Biochemical Pharmacology, Vol. 22, pp.1565–1572. Pergamon Press, 1973. Printed in Great Britain.

CORTISOL EFFECT ON PROTEIN SYNTHESIS AND RIBONUCLEIC ACID POLYMERASE ACTIVITY IN RAT THYMUS*

WILLIAM C. BUSS† and JOHN D. GABOUREL
Department of Pharmacology, University of Oregon Medical School, Portland, Ore. 97201 U.S.A.

(*Received* 23 *August* 1972; *accepted* 26 *December* 1972)

Abstract—The time course for cortisol to effect a decrease in the amount of soluble (extractable) thymic nuclear RNA polymerase activity was determined. Decreases in soluble polymerase activity were compared with the cortisol-mediated inhibition of [^{3}H]leucine ([^{3}H]leu) incorporation into cells and cell fractions. [^{3}H]leu incorporation into total cell protein was inhibited by cortisol before measurable effects on extractable nuclear RNA polymerase were detected. Cortisol inhibition of [^{3}H]leu incorporation into protein of nuclear extract (soluble at pH = 8·0) and protein associated with sucrose gradient purified RNA polymerase lagged behind [^{3}H]leu incorporation into total cell protein but preceded the decrease in extractable RNA polymerase activity. These data suggest that the decrease in extractable RNA polymerase seen 12 hr after cortisol treatment is due, at least in part, to a decreased synthesis of the enzyme. The decrease in RNA polymerase activity, however, does not occur early enough to explain the marked inhibition of protein and RNA synthesis seen early (3 hr) after cortisol injection.

INHIBITION of thymic RNA synthesis occurs within 3 hr after rats are injected with 50 mg of cortisol/kg body wt as measured by [^{3}H]uridine incorporation by intact thymocytes,[1] [^{3}H]UMP incorporation by isolated nuclei[2] and [^{3}H]UMP incorporation by aggregate enzyme preparations.[2,3] Studies on aggregate enzyme preparations[3] have indicated that cortisol inhibits the final polymerization of nucleoside triphosphates into RNA, and that this effect is not the result of a decreased availability of nucleoside triphosphates. It has also been shown that cortisol does not alter either acid or alkaline RNase activity in rat thymus.[2] More recently, Gabourel and Fox[4] demonstrated that, 12 hr after injection, cortisol was without effect on chromatin template activity when measured in the presence of added *Escherichia coli* RNA polymerase. This report also provided data showing that addition of *E. coli* RNA polymerase to aggregate enzyme preparations, obtained from rats 12 hr after cortisol treatment, could reverse the cortisol-induced inhibition of RNA synthesis. In addition, these investigators showed[4,5] that the amount of soluble RNA polymerase extractable from purified thymus nuclei was reduced 12 hr after rats were injected with cortisol.

Nakagawa and White[6] reported that addition of excess purified DNA to nuclear

* This research was supported by United States Public Health Services Grant CA 07753 from the National Cancer Institute, by United States Public Health Service Pharmacology Training Grant GM 1396 and by a grant from the Oregon Division of the American Cancer Society. A preliminary report has been published in *Fedn Proc.* **30,** 308 (1971).

† Submitted in partial fulfilment of the requirements for the Ph.D. degree in Pharmacology. Present address: Department of Pharmacology, University of New Mexico School of Medicine, Albuquerque, N.M. 87106.

lysates increased RNA synthesis by these preparations, but the rate of RNA synthesis by preparations from steroid-treated rats was always significantly slower than that of control preparations. They have also shown that steroid treatment reduces the amount of soluble RNA polymerase appearing in crude thymus cell sap after total cell disruption.[7] All of the above results suggest that cortisol inhibits RNA synthesis by altering the amount or activity of the enzyme RNA polymerase.

