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# *HYDROCORTISONE-STIMULATED SYNTHESIS OF NUCLEAR RNA IN ENZYME INDUCTION*

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Earlier reports from this laboratory have demonstrated that the hydrocortisone-mediated induction of tyrosine transaminase in rat liver involves an increased rate of enzyme synthesis.[1, 2] Other evidence[3, 4] is consistent with the conclusion that enzyme synthesis is required in similar inductions of other hepatic enzymes, and Segal and Hopper[5] recently described a direct demonstration of increased synthesis of the glutamic-alanine transaminase. There is thus ample evidence to support the hypothesis that the basis for adrenocorticoid effects on hepatic metabolism lies in enzyme induction, and the mechanism of hormonal action in these inductions thereby assumes considerable significance. Experience with microbial systems has shown that induced synthesis of enzyme proteins reflects a primary alteration in the rate of formation of a specific fraction of RNA. In a study of corticosteroid effects on RNA turnover in rat liver, Feigelson, Gross, and Feigelson[6] found that precursor incorporation into RNA was increased in all the subcellular fractions of liver. This question has been reinvestigated using pulse-labeling techniques, and the results of these investigations are reported here, together with studies on the characterization of the RNA formed in response to hormone administration. A preliminary report describing somewhat similar studies was recently presented by Garren and Howell.[7]

*Materials and Methods.*—Male Sprague-Dawley rats weighing 150–200 gm were adrenalectomized 24–48 hr, and fasted for 18–24 hr before experiments. Induced animals received 5 mg of hydrocortisone intraperitoneally. Carrier-free $Na_2HP^{32}O_4$ (1 mc) was also given intraperitoneally, and in pulse-labeling experiments was given 20 min before the animals were killed. Livers were quick-frozen and stored in liquid nitrogen for some experiments, or placed in cold 0.25 *M* sucrose–0.001 *M* $MgCl_2$ (SM) and fractionated immediately in others.

*Preparation of liver fractions:* Livers were homogenized in 4 volumes of SM, and the homogenates filtered twice through several layers of cheesecloth. The nuclear fraction was isolated by centrifugation at 600 × *g* for 5 min, resuspended in a volume of SM equal to the liver weight, and recentrifuged. This wash was added to the supernatant of the first centrifugation to constitute the cytoplasmic fraction. The nuclear fraction was washed a second time with 10 volumes of SM, then suspended in 0.01 *M* Tris, pH 7.6, 0.001 *M* $MgCl_2$, in a volume equal to twice the liver weight. Temperature was maintained at 0–3°C during all preparative steps.

*Isolation of RNA:* Early experiments showed that when RNA was isolated by either of the phenol methods described below, or by the method of Davidson and Smellie,[8] both variation in the extent of extraction of labeled RNA and inclusion of radioactive contaminants were so great that specific radioactivity measurements were not reproducible. Hence, when specific radioactivity was the parameter measured, RNA was isolated by the following hot salt-DEAE procedure.

