tions ($\sim 10^{-3}$ M) of pyruvate will induce prompt and sustained stimulation of sodium transport. Only pyruvate and, to a lesser extent, those substrates known to yield pyruvate during the course of their metabolism, i.e., glucose, lactate, and oxalacetate, have been found to possess this stimulatory effect in the aldosterone-treated bladder. Possibile mechanisms for this effect of pyruvate are considered.

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† Work done during the tenure of a Wellcome travel grant.

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A PARADOXICAL EFFECT OF ACTINOMYCIN D: THE MECHANISM OF REGULATION OF ENZYME SYNTHESIS BY HYDROCORTISONE

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It has been suggested that regulation of enzyme synthesis in microorganisms occurs by alterations in the levels of specific messenger RNA's.[1] In these terms, induction results from an increased synthesis of messenger RNA (mRNA), and repression from inhibition of mRNA synthesis. Similar phenomena in mammals have recently been explained in the same way. Thus, increases in RNA synthesis have been regularly observed in systems where protein synthesis is stimulated by hor-

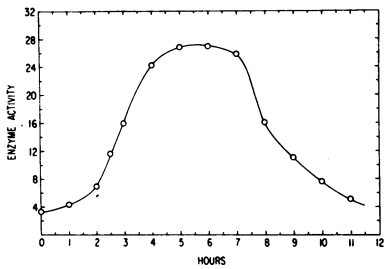

FIG. 1.—Induction of tryptophan pyrrolase by hydrocortisone. Hydrocortisone hemisuccinate (5 mg) was injected intraperitoneally to 118 rats. At least 6 rats were sacrificed each hour for the following 11 hr. The livers were rapidly removed, immediately frozen at −78°C, and stored for 24 hr, at which time they were thawed and assayed for tryptophan pyrrolase (TP). The TP activity, expressed as μM kynurenine/gm liver/hr, represents the average of at least 6 separate animals. The range of enzyme activities at each time point did not exceed 8 μM kynurenine/gm liver/hr.

mones,[2-4] and stimulation of the synthesis of specific enzymes following hormone administration is prevented by actinomycin D (e.g., ref. 5).

In the present communication, a paradoxical effect of actinomycin D on hydrocortisone-induced enzyme synthesis in liver is reported. These studies suggest that in this case, at least, other factors besides the level of messenger RNA are involved in the regulation of enzyme synthesis.

Materials and Methods.—Animals: Male adrenalectomized rats, 140–160 gm, were obtained from the Badger Research Corporation, Madison, Wisc.

Chemicals: Actinomycin D was obtained from the Cancer Chemotherapy Section of the National Institutes of Health, puromycin dihydrochloride from Nutritional Biochemicals, hydrocortisone hemisuccinate from the Mann Research Laboratories, and 5-fluorouracil (5 FU) from the Hoffman-LaRoche Co. Uniformly labeled valine-C^{14} was purchased from New England Nuclear Corp., and orotic acid-6-C^{14} from Nuclear-Chicago.

Procedure: Tryptophan pyrrolase (TP) was assayed according to the method of Civen and Knox[6] as modified by Feigelson and Greengard[7] to measure the total amount of apoenzyme. Tyrosine transminase (TT) was assayed as described by Lin and Knox.[8] Proteins were prepared for radioactive counting by the method of Siekevitz[9] and assayed in a liquid scintillation counter. Radioactive RNA was purified from liver as described by Dingman and Sporn,[10] and counted in a gas flow counter.

Results.—Course of tryptophan pyrrolase induction: As shown in numerous previous studies,[4] the intraperitoneal injection of hydrocortisone to adrenalectomized animals resulted in a rapid increase in the level of TP in the liver. In the present experiments (Fig. 1) (using hydrocortisone hemisuccinate), the maximum enzyme activity was reached in 5 hr, and this level was maintained for the next 2 hr, i.e., 5–7 hr after hormone administration.

