polymer phase systems. A preparative modification currently being explored has been successful in separating more than 100 mg of DNP amino acids.

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$$N = (4\ R/w)^2$$

where N is the number of the theoretical plates, R is the retention time referred to the peak maximum, and w is the peak width.
5. The validity of Eq. 1 has been confirmed by manometric measurement.
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Posttranscriptional Control in the Steroid-Mediated Induction of Hepatic Tyrosine Transaminase

Abstract. *The purine analog azaguanine does not inhibit the initial induction of hepatic tyrosine transaminase by hydrocortisone. However, the continued induced synthesis of tyrosine transaminase, elicited by repeated doses of hydrocortisone, is inhibited approximately 64 percent in the presence of the analog after 7 to 8 hours and appears to be almost completely inhibited by 9 to 10 hours; this suggests that the induction cycle involves the activation and renewal of a pool of preexisting messenger RNA.*

Because actinomycin D inhibits the corticosteroid-mediated inductions of hepatic tyrosine transaminase [L-tyrosine : 2-oxoglutarate aminotransferase (E.C. 2.6.1.5)] and tryptophan pyrrolase [L-tryptophan : oxygen oxidoreductase (E.C. 1.13.1.12)] (*1*), it has been inferred that the inductions require the synthesis and transport to the cytoplasm of newly formed messenger RNA (mRNA). However, actinomycin D is reported (*2*) to have a number of effects on RNA metabolism in addition to its inhibition of RNA synthesis. Therefore, we investigated the mechanism of induction of tyrosine transaminase with the purine analog 8-azaguanine, which inhibits protein biosynthesis as a result of its incorporation into mRNA (*3*).

Male Sprague-Dawley rats (250 g) were adrenalectomized 7 days before use and fasted 24 hours before the start of the experiment. 8-Azaguanine, dissolved as described previously (*3*), hydrocortisone sodium succinate (Solucortef, Upjohn), and 5-amino-4-imidazole-carboxamide (AIC) were administered intraperitoneally. Tyrosine transaminase and tryptophan pyrrolase activities were measured (*4*) in the supernatant fraction (105,000*g*) from the liver; one activity unit was defined in each case as the amount of enzyme catalyzing the formation of 1 μmole of product per hour at 25°C. Experiments designed to measure the rate of synthesis of tyrosine transaminase were performed according to standard immunochemical techniques (*5*, *6*) with minor modifications (*7*).

8-Azaguanine inhibits by 89 percent the initial induction by hydrocortisone of hepatic tryptophan pyrrolase, but not that of tyrosine transaminase; a similar differential effect of the nucleoside analog 5-azacytidine on the steroid-mediated inductions of these enzymes has also been reported (*8*). Since the induction of tryptophan pyrrolase is virtually completely inhibited, it is inferred that effective concentrations of 8-azaguanine nucleotides are present during the initial phases of the induction of tyrosine transaminase.

These results are consistent with the hypothesis that the hydrocortisone-mediated induction of tyrosine transaminase utilizes preexisting RNA. An alternative explanation is that the messenger for this enzyme can function normally even when a portion of its guanine residues are replaced by 8-azaguanine. The latter possibility was eliminated by the results of the experiment, shown in Fig. 1, which also provide information concerning the size of the preexisting pool of messenger RNA. Hydrocortisone was administered at 3-hour intervals to ensure the continued induction of tyrosine transaminase at its maximum rate in the control rats. The controls received AIC and saline, while the experimental rats received AIC and 8-azaguanine, with each dose of hydrocortisone. The AIC reduces the deamination of 8-azaguanine in rat liver (*9*) and thereby potentiates the inhibitory action of the analog (*3*); it also eliminates the superinduction of tyrosine transaminase which appears to depend on the extensive deamination of 8-azaguanine (*10*).

The continued induction of tyrosine transaminase is significantly decreased after about 6 hours in the presence of 8-azaguanine (Fig. 1). This approximately corresponds to the time at which maximum induction is attained after a single inducing dose of hydrocortisone. It appears, therefore, that after the initial stage of the induction an increasing proportion of the corticosteroid-induced synthesis of the enzyme is dependent on the synthesis of new mRNA which can be rendered defective by 8-azaguanine. That is, this messenger species does not function normally when it contains the base analog.

