Skogerson, L., and Moldave, K. (1968a), *Arch. Biochem. Biophys. 124*, 497.
Skogerson, L., and Moldave, K. (1968b), *J. Biol. Chem. 243*, 5354.
Skogerson, L., and Moldave, K. (1968c), *J. Biol. Chem. 243*, 5361.
Traugh, J. A., and Collier, R. J. (1970), *Biochem. Biophys. Res. Commun. 40*, 1437.

Changes in Labeling Pattern of Ribonucleic Acid from Mammary Tissue as a Result of Hormone Treatment*

Marie R. Green,† Sylvia L. Bunting, and Andrew C. Peacock

ABSTRACT: RNAs produced in explants of mouse mammary glands maintained on an insulin (I) or insulin plus hydrocortisone (IF) containing medium in the presence or absence of prolactin (P) have been studied. Electrophoretic analysis of the RNAs demonstrated that addition of P for 24 hr to explants incubated previously on I or IF for 4 days produced no significant alteration in the distribution of radioactivity in RNA following 2- or 4-hr incubations with tritiated uridine.

In contrast, the RNAs isolated from explants which had been on IF medium, whether P was present or not, showed a greater percentage of slowly migrating RNA species following incubation with tritiated uridine than those which had been maintained on I or IP medium.

Explants of mouse mammary glands may be maintained on a synthetic medium for several days. Depending on which hormones are used to supplement the medium, the physiological and biochemical activity of the explants may be varied. In particular, the gland may be induced to form specific milk proteins.

Proliferation of alveolar epithelial cells of mammary glands of mice in midpregnancy occurs when these cells are cultured on synthetic medium containing insulin (Stockdale *et al.*, 1966). Supplements of hydrocortisone (Mills and Topper, 1970) and prolactin (Green and Topper, 1970) to this medium do not detectably alter the proliferative response to insulin. The addition of hydrocortisone to an insulin-containing medium results in an extensive increase of the rough endoplasmic reticulum of the alveolar epithelium (Mills and Topper, 1970) but has no detectable effect on incorporation of uridine into total RNA of the epithelial cells and does not increase milk protein production in the absence of added prolactin (Turkington *et al.*, 1967). The adipose cells, a normal component of mammary explants, incorporate less uridine into RNA as a result of hydrocortisone addition (Green and Topper, 1970).

The addition of prolactin to explants previously cultured on an insulin-containing medium produces only a transient increase in incorporation of uridine into RNA of epithelial cells, and in the absence of hydrocortisone is not effective in eliciting milk protein production (Green and Topper, 1970). The addition of prolactin to explants cultured on insulin plus hydrocortisone induces the alveolar epithelial cells to assume ultrastructural characteristics typical of secretory cells (Mills and Topper, 1970). Uridine incorporation into RNA and the amount of RNA present in epithelial cells increase, but RNA metabolism of the adipose cells is unaffected (Green and Topper, 1970). The production of the milk proteins, casein (Turkington *et al.*, 1967), and the two proteins of the lactose synthetase system (Turkington *et al.*, 1968; Palmiter, 1969) is markedly increased.

The present study is an electrophoretic analysis of RNAs produced in explants cultured in the presence of insulin or insulin plus hydrocortisone with and without the addition of prolactin.

Materials and Methods

Tissue. Mammary explants were prepared from Balb/C mice 10–12 days in their first pregnancy.

Reagents. Medium 199 was purchased from Microbiological Associates. Crystalline beef insulin was a gift from the Eli Lilly Co., and ovine prolactin was a gift from the National Institutes of Health Endocrinology Study Section. Hydrocortisone was purchased from Nutritional Biochemicals, Inc. The final concentration of each hormone, when used, was 5 μg/ml. Uridine-*5-t* (Schwarz BioResearch, Inc.; specific activity 8 Ci/mmole) was used at a level of 1 μCi/ml in 24-hr incubations or 5 μCi/ml for incubations of 4 hr or less. Tris (Schwarz BioResearch, Inc.), Na_2EDTA, sodium lauryl sulfate (SLS),[1] phenol, and boric acid were analytical grade. Acrylamide, *N,N*-methylenebisacrylamide, and 3-dimethylaminopropionitrile (DMAPN), as well as *N,N,N′,N′*-tetramethylethylenediamine (TEMED) and Stains-all (Dahlberg *et al.*, 1969), were obtained from Eastman Organic Chemical Distillation Products Industries, Rochester, N. Y. SeaKem agarose was purchased from Marine Colloid, Inc., Bausch and Lomb, Distributors. NCS reagent was purchased from Amersham-Searle.