This report contains data comparing the time course of cortisol effects on extractable (soluble) RNA polymerase, total cell protein synthesis and synthesis of protein associated with RNA polymerase activity after partial purification on two sequential sucrose gradients. No decrease in extractable RNA polymerase could be seen 3 hr after cortisol treatment, and the results suggest that the decreases seen 6 and 12 hr after steroid injection may be secondary to a decreased rate of protein synthesis.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (Simonsen Laboratories, Gilroy, Calif.) weighing 60–65 g each, were adrenalectomized under ether anesthesia and maintained for 3–5 days on a standard laboratory diet and drinking water containing 1% NaCl.

Chemicals and drugs. Cortisol (Cortef, 50 mg/cm^3 in Vehicle No. 100) and Vehicle No. 100 were purchased from Upjohn. Unlabeled nucleoside triphosphates (Grade A), calf thymus DNA (Grade A), dithiothreitol (DTT) and crystalline bovine serum albumin (BSA) were obtained from CalBiochem. [^{3}H]UTP (17·1 to 25·0 Ci/m-mole in 50% ethanol as the tetralithium salt) and 4,5-[^{3}H]-L-leucine (20 Ci/m-mole or 54–58 Ci/m-mole in 0·01 N HCl) were purchased from Schwarz Bioresearch. Calf serum and Eagle's Minimum Essential Medium for suspension cultures, No. F-14 (MEM), were obtained from GibCo. In cases where it was necessary to adjust the concentration of a particular ingredient, MEM was formulated in our laboratory.

Cell culture in vitro. Thymus cells were suspended in the defined culture medium of Eagle (MEM),[8] supplemented with 6% calf serum, 0·005% sodium penicillin G, 0·005% streptomycin sulfate, 10^{-4} M L-serine and 10^{-4} M sodium pyruvate.

Preparation and extraction of labeled RNA polymerase. For each experiment, fourteen rats were injected with cortisol (50 mg/kg in Vehicle No. 100), and ten with an equivalent volume of Vehicle No. 100. Rats were sacrificed by decapitation 3, 6 and 12 hr after treatment, and the thymus glands excised, weighed and minced. Sacrifice and subsequent procedures through incubation were carried out at 37°. Cells of the minced glands were dispersed in 30 ml of complete culture medium, by three slow strokes of a loosely fitting pestle (size A) in a Dounce homogenizer. The cell suspension was filtered through gauze, allowed to stand for 15 min, and refiltered into 350–400 ml of MEM. Cell number was determined and the data were used to equalize cortisol and vehicle cultures.

After a 10- to 20-min equilibration of the cell suspension under Carbogen (5% CO_2, 95% air) in a shaking water bath at 37°, thymocytes were labeled by adding 200 μCi of [^{3}H]leucine ([^{3}H]leu) to each flask. The final concentration of L-leucine was 1×10^{-5} M. Incubations were continued for 4 hr, and the flasks were flushed with Carbogen every hr. Incorporation was essentially linear over the incubation period. Mackman *et al.*[9] have shown that, when cortisol is given *in vivo*, the same degree of

inhibition of [^{3}H]uridine incorporation into thymocytes is observed whether the cells are labeled immediately or preincubated *in vitro* for 3 hr before labeling.

Following a 4-hr incubation with [^{3}H]leu, 100 ml of ice-cold MEM containing 1×10^{-3} M non-radioactive leucine was added to each of the flasks. All subsequent operations were performed in the cold (0–4°). Cells were harvested by centrifugation for 10 min at 630 g_{max}. The cells were then washed with 20 ml of MEM and re-sedimented.

Nuclei were obtained by homogenizing washed cells in 12 ml of 2·0 M sucrose containing 0·05 M Tris, 0·025 M KCl, and 0·005 M $MgCl_2$, at pH 6·4. The degree of cell breakage was checked by light microscopy and an aliquot removed to assay DNA and [^{3}H]leu content. Nuclei were then purified by the method of Blobel and Potter.[10] Purified nuclei were homogenized in a 0·05 M Tris–HCl buffer, pH = 8·0, containing 0·005 M DTT, 0·025 M KCl and 0·1 M NH_4Cl. Aliquots were removed for DNA assays. Analysis of DNA recoveries at each step showed no significant differences in recovery between cortisol and control preparations.