Table 1. Inhibition by 8-azaguanine of the continued induced synthesis of tyrosine transaminase elicited by repeated doses of hydrocortisone. Conditions identical to those in Fig. 1, except that the rats were killed either 3 or 8 hours after the initial dose of hydrocortisone. The rats received ^{3}H-leucine (New England Nuclear, 44 c/mmole) (80 μc/250 g) 20 minutes before removal of the liver. The radioactivity in hepatic tyrosine transaminase was estimated and corrected for differences between samples with respect to the specific radioactivity of the free leucine pool in the liver (*7*). The data represent the mean ± range for two rats. The rate of change in enzyme activity during the labeling period was estimated from the curves in Fig. 1 and is included for purposes of comparison (*11*).

Addition	Time (hours)	Enzyme radioactivity: Corrected per milliliter of liver extract (count/min)	Enzyme radioactivity: Relative to that of controls	Increase in enzyme activity during labeling (units per 100 mg of protein per hour)
		Saline control		
		140 + 4	1.0	
		Hydrocortisone + AIC		
Saline	3	508 ± 28	3.6	
8-Azaguanine	3	536 ± 24	3.8	
Saline	8	444 ± 44	3.2	35
8-Azaguanine	8	260 ± 20	1.8	20

These data also suggest that the preexisting pool of inactive mRNA is sufficiently large to support the induction of tyrosine transaminase for 7 to 8 hours, during which time increasing amounts of defective messenger enter the pool in the rats treated with 8-azaguanine. These conclusions are supported by the finding (Fig. 1) that the enzyme is not inducible by hydrocortisone 9 hours after the initiation of the induction in the presence of 8-azaguanine, but that it is still responsive at this time to the administration of the hormone when it has been continually induced in the absence of the base analog. However, when a second attempt is made to reinduce the enzyme with hydrocortisone, 3 hours after the initial unsuccessful attempt (at 12 hours), the enzyme appears to be fully inducible in the rats treated with 8-azaguanine. It is inferred that the administration of hydrocortisone alone at 9 hours causes the renewal of a pool of mRNA for tyrosine transaminase (that is, replacement of defective mRNA by species which contain little or no 8-azaguanine). The new, functional mRNA is then activated by the administration of the hormone at 12 hours.

The kinetic results (Fig. 1) have been confirmed by immunochemical measurements of the rate of synthesis of tyrosine transaminase. Three hours after the administration of hydrocortisone, the rate of synthesis of the enzyme was increased almost 300 percent over the basal rate both in the presence and absence of 8-azaguanine (Table 1). However, by 8 hours after the first dose of hydrocortisone, the rate of synthesis in the controls was still 220 percent above the basal rate, but was only 80 percent above the basal rate in the rats treated with 8-azaguanine; this indicates that the base analog has inhibited the steroid-induced synthesis approximately 64 percent by this time. Furthermore, a comparison (*11*) of the last two columns in Table 1 demonstrates that the decrease in the rate of rise of enzyme activity in the rats treated with 8-azaguanine can be fully accounted for by this inhibition of the rate of synthesis, an indication that the decrease in the rate of rise is not attributable to enzyme degradation. The results of immunochemical studies (*7*) on the rate of degradation of tyrosine transaminase show that the destruction of the corticosteroid-induced enzyme is completely inhibited for up to 10 hours after the administration of 8-azaguanine. This finding explains the failure of the enzyme activity to return to the basal activity in the presence of the analog (Fig. 1), and confirms that the almost complete halt in the rise in enzyme activity seen at 9 to 12 hours results from further inhibition of enzyme synthesis.

The effect of prior treatment of rats with 8-azaguanine and AIC on the steroid-mediated induction of tyrosine transaminase was tested by giving rats either saline or 8-azaguanine (100 mg/kg) together with AIC (50 mg/kg) at 0, 3, and 6 hours. All the rats received hydrocortisone (40 mg/kg) at 6 hours and were killed at 10 hours. The tyrosine transaminase activity (units per 100 mg of protein; basal enzyme activity is 51 ± 4 units per 100 mg of protein) was 244 ± 15 for the rats given saline and 227 ± 15 for those given 8-azaguanine. Thus, the inhibition observed (Fig. 1 and Table 1) does not result from nonspecific effects after the prolonged treatment with 8-azaguanine. These data also suggest that the preexisting pool of mRNA for tyrosine transaminase is stable in the absence of an inducing dose of hydrocortisone.