Tryptophan pyrrolase degradation: Previous studies have shown that the hormone-induced increase in TP activity when assayed as in the present studies results from an increase in the rate of synthesis of the enzyme and is not due to activation of an inactive precursor.[4, 11] As illustrated in Figure 1, the induced level of TP falls to the basal level after several hours. Therefore, as previously suggested,[12, 13] enzyme degradation also plays a role in determining its intracellular concentration, and recently it has been suggested that regulation of enzyme levels may be mediated by changes in rates of degradation.[14, 15]

The possibility was therefore investigated that the time course of TP appearance

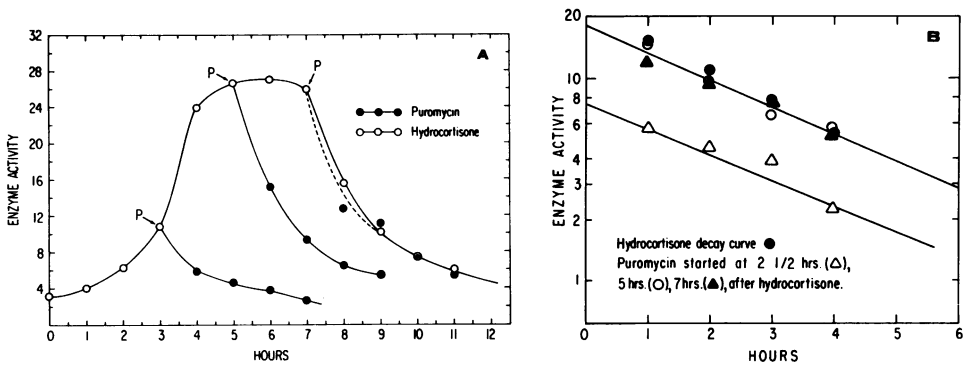

FIG. 2.—Degradation of tryptophan pyrrolase following puromycin administration. (*A*) Hydrocortisone was administered to rats and tryptophan pyrrolase assayed in the livers as previously described. Puromycin was administered intraperitoneally to 3 groups of rats (each containing 24 animals) beginning at the times indicated by *P*, i.e., at 2.5, 5, and 7 hr after hormone administration. Puromycin was given according to the following schedule: initial dose, 20 mg/rat; 1 hr later, 15 mg/rat; then 10 mg/rat each hour for the next 2 hr. Six rats from each group were sacrificed each hour after puromycin administration was started for the next 4 hr. The TP activity is expressed as in Fig. 1 with a range of enzyme activities at each point that did not exceed 9 enzyme units. (*B*) The data from (*A*) plotted on a semilogarithmic scale.

and disappearance following induction was due to changes in its rate of degradation during the experimental period. For this purpose, puromycin was administered at different times after hormone administration (Fig. 2).[16]

Figure 2*A* depicts the decline in TP activity when puromycin was administered during the course of induction, and 2*B* shows a semilogarithmic plot of these data which illustrates that the decline in activity followed first-order kinetics with a $T_{1/2}$ of about 2.5 hr at all times during the course of induction. This value is very close to that previously reported for the degradation of basal and induced enzyme.[11, 17] Since the rate constant of degradation was unchanged during the experiment, the changes in enzyme levels illustrated in Figure 1 were due entirely to changes in the rate of enzyme synthesis which slowed abruptly at 5 hr, and stopped completely 7 hr after hormone injection.

Effect of tryptophan on TP activity: The effect of tryptophan on the kinetics of TP induction also suggested that decreased enzyme synthesis, and not enhanced degradation, produced the plateau and decline of TP level beginning at 5 hr.