The nuclear lysate was incubated for 10 min at 25°. This procedure resulted in the solubilization of 70 per cent of the original nuclear RNA polymerase activity.[4] The extraction procedure solubilized only a very small fraction of [^{3}H]leu labeled nuclear proteins, and no measurable amount of DNA. Glycerol was then added to the incubated nuclear lysate to a final concentration of 15–18% (w/v). The nuclear lysate was centrifuged for 30 min at 280,000 g_{max}. The supernatant contained a high level of RNA polymerase activity, and was referred to as nuclear extract.

Sucrose gradient purification of RNA polymerase. One-ml aliquots of the nuclear extracts were layered onto 5–20% (w/v) linear sucrose gradients containing 20% glycerol (w/v), 100 μg of BSA/ml, 0·005 M DTT, 0·025 M KCl, 0·1 M NH_4Cl and 0·05 M Tris–HCl, pH 8·0. The gradients (G–I) were centrifuged at 270,000 g_{max} for 24 hr at −5°. Gradients were collected fractionally and aliquots assayed for [^{3}H]leu content and RNA polymerase activity. [^{3}H]leu incorporation was expressed as counts/min of [^{3}H]leu/100 μg of nuclear lysate DNA layered onto the gradient.

The fractions containing peak enzyme activity were combined and 1·0–1·5-ml aliquots applied to linear 7·5–20% sucrose gradients (G-II) containing the same ingredients as G-I. The gradients were centrifuged for 32 hr under the same conditions as those for G-I, then analyzed for [^{3}H]leu and RNA polymerase activity. When peak enzyme fractions of G-I were aggregated and relayered onto G-II, the fraction of the total enzyme peak removed from G-I was used to calculate a new value for DNA equivalents of nuclear extract layered onto G-II.

RNA polymerase assay system. Incorporation of [^{3}H]UMP into cold acid insoluble material was used to assay RNA polymerase activity as previously described.[4] The specific activity of UTP in the assay tube was always 0·9 μCi/mμmole. Assays were carried out in the presence of excess native DNA template (50 μg/assay) prepared by dissolving calf thymus DNA in 0·01 M Tris–HCl (pH 8·0). It should be noted that RNA polymerase activity, as measured by [^{3}H]UMP incorporation into cold acid insoluble material, was calculated on the basis of the amount of DNA in the original material (nuclear lysate) from which it was extracted, and not on the amount of DNA template added to the assay.

Recovery of RNA polymerase activity from thymic nuclei. It has been impossible to determine the recovery of RNA polymerase from thymic nuclei because the activity

of the enzyme is dependent upon the nature of the DNA template in the assay system. Using purified native calf thymus DNA as template, nuclear extract incorporates about 150 p moles of UMP into RNA/mg of nuclear DNA from whence it came. Using endogenous (repressed) DNA as template, intact nuclei and the residual pellet (remaining after preparation of nuclear extract) incorporate only 40 p moles of UMP and 12 p moles of UMP/mg of DNA, respectively. The fact that only about 30 per cent of the original nuclear activity remained in the pellet after extraction suggests that the nuclear extract contains about 70 per cent of the enzyme activity present in intact nuclei.

The amount of RNA polymerase activity extractable from nuclei of thymocytes which were incubated *in vitro* at 37° for 4 hr (95 p moles of UMP incorporated/mg DNA; S.D. = 27; N = 5) was not significantly different ($P > 0{\cdot}1$) from the amount of activity found when the enzyme was extracted immediately without incubation (143 p moles of UMP incorporated/mg of DNA; S.D. = 22; N = 4).

Nuclear extract containing 20% glycerol can be stored at −20° for 1 week without appreciable loss of activity. Sucrose gradient purified RNA polymerase can be stored for 2–3 weeks at −20° without appreciable loss of activity. All results presented in this report were obtained on preparations stored no longer than 7 days.