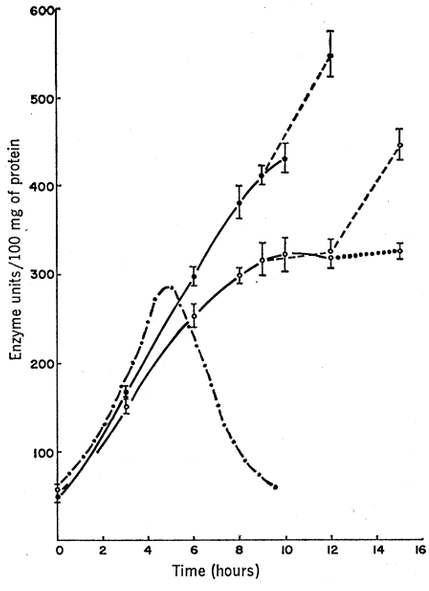

Fig. 1. Inhibition by 8-azaguanine of the continued induction by hydrocortisone of hepatic tyrosine transaminase. Rats received (group 1) hydrocortisone (40 mg/kg), AIC (50 mg/kg), and saline (●——●), or (group 2) hydrocortisone, AIC, and 8-azaguanine (100 mg/kg) (○——○), at zero time and again at 3 and 6 hours. Five rats in each group were killed at 0, 3, 6, 8, 9, or 10 hours, and five rats from group 2 were killed at 12 hours. Attempts to reinduce were begun at 9 hours, and the results are shown by the dashed lines (– – –). For the latter experiments, the remainder of the rats from groups 1 and 2 (which had received the three series of injections) were given a fourth dose of hydrocortisone at 9 hours, and five rats from each group were killed at 12 hours. Since the enzyme was not reinduced in group 2, rats from this group received a fifth dose of hydrocortisone at 12 hours and were killed at 15 hours [controls for the 15-hour point received saline at 9 hours and hydrocortisone at 12 hours (• • • • •)]. The activity of tyrosine transaminase was estimated in the liver of each rat. The data at each point represent the mean ± S.E. for five rats. The induction curve elicited by a single dose of hydrocortisone (•–•–•–•) was established previously (*10*) and is shown for reference purposes.

Since the steroid induction of tryptophan pyrrolase is inhibited almost 90 percent by simultaneous or prior treatment with 8-azaguanine or 5-azacytidine (*8*), it is inferred that the pool of preformed messenger for this enzyme must be small as compared with that for tyrosine transaminase. However, the possibility that the analogs are affecting the induction of tryptophan pyrrolase indirectly, for example through the reticuloendothelial system (*12*), cannot be ruled out.

It is necessary to reconcile these results with the observation (*1*) that actinomycin D inhibits the steroid-mediated induction of tyrosine transaminase. There are at least two reasonable explanations for this apparent discrepancy. It is possible that the preexisting pool of inactive mRNA resides within the nucleus and that actinomycin D causes its destruction, as this drug is known to accelerate the breakdown of nuclear RNA (*2*). Alternatively, the transport of mRNA out of the nucleus may be coupled to its synthesis (*2*). Thus, in the presence of actinomycin D, the preexisting messenger may not leave the nucleus because it cannot be replaced through new synthesis. Although there is evidence that some ribosomal RNA can leave the nucleus in the presence of actinomycin D, a large proportion of the RNA which could qualify as mRNA is degraded to acid-insoluble material in the nucleus after administration of the antibiotic (*13*). Furthermore, the functional integrity of the RNA which does leave the nucleus under these conditions is yet to be established.

The fate of mRNA between the time of its synthesis in the nucleus and its subsequent translation in the cytoplasm is virtually unknown, although the available evidence suggests that complex processing and transport mechanisms

are involved (*14*). Similarly the site of action of hydrocortisone in the induction of tyrosine transaminase is far from clear, although it seems possible from our results that the hormone affects the nucleocytoplasmic barrier, allowing the transport to the cytoplasm of certain RNA species which are normally restricted to the nucleus. Hybridization studies do suggest that certain nuclear RNA species are not normally present in liver cytoplasm (*15*), and that the nucleocytoplasmic barrier may be defective in several hepatomas (*15*).