Aliquots of each fraction containing 600–900 $\mu$g RNA were extracted three times in 2.0 *M* NaCl–0.015 *M* phosphate, pH 7, at 100°. Extracts were pooled, 2 volumes of cold ethanol added, and the nucleic acids were collected after $1^1/_2$ hr precipitation at 4°. The yield of RNA at this point, calculated from average values,[9] ranged from 80 to 108% and averaged over 90%. Removal of radioactive contaminants from these preparations was based on the observations of Jacobson and Nishimura[10] concerning the behavior of RNA on DEAE paper. Aliquots containing 200–500 $\mu$g RNA were spotted on 15 × 7-cm strips of DEAE paper (chloride form, Whatman) cut to form a point at one edge. These were then irrigated with 0.4 *M* NaCl for 12 hr, then briefly with 70% ethanol to remove salt, and finally with ether:ethanol:chloroform (2:2:1), again for 12 hr. RNA was then eluted from the paper with 0.5 *N* KOH; ultraviolet absorbancy was completely removed after 3–4 ml were collected. The KOH eluate was incubated at 37° for 18 hr, and the hydrolysate was then acidified to 0.5 *N* $HClO_4$ to remove DNA and any residual protein. The clear solutions were analyzed for RNA by ultraviolet measurements and for $P^{32}$ by liquid scintillation counting. Virtually all of the RNA added to the paper was recovered as nucleotides. Acid-washed Norit removed 95–100% of the radioactivity, indicating that labeled contaminants were negligible. The mononucleotides were eluted from the charcoal and chromatographed on DEAE paper as described by Jacobson;[11] no radioactive areas were detected except those corresponding to the carrier nucleotides added prior to chromatography. These areas were cut out and counted directly by liquid scintillation counting to estimate the base ratios of the newly synthesized RNA. RNA-specific radioactivity measurements were corrected for variation in the specific radioactivity of the acid-soluble phosphate of each homogenate. In some experiments, radioactivity of the phosphate adsorbed by charcoal and released in 1 *N* HCl at 100° for 10 min was determined. Specific radioactivity of this phosphate was close to that of the inorganic phosphate, and variation in one was always paralleled by the other, and hence the measurements of total acid-soluble phosphate were considered sufficient.

For studies of size distribution and biological activity, nuclear RNA was usually prepared by a method (phenol extraction) similar to that described by Hiatt,[12] employing dodecyl sulfate. In the experiments described, no attempt was made to remove the products of limited (3–5 min) DNAase digestion, and DNA (measured by diphenylamine[13]) was present in amounts roughly equivalent to RNA. The specific radioactivity of the product was usually, but not always, approximately that expected from the results of the hot salt-DEAE procedure. Radioactivity of DNA was assumed to be negligible. For some experiments, deoxycholate was employed in place of dodecyl sulfate. Under these conditions little or no DNA was present in the product, but only 50–60% of the radioactive RNA was extracted. Cytoplasmic RNA was prepared by phenol-dodecyl sulfate extraction, DNAase treatment and ancillary steps being omitted. All the RNA preparations obtained by phenol extraction had a 260/280 ratio greater than 1.9.

*Analytical procedures:* Sucrose gradients were prepared and sampled as described by Britten and Roberts.[14] Aliquots containing 0.6–1 mg RNA were layered over 4.7 ml of a linear gradient 3–20% sucrose, containing 0.01 *M* Tris, pH 7.6, and 0.001 *M* $MgCl_2$. Centrifugation was carried out for 3.75 hr at 4° at 39,000 rpm. Aliquots of each 2-drop fraction were pipetted onto filter paper disks and then washed and counted as described by Bollum.[15] The portion of each fraction remaining was diluted to 2.5 ml for absorbancy determinations at 260 m$\mu$. Cytoplasmic fractions were assayed for tyrosine-$\alpha$-ketoglutarate transaminase as described previously.[16] Ribosomes and soluble fractions used in amino acid incorporation studies were prepared from the livers of fasted, intact rats by modifications of the procedure of Robinson and Novelli.[17] The incorporation assay mixture contained, in 1.0 ml: 50 $\mu$moles Tris, pH 7.6; 9 $\mu$moles $MgCl_2$; 50 $\mu$moles KCl; 20 $\mu$moles $\beta$-mercaptoethanol; 1 $\mu$mole ATP; 0.5 $\mu$mole GTP; 10 $\mu$moles phosphopyruvate; 50 $\mu$g pyruvic kinase; 0.5 $\mu$c $C^{14}$-leucine, 25 $\mu$c per $\mu$mole; 30 $\mu$g of each of 19 amino acids (leucine omitted); 200 $\mu$g sRNA, 10 $\mu$g DNA-ase, 1.0 mg ribosomal protein, and 2.75 mg soluble protein. Rat liver sRNA was prepared as described by Brunngraber,[18] except that the product was treated with

DNAase and rechromatographed on DEAE. When tested under conditions used for incorporation assay (ribosomes omitted), this sRNA accepted 2 mμmoles $C^{14}$-leucine per mg, dilution of isotope by endogenous leucine being ignored. Amino acid incorporation was assayed by the method of Mans and Novelli.[19]