Determination of radioactivity. Duplicate or triplicate aliquots were removed from thymocyte and nuclear suspensions after incubation with [^{3}H]leu for assay of [^{3}H]leu incorporation. Proteins were precipitated with 6% TCA, sedimented at 17,300 g_{max}, and then washed twice with 6% TCA and resedimented. The resulting pellets were dissolved in Soluene. Cold acid insoluble pellets containing [^{3}H]UMP obtained from RNA polymerase assays were also dissolved in soluene. Fifteen ml of toluene scintillation mixture was then added, and radioactivity determined in a Packard Tri-Carb liquid scintillation spectrometer. Background, zero time incubation, and quenching corrections (Automatic External Standard System) were made as required.

DNA determinations. Duplicate aliquots of thymocytes, nuclei and nuclear lysates were precipitated with cold 6% perchloric acid (PCA). The precipitates were washed and resuspended in 2·0 ml 6% PCA and incubated at 70° for 20 min.[11] The resulting solution was chilled in ice, centrifuged, and the supernatant assayed for DNA in triplicate by the method of Burton.[12]

RESULTS

Table 1 provides comparative data of cortisol effects on thymus weight, RNA polymerase activity extractable from thymus nuclei and [^{3}H]leu incorporation into proteins of nuclear extract, whole nuclei and whole cells. Measurements were made on preparations obtained from rats sacrificed 3, 6 and 12 hr after injection with cortisol (50 mg/kg) or an equivalent volume of vehicle. Extractable RNA polymerase activity decreased as a function of time after cortisol treatment, and wet thymus weight decreased in a parallel fashion. The reduction in incorporation of [^{3}H]leu into proteins of the nuclear extract, nuclei and thymocytes was greater at 3, 6 and 12 hr after cortisol treatment than the corresponding decreases in RNA polymerase activity.

Figure 1 shows the distribution of RNA polymerase activity and [^{3}H]leu labeled proteins in a typical G-I fractionation of nuclear extract from cortisol- and vehicle-treated rats. The nuclear extracts were obtained from thymocytes labeled 12 hr after

TABLE 1. THYMUS WEIGHTS AND RNA POLYMERASE ACTIVITY OF NUCLEAR EXTRACTS AND [^{3}H]LEUCINE INCORPORATION INTO THYMOCYTES, NUCLEI AND NUCLEAR EXTRACT

Hr after cortisol treatment (No.)	mg Thymus/rat (% of control)	RNA polymerase activity* (% of control)	[^{3}H]leu incorporation† (% of control)		
		Nuclear extract	Nuclear extract	Nuclei	Cells
3 (1)	92·2	102·4	89·6	98·0	72·7
6 (1)	89·8	86·7	71·5	61·0	59·6
12 (4)	71·2 ± 2·8	63·9 ± 3·4	54·3 ± 2·0	36·3 ± 1·4	41·3 ± 1·8

* Assay tubes contained nuclear extract derived from 0·6 to 0·8 mg of nuclear DNA. Average incorporation of [^{3}H]UMP into RNA by control nuclear extract preparations was 47,900 ± 6800 cpm/mg of DNA (standard error). Effects of cortisol treatment are expressed as per cent of control incorporation, with paired comparisons used for each experiment. Values shown at 3 and 6 hr are means of triplicate assays from one experiment; the average standard error for such triplicate assays was ± 3 per cent. Values shown at 12 hr are means of triplicate assays from each of four experiments, ± S. E. M. for all twelve assays. All data are zero time corrected.