It has recently been demonstrated[14] that tryptophan administration to rats stops the degradation of TP *in vivo* without affecting its rate of synthesis. This makes possible an evaluation of TP synthesis without having to account for its degradation. Tryptophan was administered to untreated adrenalectomized rats and similar animals which had been injected with hydrocortisone 7 hr previously, and the resulting levels of tryptophan pyrrolase were compared (Fig. 3). In the controls, not treated with hormone, tryptophan caused the expected increase in the basal level of TP, presumably by inhibiting TP inactivation and allowing the basal rate of synthesis to proceed. However, a similar dose of tryptophan, administered to the rats injected with hydrocortisone 7 hr previously, did not increase the enzyme level, although the rate of enzyme degradation was greatly decreased by tryptophan as previously shown.[14] This failure of tryptophan to increase the TP level after hydrocortisone treatment was consistent with the absence or marked decrease of enzyme synthesis 7 hr after induction.

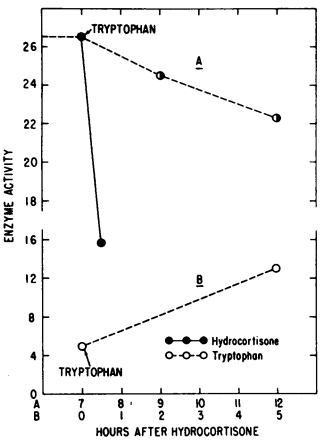

FIG. 3.—Effect of tryptophan administration on tryptophan pyrrolase activity 7 hr following hydrocortisone. (*A*) Twenty-four rats were injected with hydrocortisone hemisuccinate as described. Seven hr later 120 mg of L-tryptophan, dissolved in 0.15 *M* NaCl, was administered intraperitoneally to 12 rats, 6 of which were sacrificed 2 hr later, i.e., 9 hr after hydrocortisone. The remaining 6 rats were sacrificed 5 hr later, i.e., 12 hr after hydrocortisone. (*B*) L-Tryptophan (120 mg) was administered as in (*A*) to 6 adrenalectomized rats which were killed 5 hr later. Each point represents the average tryptophan pyrrolase activity of 6 rats as in Fig. 1 with a range of enzyme activities at each time point of less than 8 enzyme units.

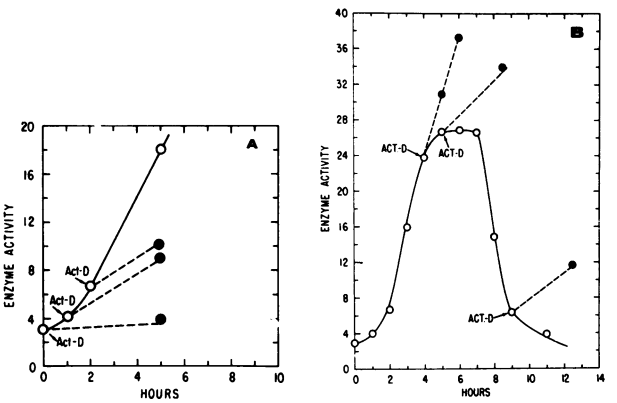

FIG. 4.—Effect of actinomycin D on tryptophan pyrrolase induction. (*A*) Following the injection of 5 mg hydrocortisone hemisuccinate intraperitoneally to rats at zero time, 0.6–1.0 mg actinomycin D was administered intraperitoneally at zero time, 1, and 2 hr after hormone administration at points marked "Act-D." Tryptophan pyrrolase activity was assayed in each case 5 hr after hormone injection. Values from actinomycin-treated animals (●) were compared with hormone-treated animals which did not receive actinomycin D (○). Each point represents the average tryptophan pyrrolase activity of 6 rats. The range of enzyme activities at each time point did not exceed 8 units. (*B*) The experimental conditions are the same as described under (*A*) except that actinomycin D was administered at 4, 5, and 9 hr after hormone treatment (●), and the values were compared with uninjected animals (○). Each point represents the average of at least 6 rats. The range of enzyme activities at each time point did not exceed 6 units.