*Results.—Rate of RNA synthesis in nuclear and cytoplasmic fractions:* Figure 1 demonstrates the effect of hydrocortisone on the rate of RNA synthesis, as measured by pulse labeling, during enzyme induction. The rate of synthesis of cytoplasmic RNA is unchanged by the hormone treatment. Nuclear RNA synthesis in noninduced (0 time) animals is 3–4 times faster than that of cytoplasmic RNA, as has been repeatedly observed by others. Hydrocortisone administration increases the rate of synthesis of nuclear RNA, the effect becoming marked after about 60 min, and increasing to about 3-fold by 4 hr. The rapid rise in the rate of nuclear RNA synthesis after 60 min is accompanied by a gradual accumulation of the induced transaminase. After 2 hr, while the rate of nuclear RNA synthesis increases more gradually, enzyme begins to accumulate rapidly. These results are consistent with the concept that enzyme induction is the secondary result of a primary hormonal effect on the synthesis of nuclear RNA. The delayed response in enzyme level is not unexpected in that an increase in this parameter requires enzyme accumulation.

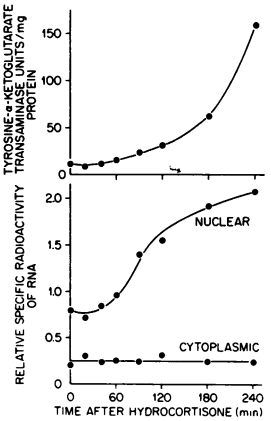


FIG. 1.—Rates of synthesis of nuclear and cytoplasmic RNA during enzyme induction. Each point represents a determination on a single animal; hydrocortisone was given to all at 0 time, and $P^{32}$ to each animal 20 min before it was killed. Relative specific radioactivity is cpm per mg RNA/cpm per μmole acid-soluble Pi × 100. RNA was prepared and analyzed by the hot salt-DEAE method.

Since the rapidity with which hydrocortisone stimulates RNA synthesis is of importance in consideration of the detailed mechanisms of hormone action, the earlier effects of the hormone were examined more closely. Figure 2 presents the data of such analyses, and also demonstrates the reproducibility of these measurements. Hydrocortisone administration appears to cause a slight (but reproducible, cf. Fig. 1) immediate depression in nuclear RNA synthesis. Increased synthesis of nuclear RNA begins after a delay of about 30 min. Enzyme activity is increased over the control level after 60 min. Since, as stated above, this requires an accumulation of newly synthesized enzyme, these data suggest that nuclear RNA synthesized in response to hydrocortisone is quite rapidly utilized in enzyme synthesis.

When $P^{32}$ is present for 2 hr or longer during hormone treatment, an effect on labeling of cytoplasmic as well as nuclear RNA is seen (Fig. 3), a result in agreement with that of Feigelson *et al.*[6] From the data of Figures 1 and 2, it is clear that hormonally increased labeling of cytoplasmic RNA reflects RNA synthesized in the nucleus. These results can be interpreted as indicating that the nuclear RNA detected by pulse-labeling passes, in time, into the cytoplasm. That the induced transaminase is synthesized on cytoplasmic ribosomes was suggested by the detection of an enzymically inactive, immunologically crossreactive material bound to this fraction.[1]

*RNA characterization:* (1) *Sucrose gradient analysis:* The sedimentation of

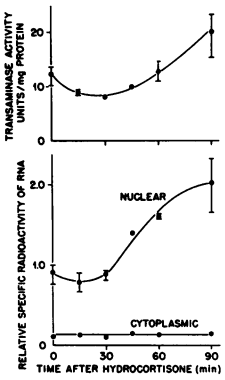


FIG. 2.—Early time-course of the hydrocortisone effect on enzyme and RNA synthesis. Vertical bars indicate the range of values from determinations on 2 or 3 animals. Other details as in Fig. 1.

