. However, in four animals aged less than 6 weeks, which were used in an early experiment, up to 19 percent of the cells were labeled; consequently, only animals aged 3 to 6 months were used in subsequent experiments.

In control mice which received either no infusions or saline, the majority of regenerating liver cells were labeled 72 hours after hepatectomy, as described by Bryant (*2*). In contrast, animals which received cold thymidine infusions showed only a minimal increase over the baseline in the percentage of labeled liver cells, with the exception of two mice which are indicated by asterisks