Messenger RNA (mRNA): In microorganisms, it is generally considered that the rate of enzyme synthesis is regulated by the concentration of mRNA. In the present case, abrupt variations in mRNA levels would have had to occur to account for the relatively sudden changes in the rate of TP synthesis observed after hormone induction.[18] Therefore, the role of RNA synthesis in TP induction was investigated by administering actinomycin D[19, 20] at different times after hydrocortisone injection, and observing the resulting levels of TP in the liver.

Basal messenger: The following studies indicate that the mRNA for TP synthesis in the absence of hormone, i.e., basal messenger, was not rapidly degraded. The administration of 1 mg of actinomycin D, which blocked orotic acid-6-C^{14} incorporation into RNA by more than 90 per cent, did not affect the basal level of TP for at least 6 hr, although puromycin (which inhibits enzyme synthesis) caused about a 75 per cent decrease in the levels of TP during this time.

Furthermore, the same dose of actinomycin did not prevent tryptophan from causing the expected increase in TP activity when followed for 7 hr after trypto-

phan administration, confirming the results of Greengard and Acs.[5] Since the rise in TP due to tryptophan administration results from inhibition of enzyme degradation, it reflects only the rate of TP synthesis. The lack of an actinomycin effect on this process indicates, again, that the level of basal mRNA remained constant despite the fact that RNA synthesis had been almost totally inhibited.

Effect of actinomycin D on TP induction by hydrocortisone: When 0.6–1.0 mg of actinomycin D was administered together with hydrocortisone, TP induction did not occur.[5, 21] The same doses of actinomycin, given 1 or 2 hr after the hormone, likewise inhibited TP synthesis (Fig. 4*A*). These observations are consistent with a requirement for (DNA-dependent) RNA synthesis in the induction process.

Surprisingly, however, when the same dose of actinomycin was administered 4 hr after hydrocortisone (Fig. 4*B*), there was an increase in enzyme activity for the succeeding 2 hr, rather than the expected plateau seen without actinomycin. Sim-

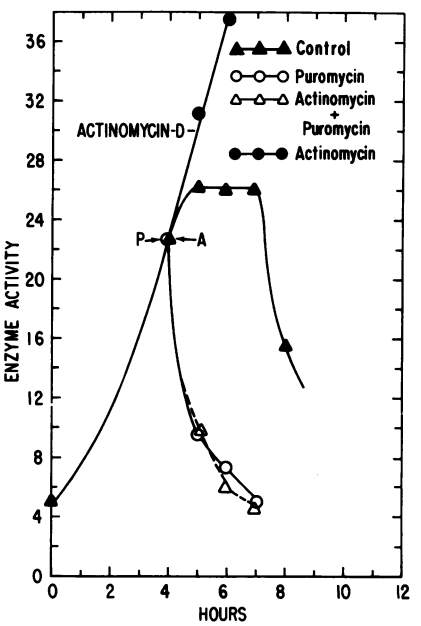

FIG. 5.—Effect of puromycin and actinomycin D on tryptophan pyrrolase. Hydrocortisone hemisuccinate (5 mg) was administered to rats as described under Fig. 1. Four hr after hormone administration, they were divided into 4 groups (italicized): *Actinomycin D* (0.8 mg) was injected and following this, points were taken 1 and 2 hr later; *puromycin* was administered according to the schedule described under Fig. 2 with points taken 1, 2, and 3 hr after puromycin, and *actinomycin D + puromycin* as above with points taken 1, 2, and 3 hr later. *Control* was given only hydrocortisone at zero time. Each point represents the average tryptophan pyrrolase activity from 6 rats. The range in enzyme activities was less than 7 units at each time point.