rapidly labeled nuclear RNA from induced and noninduced liver is seen in Figure 4. The products of limited DNAase digestion appear as a large peak overlying the 5 S (Svedberg units) component of nuclear RNA, which normally appears as a small peak in fractions 25–27 under these conditions. Experiments wherein the partially degraded DNA was removed, as described by Hiatt,[12] established that sedimentation of both bulk and labeled RNA was unchanged by the presence of DNA. As shown by Hiatt, the pulse-labeled nuclear RNA is heterogeneous, ranging in size from about 7–40 S. The pulse-labeled RNA from induced livers sediments much like that prepared from noninduced livers; the apparent shift toward higher molecular weight cannot be considered significant in view of the extensive variation that we have observed in many similar analyses. No indication of an increase in a specific fraction of the rapidly labeled RNA was found. Similarly, when RNA was prepared using deoxycholate there was no change due to induction (Fig. 5).

In sucrose gradient experiments livers were cooled in an ice bath, rather than quick-frozen in liquid nitrogen as was done in the previous analytical experiments. The slower cooling rate appears to permit passage of some of the pulse-labeled nuclear RNA into the cytoplasm, as is indicated by the analysis of cytoplasmic RNA prepared from these livers (Fig. 6). Sedimentation of the RNA from control livers yields a labeling pattern similar to that observed by Hiatt,[12] with nearly all the radioactivity being associated with the slowest absorbancy peak, presumably containing transfer RNA. In the preparations analyzed here, the specific radioactivity of the cytoplasmic RNA from induced livers was nearly twice that from control livers, and the induced nuclear RNA was correspondingly lower in radioactivity than expected. The excess-labeled cytoplasmic RNA, known to be of nuclear origin since its formation is stimulated by induction, can be seen to sediment in a broad peak beginning at about fraction 16 (*ca.* 20 S), then increasing in amount and forming a shoulder (fraction

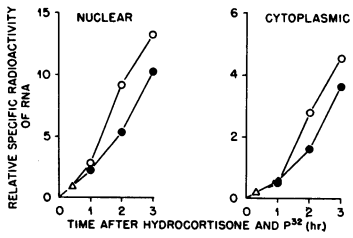


FIG. 3.—Hydrocortisone effects on long-term $P^{32}$-labeling of RNA. Isotope was given to all animals at 0 time; induced animals (open circles) received hydrocortisone simultaneously. The triangles represent points from pulse-labeling experiments. Other details as in Fig. 1.

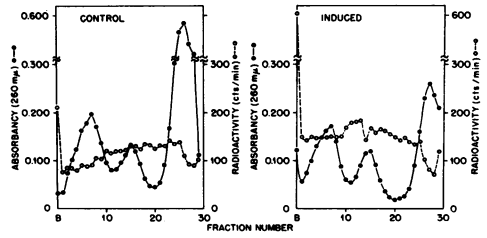


FIG. 4.—Sucrose gradient analysis of pulse-labeled nuclear RNA. Livers from 6 similarly treated animals were pooled for each group. Induced animals were given hydrocortisone 2 hr, and $P^{32}$ 20 min, before death; controls received only $P^{32}$. RNA was prepared by the phenol-dodecyl sulfate method. Specific radioactivity of the induced preparation was 1.57 times that from controls.

21) on, and merging with, the peak of labeled transfer RNA. This sedimentation pattern is similar to that of the cytoplasmic RNA recently described by Hoagland and Askonas,[20] which these authors found to have certain properties in common with bacterial messenger RNA. The discrepancy in size between the rapidly labeled RNA of the nucleus (7 to >40 S) and the same RNA when found in the cytoplasm (<20 S) may reflect some degradation of this RNA by nucleases.