* From the Laboratory of Biochemistry and Metabolism, National Institute of Arthritis and Metabolic Diseases, and the Chemistry Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014. *Received January 11, 1971.*

† To whom correspondence should be addressed. Present address: Chemistry Branch, National Cancer Institute.

[1] Abbreviations used are: SLS, sodium lauryl sulfate; I, insulin; IF, insulin plus hydrocortisone; P, prolactin; SDS, sodium dodecyl sulfate.

TABLE I: Hormone Supplementation Schedule.

First Incubn 4 Days[a]	Second Incubn 1 day (5th Day)[b]	Designation
Insulin	I + (no prolactin)	I
	I + prolactin	IP
Insulin + hydrocortisone	IF + (no prolactin)	IF
	IF + prolactin	IFP

[a] The medium was changed at the start of the second and fourth day. [b] Uridine incorporation was studied on the fifth day. The end of the incubation period (2–24 hr) coincided with the end of the fifth day.

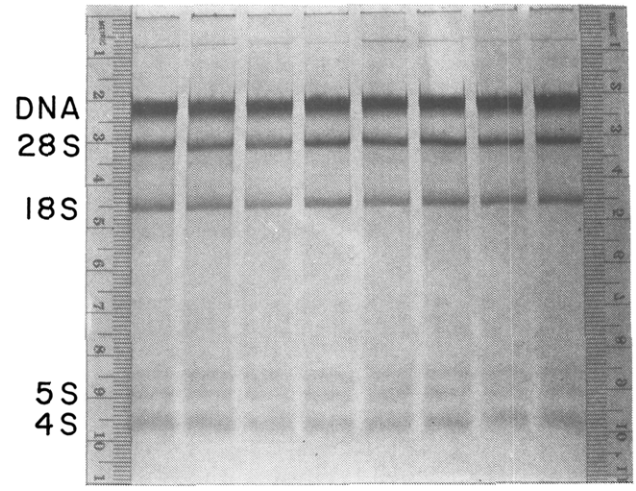

FIGURE 1: Photograph of a stained gel. The migration is from top to bottom. Labels indicate zones occupied by DNA, 28S RNA, 18S RNA, 5S RNA, and 4S RNA. The samples studied were, left to right (slots 1–4), I, IP, IF, and IFP, 2-hr incubations with [^{3}H]-uridine; (slots 5–8) similar explants (I, IP, IF, IFP) incubated for 4 hr with [^{3}H]uridine.

Culture Conditions. Explants weighing approximately 1 mg from abdominal mammary glands were cultured on 2 ml of Medium 199 in disposable Falcon plastic diffusion dishes, using a modification (Elias, 1957) of the procedure of Chen (1954).

The explants were cultured for 4 days on medium containing insulin (I) or insulin plus hydrocortisone (IF) (the medium was changed every other day). The 4-day period was chosen to provide epithelial cell populations (Lockwood *et al.*, 1967) in which the burst of DNA synthesis had largely subsided (Green and Topper, 1970). At the beginning of the fifth day, one-half of the explants from each system was transferred to media containing prolactin (P) in addition to insulin or insulin plus hydrocortisone. The explants were then transferred to these media containing tritiated uridine (5 μCi/ml) and incubated for periods of 2 or 4 hr at the end of the fifth day. When tritiated uridine was present for 24 hr, it was added to the medium at the beginning of the fifth day. These conditions are summarized in Table I.

Extraction of RNA. The method of isolation and preparation of RNA used in this study was chosen to minimize degradation of RNAs as much as possible. To this end, extractions were made as rapidly as possible and all solutions were kept at 0–4°. Crystallization of SLS did not occur if the SLS was used shortly after the solution reached 0°.

Explants were removed from the culture medium and blotted. Tissue (20 mg) was homogenized with 20 up-and-down strokes of a motor-driven Teflon glass homogenizer in 200 μl of ice-cold Tris–EDTA–borate buffer (pH 8.3; as used in electrophoresis, see below), containing 0.5% SLS.

The homogenate was transferred to a 500-μl centrifuge tube and mixed for 15 sec on a vibration mixer. Water-saturated phenol (200 μl) was added, and following another mixing, the solution was centrifuged in a Beckman microfuge for 2 min at full speed (approximately 15,000*g*). The top aqueous layer containing RNA and DNA was pipetted off and kept at 0°. A portion of this aqueous layer was diluted 4:5 with a 40% sucrose–bromophenol blue solution, and 25-μl aliquots were applied to the gel within 1 hr of extraction of the RNA.

Resolution of RNAs by Electrophoresis on Polyacrylamide–Agarose Gels. Electrophoresis of the RNA was performed as previously described (Peacock and Dingman, 1967; Dingman and Peacock, 1968). Composite gels, 1.7% acrylamide–bisacrylamide, 0.5% agarose, were used.