† The average incorporation of [^{3}H]leu into protein for control preparations ± S. E. M. was as follows: nuclear extract 44,200 ± 8900 cpm/mg of DNA; nuclei, 78,601 ± 9900 cpm/mg of DNA; cells, 366,050 ± 56,000 cpm/mg of DNA. Effects of cortisol treatment are expressed as percent of control incorporation with paired comparisons used for each experiment. Values shown at 3 and 6 hr are means of duplicate determinations from one experiment. Duplicate determinations always agreed within ± 2 per cent. Values shown at 12 hr are means of duplicate determinations from each of four experiments, ± S. E. M. for all eight assays. All data are background corrected.

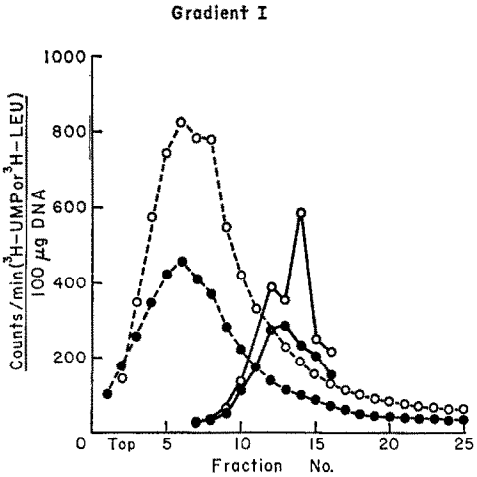

FIG. 1. Gradient I fractionation of nuclear extract proteins. Thymocytes were labeled *in vitro* 12 hr after treatment with cortisol or vehicle *in vivo*. One-ml aliquots of the nuclear extracts were layered onto linear 5–20% sucrose gradients containing 20% glycerol and 100 µg of BSA/ml in DTT-Tris, pH 8·0, and centrifuged for 24 hr at −5° and 270,000 g_{max}. The distribution of RNA polymerase activity and [^{3}H]leu incorporation is shown for cortisol and vehicle treatments (○——○ RNA polymerase control; ●——● RNA polymerase cortisol; ○- - -○ [^{3}H]leu control; ●— —● [^{3}H]leu cortisol). Percentage recoveries of nuclear extract RNA polymerase activity applied to the gradients averaged 40 per cent, and did not significantly vary between cortisol and vehicle preparations.

rats were injected with cortisol or vehicle. It is noteworthy that most of the radioactivity sediments more slowly than does RNA polymerase activity. Four fractions containing the bulk of the enzymatic activity were combined and applied to G-II. Figure 2 shows a typical distribution pattern of [^{3}H]leu and RNA polymerase activity on G-II. It is evident that a further separation of [^{3}H]leu from enzyme activity has occurred at this step. The [^{3}H]leu activity found in proteins associated with RNA polymerase after G-II purification represented only 0·1–0·15% of the [^{3}H]leu incorporated into total cell protein.

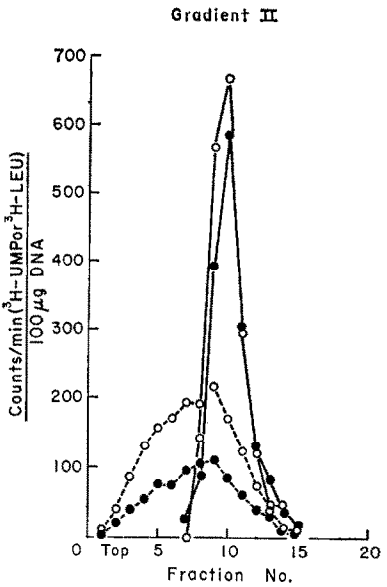

Fig. 2. Gradient II fractionation of gradient I RNA polymerase activity. This figure demonstrates a typical G-II centrifugation of combined fractions of labeled proteins associated with G-I RNA polymerase activity Gradient I (Fig. 1) fractions combined and recentrifuged on G-II were: cortisol and vehicle fractions 11–14 inclusively (○——○ RNA polymerase control; ●——● RNA polymerase cortisol; ○- - -○ [^{3}H]leu control; ●- - -● [^{3}H]leu cortisol). One-ml aliquots were layered onto G-II gradients as described in Methods. Percentage recoveries of G-I RNA polymerase activity applied to the gradients averaged 82 per cent and did not significantly vary between cortisol and vehicle preparations.