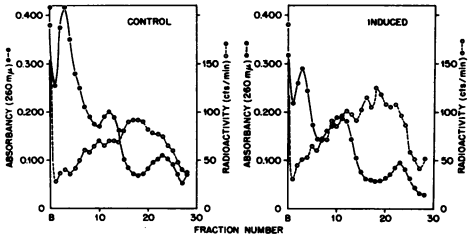


FIG. 5.—Sucrose gradient analysis of pulse-labeled nuclear RNA prepared without dodecyl sulfate. Centrifugation was at 10°. Specific radioactivities of induced/control = 1.78. Other details as in Fig. 4.

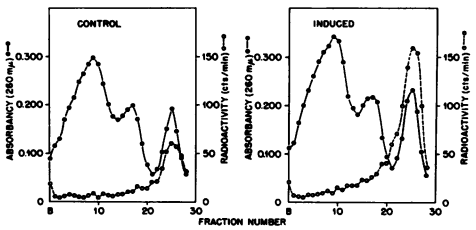


FIG. 6.—Sucrose gradient analysis of pulse-labeled cytoplasmic RNA. Specific radioactivities of induced/control = 1.87. Other details as in Fig. 4.

(2) *Activity in amino acid incorporation:* Nuclear RNA prepared by the phenol-dodecyl sulfate method was found to have little or no effect on amino acid incorporation in the rat liver system described. Hoagland and Askonas have suggested[20] that such inactivity may be due to this detergent, and accordingly we have employed nuclear RNA prepared using deoxycholate in place of dodecyl sulfate. These RNA preparations were somewhat active in stimulating leucine incorporation into protein, and response was approximately linear with amounts up to 1 mg (Fig. 7). RNA from induced livers consistently was slightly more active (5–10 per cent) in these assays, but never to the extent of the difference in specific radioactivity due to induction (2- to 3-fold). When assayed for charging capacity, nuclear RNA preparations were found to stimulate leucine incorporation into RNA with an efficiency about 10 per cent of that of highly purified sRNA, and there was no change due to induction. Assays of leucine incorporation into protein were done with sRNA added at twice the saturating level, and hence it is felt that stimulation by nuclear RNA could not be due to addition of transfer RNA. That the system employed is responsive to addition of messenger RNA was demonstrated in experiments wherein $C^{14}$-phenylalanine incorporation was doubled by addition of 40 μg polyuridylic acid (Miles Laboratories), while $C^{14}$-leucine incorporation was unchanged.

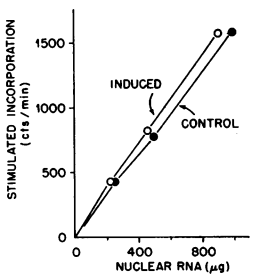


FIG. 7.—Stimulation of $C^{14}$-leucine incorporation into protein by nuclear RNA. Isotope incorporation by the system without added nuclear RNA was 11,060 cpm in a 60 min incubation.

(3) *Base composition:* The base ratios of the pulse-labeled RNA, as determined by isotopic labeling, are indicated in Table 1. The data presented are average values from 2 groups of rats, 1 untreated and the other given hydrocortisone for 1 hr. Other groups were analyzed over a 4-hr period of hormone treatment, and in no case was there a marked change from the values in Table 1. The composition of the rapidly labeled nuclear RNA is

TABLE 1

BASE COMPOSITION OF PULSE-LABELED RNA*

| Fraction | Per Cent of Total Radioactivity in— | | | | Ratio |
|---|---|---|---|---|---|
| | Cytidylic | Guanylic | Adenylic | Uridylic | (A + U/C + G) |
| Nuclear | 26.5 | 27.0 | 21.1 | 25.2 | 0.87 |
| Cytoplasmic | 22.6 | 23.2 | 29.0 | 24.9 | 1.18 |

* RNA prepared and analyzed by the hot salt-DEAE method. The numbers are the averages of duplicate determinations on 2 groups of 3 rats given $P^{32}$ for 20 min. Variation between groups was about ±2%.

similar to that of the total nuclear RNA, determined chemically (ratio of A + U/C + G = 0.81; ref. 21). Composition of the pulse-labeled cytoplasmic RNA is not consistent with labeling owing to turnover of the terminal cytidylic and adenylic residues of transfer RNA. The composition is also unlike that of the nuclear RNA, analyzed from the same livers, nor is it like either microsomal or cell sap RNA determined chemically (A + U/C + G = 0.60 and 0.63, respectively[21]).