The electrophoresis was carried out for 1–2 hr at 0° and 250 V. The gels were removed from the electrophoresis apparatus, placed into a tray containing Stains-all, and left covered overnight (Dahlberg *et al.*, 1969). The background stain was removed by rinsing the gel in running-tap water. Gels were photographed with a Polaroid MP-3 Land camera. The gels were cut into strips and scanned in a Gilford 240 spectrophotometer at 570 nm. The scanned strips were cut by means of a Lucite block which contained 150 stainless steel blades set 1 mm apart. Approximately 100 1-mm slices were obtained per sample, and these were placed into scintillation vials with 0.2 ml of 0.5 N NaOH overnight at room temperature. They were counted in a Beckman or a Packard scintillation counter following the addition of 1 ml of NCS and 10 ml of scintillation fluid as described previously (Peacock and Dingman, 1967).

To investigate whether all the radioactivity on the gel slices was due to RNA, control experiments in which the

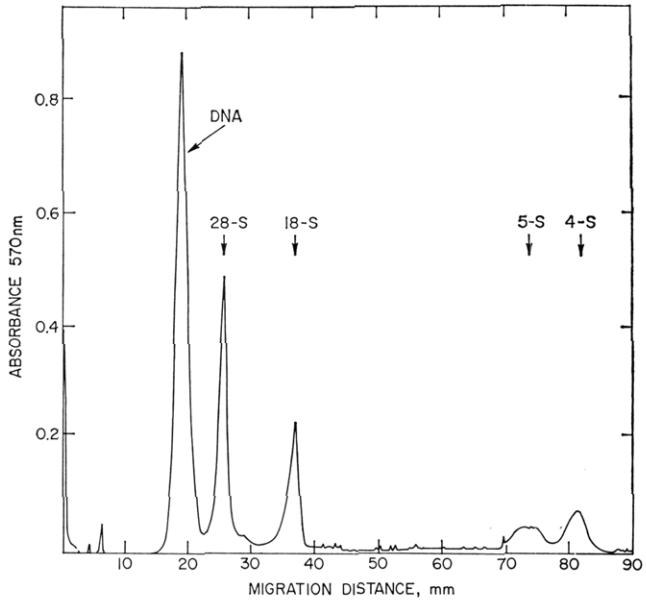

FIGURE 2: Representative scan at 570 nm of stained gel. The migration is from left to right. The migration distance was shorter than usual so that the gel would fit in the gel-scan cuvet.