Table 2 shows the effect of cortisol on RNA polymerase activity and [^{3}H]leu incorporation into proteins associated with this activity at various stages of purification, i.e. nuclear extract and after fractionation on sucrose gradients G-I and G-II. A significant decrease in RNA polymerase activity was seen only at 12 hr after cortisol injection, and this decrease was evident at all stages of purification. In contrast, [^{3}H]leu incorporation into proteins migrating with RNA polymerase through two sequential sucrose gradients (G-I and G-II) was decreased to 84 per cent of control by 3 hr and 58 per cent of control by 6 hr after steroid treatment. Even at 12 hr the degree of inhibition of [^{3}H]leu incorporation exceeded the inhibition of RNA polymerase activity at all stages of purification.

TABLE 2. TOTAL CORTISOL-TREATED RNA POLYMERASE ACTIVITY AND [^{3}H]LEUCINE INCORPORATED IN PEAKS OF RNA POLYMERASE ACTIVITY FOR GRADIENTS I AND II, AS COMPARED TO NUCLEAR EXTRACTS*

	Nuclear extract		Gradient I (No.)		Gradient II (No.)	
Hr after cortisol treatment (No.)	RNA polymerase activity (Control %)	[^{3}H]leu (Control %)	RNA polymerase activity (Control %)	[^{3}H]leu (Control %)	RNA polymerase activity (Control %)	[^{3}H]leu (Control %)
3	102·4	89·6	108·3 ± 8·0	94·3	92·9 ± 4·5	84·8 ± 6·9
(1)	(1)	(1)	(3)	(1)	(2)	(2)
6	86·7	71·5	85·7 ± 1·2	62·0	97·4 ± 0·9	58·2 ± 1·8
(1)	(1)	(1)	(3)	(1)	(2)	(2)
12	65·2 ± 4·3	54·1 ± 2·6	68·9 ± 3·0	51·8	73·6 ± 2·4	48·7 ± 1·4
(3)	(3)	(3)	(5)	(1)	(5)	(5)

* Data for this table were calculated by determining the total areas under peaks of RNA polymerase activity and the total amount of [^{3}H]leucine activity associated with these fractions (Figs. 1 and 2). The number of experiments is shown in parentheses in the left-hand column. The number of individual determinations is shown in parentheses beneath each value. Values for more than one determination are ± S. E. M.

DISCUSSION

The time course for cortisol to effect a decrease in the amount of extractable thymic nuclear RNA polymerase activity was determined at 3, 6 and 12 hr. These data were then compared with the ability of cortisol to inhibit [^{3}H]leu incorporation into whole cell protein, nuclear extract protein and protein associated with RNA polymerase activity after purification by two sequential sucrose gradients (Tables 1 and 2, Figs. 1 and 2).

The data in Table 1 show that [^{3}H]leu incorporation into whole cell protein was inhibited by cortisol before measurable effects on extractable nuclear RNA polymerase activity could be seen. Cortisol inhibition of [^{3}H]leu incorporation into proteins of nuclear extract appeared to lag behind [^{3}H]leu incorporation into whole cell protein, but preceded the decrease in extractable RNA polymerase activity. Nuclear extracts from the above experiments (containing [^{3}H]leu labeled protein) were subjected to sucrose gradient centrifugation. The distribution of label from [^{3}H]leu and RNA polymerase activity is shown in Fig. 1. The polymerase activity shown in Figs. 1 and 2 is the more rapidly sedimenting nucleoplasmic RNA polymerase activity, which is the thymocyte form affected by cortisol treatment.[13] The slower moving nucleolar RNA polymerase activity was generally not detectable in these experiments due to the high background of [^{3}H]leu labeled proteins, and was eliminated on G-I. Fractions containing peak RNA polymerase activity were combined and relayered on a second, similar sucrose gradient. The distribution of label from [^{3}H]leu and RNA polymerase activity on G-II from a typical cortisol and vehicle preparation is shown in Fig. 2.