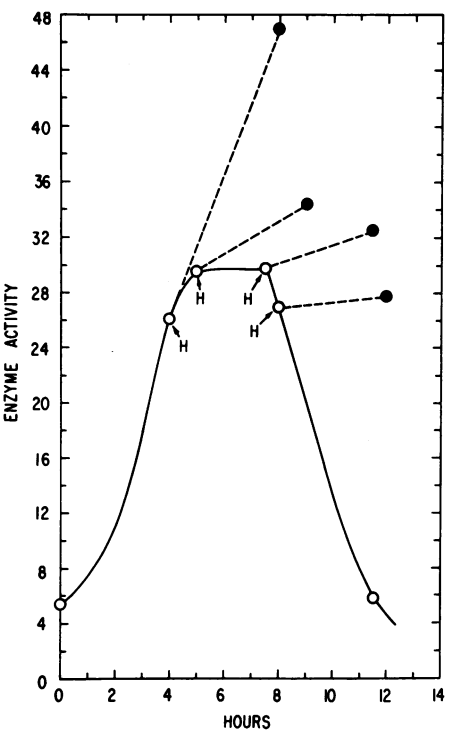

FIG. 6.—Effect of second dose of hydrocortisone following induction by hydrocortisone. Hydrocortisone hemisuccinate (5 mg) was administered to rats as described under Fig. 1. A second injection (*H*) of 5 mg hydrocortisone hemisuccinate was given 4, 5, 7, and 8 hr after the initial dose. Tryptophan pyrrolase activity was assayed 4 hr after each of the second doses of hormone (●). Each point represents the average TP activity from the livers of 6 rats. The range of enzyme activities was less than 10 units (○, enzyme activity following initial dose of hydrocortisone).

ilarly, when actinomycin was given at 5, 6, 7, 8, or 9 hr after hydrocortisone, the expected fall in enzyme level did not occur, and, indeed, frequently an increase in TP activity was seen. Figure 4*B* illustrates typical experiments in which actinomycin was given at 4, 5, or 9 hr after hormone administration.

The rise in TP activity produced by actinomycin was blocked by the simultaneous administration of puromycin (Fig. 5), suggesting that actinomycin stimulated TP synthesis, rather than its activation.

In another experiment, TP activity fell from 24 to 14 units in the interval between 5 and 8 hr after hydrocortisone (5 mg) administration. When tryptophan (120 mg) was given at 5 hr, there was a slight rise from 24 to 37 units in this period, as expected. However, when tryptophan was given together with actinomycin (1.0 mg) at 5 hr, the enzyme rose to 50 units. This again indicates that actinomycin administration results in an increased TP synthesis when given later than 4 hr after hydrocortisone.

Another inhibitor of RNA synthesis, 5-fluorouracil (5-FU), also appeared to increase TP synthesis or prevent its fall when given 5 hr after hydrocortisone administration. Table 1 illustrates that in the interval between 7 and 9 hr after hormone treatment, the control levels of TP fell, while it apparently rose in rats which had also received 5-FU.

TABLE 1

EFFECT OF 5-FLUOROURACIL ON INDUCED SYNTHESIS OF TRYPTOPHAN PYRROLASE

Hr after hydrocortisone	No. of animals	μM Kynurenine/gm liver/hr Control	5-FU
7	12	12.7 (9–16)*	10.2 (7–14)
9	12	5.9 (3–8)	12.0 (9–16)

* Range.

Hydrocortisone hemisuccinate (5 mg) was administered to 24 rats. Starting at 5 hr after hydrocortisone administration, 50 mg 5-FU was injected hourly to each of 12 rats. Points were taken and the liver was assayed 2 and 4 hr later, i.e., 7 and 9 hr after hydrocortisone.

TABLE 2

EFFECT OF 5-FLUOROURACIL ON INDUCED TYROSINE TRANSAMINASE SYNTHESIS

Hr after hydrocortisone	No. of animals	μM p-hydroxyphenyl pyruvic acid/hr/gm liver Control	5-FU
7	12	48 (37–58)*	48 (35–60)
9	12	23 (15–30)	138 (110–150)

* Range.

The experimental procedure is the same as described for Table 1 with the exception that tyrosine transaminase is assayed instead of tryptophan pyrrolase.