*Discussion.*—The functional nature of the nuclear RNA formed under hormonal influence is difficult to assess at this time. That an increased synthesis of nuclear RNA precedes increased enzyme synthesis suggests, in the light of current theories of enzyme induction,[22] that the hormone stimulates formation of messenger RNA. The rapidly labeled RNA of the nucleus shares some characteristics with microbial messenger (cf. Hiatt[12]), and hydrocortisone stimulates the synthesis of this fraction. However, that the induced RNA could be wholly or even largely functional as messenger is not entirely in accord with our present knowledge of hydrocortisone effects on hepatic protein synthesis. Isotope incorporation into soluble liver proteins *in vivo* is increased 10–20 per cent in the first hours of hormone treatment,[2] a result consistent with the selective induction of a few enzymes, but not proportional to the large increase in nuclear RNA synthesis observed here. These considerations point to the possibility that only a small fraction of the induced RNA is active as messenger, as suggested by our results in assays for messenger activity *in vitro*.

*Summary.*—Hydrocortisone administration to adrenalectomized rats results in a 2- to 3-fold stimulation of the rate of liver nuclear RNA synthesis, as measured by pulse-labeling techniques. Labeling of cytoplasmic RNA is unchanged in pulse experiments, but is increased by the hormone if isotope is given for longer periods of time. Stimulation of nuclear RNA synthesis begins after a lag of about 30 min duration, and accumulation of the induced tyrosine transaminase begins about 30 min after the effect on RNA is apparent. The sedimentation distribution and base composition of the induced RNA are indistinguishable from those of RNA that is pulse-labeled in the absence of hormone. Nuclear RNA from induced livers was slightly more active than that from noninduced controls in stimulating amino acid incorporation *in vitro*.

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† Operated by Union Carbide Corporation for the U.S. Atomic Energy Commission.

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# *STUDIES ON THE GRAM-NEGATIVE CELL WALL, I. EVIDENCE FOR THE ROLE OF 2-KETO-3-DEOXYOCTONATE IN THE LIPOPOLYSACCHARIDE OF SALMONELLA TYPHIMURIUM**

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*Communicated by B. L. Horecker, July 24, 1963*

The cell wall lipopolysaccharides which determine the somatic (o) antigen specificities of *Salmonella typhimurium* and *E. coli* consist of highly branched, complex polysaccharides linked to a glucosamine-containing lipid.[1] The polysaccharide moiety may contain as many as 6 neutral sugars; for example, L-glycero-D-mannoheptose, glucose, galactose, mannose, rhamnose, and abequose (3,6-dideoxy-D-galactose) have been identified[2, 3] as components of the polysaccharide of *S. typhimurium*. The polysaccharides are thought[3–5] to consist of an internal core structure, possibly similar in all enteric bacteria, to which are attached complex side chains bearing group or species specific antigenic determinants. New insight into both structure and mechanism of biosynthesis of these polysaccharides has recently been obtained[3, 5] through the use of mutant organisms deficient in the synthesis of specific nucleotide sugars. It was first shown by Nikaido[5, 6] that incomplete polysaccharides are formed by mutants which are unable to synthesize UDP-galactose as a result of loss of the enzyme, UDP-galactose-4-epimerase. The polysaccharides formed by these mutants characteristically lack not only galactose, but also certain other sugars present in the normal polymers, and appear to represent the innermost core region of the wild-type polysaccharide. Previous studies in our laboratory[3] on a UDP-galactose-4-epimeraseless strain of *S. typhimurium* have shown that the incomplete "core" polysaccharide formed by this mutant contains glucose, L-glycero-D-mannoheptose, and phosphate. It has now been found that this polysaccharide also contains 2-keto-3-deoxyoctonate (KDO),[7] recently identified by Heath and Ghalambor[8] as a component of the lipopolysaccharide of