Whether the use of such analogs as 8-azaguanine will provide a general functional test for the presence of inactive messenger RNA must await further study. However, our results and those of others (*16*) suggest that the investigation of cellular controls (other than the half-life of cytoplasmic RNA) by means of actinomycin D alone may be insufficient; a comparison of the effects of the latter drug with those of 8-azaguanine and 5-azacytidine might be more meaningful.

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Growth in vitro of Cells from Hyperplastic Nodules of Liver Induced by 2-Fluorenylacetamide or Aflatoxin B_1

Abstract. *Cell suspensions obtained from hyperplastic nodules induced in rat liver by either of the two hepatic carcinogens, 2-fluorenylacetamide or aflatoxin B_1, show growth when cultured in vitro. No growth of cells from liver adjacent to the hyperplastic nodules or from liver of control rats has been obtained so far under comparable conditions. Hepatocarcinoma cells induced by 2-fluorenylacetamide grow readily in vitro but behave differently. These findings suggest that some nonmalignant cells capable of growth in vitro arise during liver carcinogenesis prior to the appearance of unequivocal cancer. Cultures of such cells may offer new avenues for the study of liver carcinogenesis.*

The appearance of overt experimental liver malignancy is preceded by the occurrence of a new population of hepatocytes organized into discrete hyperplastic liver nodules (HLN) (*1, 2*) and showing reproducible biochemical and morphologic characteristics (*2*). Such nodules appear to be one site of origin of liver cancer (*1, 2*), since (i) they are seen regularly during carcinogenesis with 2-fluorenylacetamide (2-FAA), aflatoxin B_1, and virtually every hepatic carcinogen, (ii) unequivocal cancer can be observed within nodules in livers without identifiable cancer elsewhere, and (iii) glycogen containing a bound metabolite of 2-FAA is present in nodules and in cancers but not in liver surrounding these lesions. The latter finding is consistent with the hypothesis that the nodules are part of a linear sequence between original liver and hepatic cancer cells. Since these nodules grow and become labeled with radioactive thymidine to a degree far in excess of the surrounding liver (*3*), it became important to study the growth potential of such cells under better controlled conditions in vitro in the hope of not only defining some of the parameters of their growth characteristics but also developing an in vitro system that can be exploited for the biochemical analysis of carcinogenesis.

Male white Wistar rats (Carworth Farms) weighing 150 to 200 grams were used. We induced HLN by feeding the rats a 2-FAA dietary regimen for 15 weeks (*2*) or aflatoxin B_1 for 15 weeks followed by the basal diet. Control animals were given only the basal diet (*2*). Hepatocellular carcinomas were obtained from animals maintained on the 2-FAA regimen (*2*) for 9 months.

In 14 separate experiments conducted over a 1½-year period, rats (16 on 2-FAA regimen and 3 control for each experiment) were killed by ether anesthesia or decapitation. Specimens of readily identifiable HLN, hepatocellular carcinomas, liver adjacent to both these lesions, and control rat liver were aseptically collected. We also obtained HLN and adjacent liver from two rats ingesting 3 parts per million of aflatoxin B_1 and from six fed aflatoxin B_1 at 2 parts per million. Multiple aliquots of tissues used for culture were taken for histologic study (*2*) with no evidence of malignancy observed in nodules by light microscopy. Suitably prepared tissue aliquots were repeatedly trypsinized and suspended in Roswell Park Memorial Institute medium 1629 with added 20 percent fetal calf serum, 1 percent glutamine, penicillin (100 unit/ml), and streptomycin (100 mg/ml) and were inoculated into 4-oz (118-ml) sterile plastic flasks (Falcon) or glass prescription bottles. Collagen-coated cellulose sponges were prepared and used as described by Leighton *et al.* (*4*). The antibiotic tylosin tartrate (*5*) was added to prevent mycoplasma contamination. The procedures of Stulberg *et al.* (*6*) and of Lovelock *et al.* (*6*) were used to preserve the HLN cells at low temperatures.

All cell cultures were incubated at 37°C. Cultures were examined twice weekly when the mediums were changed. The multiplication rate of the cells