As a result of these findings, it seemed that the decrease in the rate of TP synthesis which occurs between 4 and 5 hr after hydrocortisone treatment required RNA synthesis. Furthermore, when TP synthesis had slowed (at 5 hr) or even had stopped completely (at 7 hr), it could be restarted by inhibitors of RNA synthesis, indicating that TP mRNA was present, but not functional, at these later times. It seemed that inhibition of RNA synthesis by actinomycin D (or 5-FU) later than 4 hr after hydrocortisone prevented the formation of a substance "TP repressor" which inhibited further TP synthesis.[22] In addition, the "TP repressor" would have had to turn over considerably more rapidly than TP messenger, since when repressor synthesis was blocked by actinomycin or 5-FU, the repression disappeared, allowing TP synthesis to continue.

On the basis of these considerations, the appearance of "TP repressor" (i.e., about 4 hr after hydrocortisone) should inhibit the stimulation of TP synthesis by a second injection of hydrocortisone given after that time. As shown in Figure 6, a second dose of hydrocortisone, 4 hr after the first, stimulated TP synthesis as effectively as the original injection. However, when the second dose of hormone

was given later than 4 hr (i.e., 5, 7, or 8 hr) after the initial injection (when TP repressor was presumed to have appeared), the hormone was much less effective in stimulating TP synthesis.

Tyrosine-α-ketoglutarate transaminase (TT): Hydrocortisone also induces TT in rat liver,[8, 24] and the experiments described below indicate that repression of TT synthesis occurred by a mechanism similar to that of TP. Actinomycin D did not cause a fall in the basal level of the enzyme for at least 7 hr. Puromycin, however, produced a significant decrease in enzyme level during this time, indicating that the basal messenger for TT is relatively stable. Figure 7 illustrates the effects of actinomycin D on TT synthesis induced by hydrocortisone. As with TP, actinomycin D blocked TT induction when given simultaneously with the steroid,[5] and induced TT synthesis was also inhibited when actinomycin D was administered 1 or 2 hr after hydrocortisone. Again, as with TP, actinomycin stimulated enzyme activity when given 4 hr or later after hormone treatment (Fig. 7). 5-FU markedly stimulated TT synthesis when administered 5 hr after the hydrocortisone injection (Table 2).

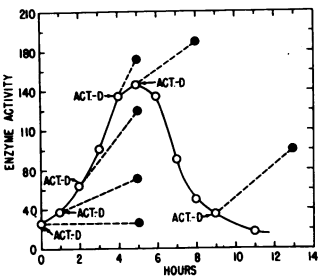

FIG. 7.—Effect of actinomycin D on tyrosine transaminase induction. After 5 mg hydrocortisone hemisuccinate was injected, 1 mg of actinomycin D was administered at zero time, 1, 2, 4, 5, and 9 hr after hormone administration. Each point represents the average tyrosine transaminase activity from the livers of 6 rats, expressed as μM p-hydroxyphenylpyruvic acid/hr/gm liver. The range of enzyme activities at each time point was less than 20 units.

TT responded similarly to TP to additional doses of hydrocortisone, that is, a second dose given 4 hr after the first, was as effective as the original injection, but when administered 5, 7, or 8 hr later, the response was markedly inhibited. Therefore, similar mechanisms may operate in the regulation of both TT and TP synthesis, although we have not yet studied TT induction as extensively as that of TP.

Discussion.—The findings presented above suggest the following hypothesis to explain the kinetics of tryptophan pyrrolase induction by hydrocortisone. (1) The hormone initially stimulates enzyme formation by an actinomycin-sensitive process presumably involving RNA synthesis.[2, 5] (2) About 4 hr after hormone administration, when the rate of enzyme synthesis is maximal, a repressor appears which inhibits further enzyme synthesis by inhibiting the function of existing mRNA. (3) The appearance of the repressor is likewise actinomycin-sensitive and presumably represents a product (or products) from genes different from the structural genes of TP. (4) TP messenger RNA is relatively stable while the repressor has a rapid rate of turnover.