All data from Fig. 2 were corrected for any differences in the percentage of total RNA polymerase activity that was relayered from the first gradient and for slight differences in recovery. Recovery from G-II was about 80 per cent and did not significantly vary between preparations from cortisol- or vehicle-treated rats. Purifica-

tion of RNA polymerase was substantial as compared to that found in whole cells, intact nuclei or aggregate enzyme preparations. Quantitative comparison of the amount of RNA polymerase activity, and [^{3}H]leu incorporation into protein associated with RNA polymerase activity, was made by measuring the areas under the polymerase peak and the [^{3}H]leu incorporation curve associated with this peak for five separate experiments. These data are given in Table 2. It is apparent that the 12-hr cortisol-induced reduction in RNA polymerase activity is maintained throughout the purification procedure. The data in Table 2 also indicate that inhibition of [^{3}H]leu incorporation into proteins associated with RNA polymerase activity precedes the observed decrease in extractable nuclear enzyme, and continues to do so with enzyme purification.

These data suggest that the decrease in extractable RNA polymerase activity seen 12 hr after cortisol treatment is due at least in part to a decreased synthesis of the enzyme. This effect, however, does not occur early enough to explain the marked inhibition of protein synthesis seen 3 hr after cortisol treatment or the early (3-hr) inhibition in RNA synthesis by whole thymocytes, isolated nuclei and aggregate enzyme.[3,6,9] The fact that RNA synthesis by whole nuclei or crude aggregate enzyme is inhibited as early as 3 hr after cortisol treatment, whereas a decrease in extractable RNA polymerase activity can only be seen 6–12 hr after treatment, might be explained by the presence of cortisol-sensitive factors which modify RNA polymerase activity in whole nuclei or nuclear lysate (i.e. aggregate enzyme), but which either do not appear in soluble nuclear extract or are undetected in the assay system used for these experiments. Gabourel *et al.*[14] have reported that cortisol (50 mg/kg) alters the template preference of soluble RNA polymerase extracted from thymic nuclei within 3 hr, providing additional support for this hypothesis.

Acknowledgement—We would like to express appreciation for the suggestions and excellent technical assistance of Miss Margie Frichtl and Mrs. Gerd (Gauslaa) Felt.

REFERENCES

1. M. H. Mackman, B. Dvorkin and A. White, *J. biol. Chem.* **241,** 1646 (1966).
2. S. Nakagawa and A. White, *Endocrinology* **81,** 861 (1967).
3. K. E. Fox and J. D. Gabourel, *Molec. Pharmac.* **3,** 479 (1967).
4. J. D. Gabourel and K. E. Fox, *Biochem. Pharmac.* **20,** 885 (1971).
5. J. D. Gabourel and K. E. Fox, *Pharmacologist* **12,** 283 (1970).
6. S. Nakagawa and A. White, *J. biol. Chem.* **245,** 1448 (1970).
7. S. Nakagawa and A. White, *Biochem. biophys. Res. Commun.* **43,** 239 (1971).
8. H. Eagle, *Science* **130,** 432 (1959).
9. M. H. Mackman, B. Dvorkin and A. White, *J. biol. Chem.* **243,** 1485 (1968).
10. G. Blobel and V. R. Potter, *Science* **154,** 1662 (1966).
11. K. Burton, *Methods in Enzymology* (Eds. L. Grossman and K. Moldave), Vol. 12 (B), p. 163. Academic Press, New York (1968).
12. K. Burton, *Biochem. J.* **62,** 315 (1956).
13. K. E. Fox and J. D. Gabourel, *Endocrinology* **90,** 1388 (1972).
14. J. D. Gabourel, K. E. Fox and G. Felt, *Fedn. Proc.* **31,** 283 (1972).