Obviously, the effects of hydrocortisone need not be due directly to the hormone but could result from secondary metabolic effects.

The possibility that the repressor acts to destroy mRNA seems unlikely since actinomycin D stimulated enzyme formation when enzyme synthesis had been either inhibited or completely arrested, indicating that mRNA was present, but not functioning, at these times.

The early action of hydrocortisone in stimulating enzyme synthesis does not seem

to be due to reversing the effect of the repressor described above, since neither actinomycin D nor 5-FU, given without the hormone, stimulated enzyme production. Furthermore, the effectiveness of the first injection of hydrocortisone when compared with the relative ineffectiveness of a second injection, given after repressor appears, argues against the presence of repressor prior to steroid administration.

Recently it has been found that actinomycin D accelerates the appearance of intestinal phosphatase in young mice.[25] This increase in enzyme activity is steroid-dependent, but unlike the present case, puromycin also stimulates phosphatase appearance, indicating that the process involves enzyme activation rather than *de novo* synthesis.

However, a phenomenon apparently very similar to the present findings has been reported by McAuslan,[26] in which the inhibition of pox-virus-induced thymidylate kinase synthesis was also reversed by actinomycin D. The results in this study were explained by postulating that actinomycin either inhibited the formation of a substance which destroyed mRNA, or operated by a mechanism similar to that suggested by the present study.

The present experiments imply that there is rapid turnover of the repressor, and it is interesting that Gallant and Stapleton[27] have suggested that the repressor for alkaline phosphatase in *E. coli* also turns over rapidly. In mammals, since mRNA's are relatively stable compared with microorganisms,[28–30] it might be expected that, as in the present case, inhibition of enzyme synthesis operates on messenger translation rather than inhibition of mRNA synthesis.

Summary.—(1) The mechanism of hydrocortisone-induced tryptophan pyrrolase and tyrosine transaminase synthesis in the liver of adrenalectomized rats has been studied. (2) Actinomycin D did not inhibit synthesis of the basal enzymes, but inhibited the induction of these enzymes when injected at early times after hormone administration. (3) Actinomycin D and 5-fluorouracil stimulated tryptophan pyrrolase and tyrosine transaminase synthesis when injected 5 hr or later after the hormone. (4) It is proposed that repression of the synthesis of these enzymes occurs at the level of messenger RNA translation.

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[18] The hypothetical time course of mRNA levels following induction can be calculated if it were assumed that the rate of TP synthesis dE/dt were determined both by the amount of mRNA (M) and the rate of TP decay [$k_2(E)$], thus: $dE/dt = k_1(M) - k_2(E)$, where k_1 which expresses the conditions for enzyme synthesis unrelated to mRNA is assumed to remain constant during induction. It has already been shown that k_2 does not vary under these conditions. The application of the differential equation to the kinetics of TP induction therefore requires that the mRNA level increase rapidly for the first 4 hr after hormone treatment. From 5 to 7 hr, when $dE/dt = 0$, mRNA would fall to a new steady-state level, and at 7 hr, when $dE/dt = -k_2(E)$, mRNA would have completely disappeared.

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[21] Inhibition of induction of TP was observed with as little as 0.015 mg of actinomycin D given at the same time as hydrocortisone.

[22] The term "repressor" is used to denote an inhibitor of enzyme synthesis[23] and does not imply a mechanism of action. However, it appears that "TP repressor" does not function by affecting mRNA *synthesis*, as proposed for microbial systems,[1] but operates at another locus. TP repressor need not inhibit directly but could antagonize the stimulatory action of the hormone.

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ERRATUM

In the article "Behavior of Solutions of a Linear Differential Equation of Second Order," by Walter Leighton, which appeared in the September issue of volume 52 (1964), pages 830–832, the proof of the theorem is incomplete. The hiatus was discovered just as the article appeared in print. Whether or not the stated theorem is valid remains, at least for the time being, an open question.