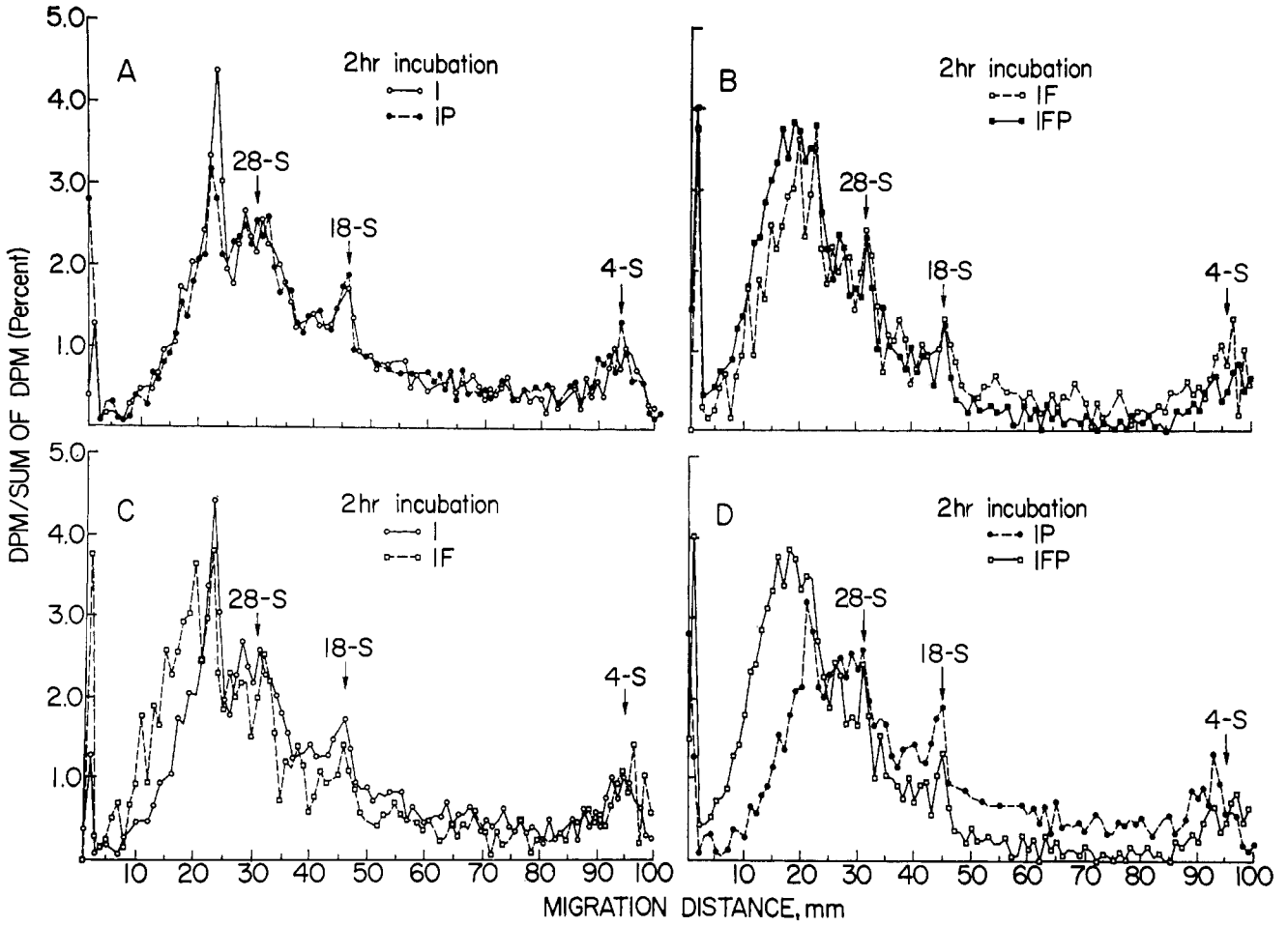

FIGURE 3: Distribution of radioactivity after 2-hr incubation with [^{3}H]uridine. The plots show comparison between I, IP (A); IF, IFP (B); I, IF (C); and IP, IFP (D). In this and succeeding figures, some experimental points have been omitted for clarity.

samples were treated with RNase prior to electrophoresis were performed. For the enzymatic studies, because phenol and SDS in the samples prevented the normal action of RNase and DNase, RNA was precipitated from the aqueous phenol-saturated layer by the addition of 0.1 volume of 1.0 M NaCl and 3 volumes of chilled ethanol. The mixture was allowed to stand at −20° for 16 hr and the precipitate was collected by centrifugation and dissolved in one-half the starting volume of 0.02 M NaCl. An equal volume of RNase solution (20 μg/ml, in 0.01 M phosphate buffer, pH 7.0) was added, and the mixture was incubated for 3 min at 37°. The incubated sample was chilled and the electrophoretic analysis promptly performed. The amount and distribution of radioactivity were little affected by the precipitation step or by incubation in the absence of enzymes, but treatment with RNase resulted in complete loss of radioactivity from the gel in the region from the origin to the 4S RNA, as well as loss of the RNA staining pattern. Stained zones at 8 mm and at 85 mm did not show [^{3}H]uridine incorporation, were not degraded by RNase or DNase, and remained unidentified. DNase treatment was performed similarly; its action did not affect the recovery of radioactivity.

Counts per minute were corrected for efficiency of counting (46%) by means of an internal standard, and background counts of gel slices in the scintillation fluid were subtracted.

The radioactivity recovered from all slices was totaled, and the percentage found in each slice was calculated. This percentage was plotted against migration distance in millimeters.

Results

A photograph of a stained gel is shown in Figure 1. DNA, 28S RNA, 18S RNA, 5S RNA, and 4S RNA are easily identifiable. The various hormone supplementation schedules appear not to affect the distribution of r-, t-, or 5S RNAs, nor is there in any case production of a new species of RNA in amounts sufficient to be detected by this staining technique. Scans (Figure 2) showed that approximately the same amount of RNA was recovered in each sample studied under the standard schedule, where P was present for only 24 hr.

In addition to the information described above, it was possible to examine each RNA preparation for degradation. Degraded specimens have many stained bands between the 28S RNA and the 4S RNA and, in some cases, exhibit a band of nucleotides migrating somewhat faster than the 4S RNA. These signs of degradation were not observed. In addition, the stained gel was used to verify that migration was uniform throughout the gel and to provide markers (28S, 18S, 4S RNA) when these were not clearly defined by the labeling patterns.

In contrast to the similarities in the amount of the stable RNAs described above, the amount of radioactivity in the RNA varied over a twofold range, depending on the hormone supplementation schedule. These quantitative variations made

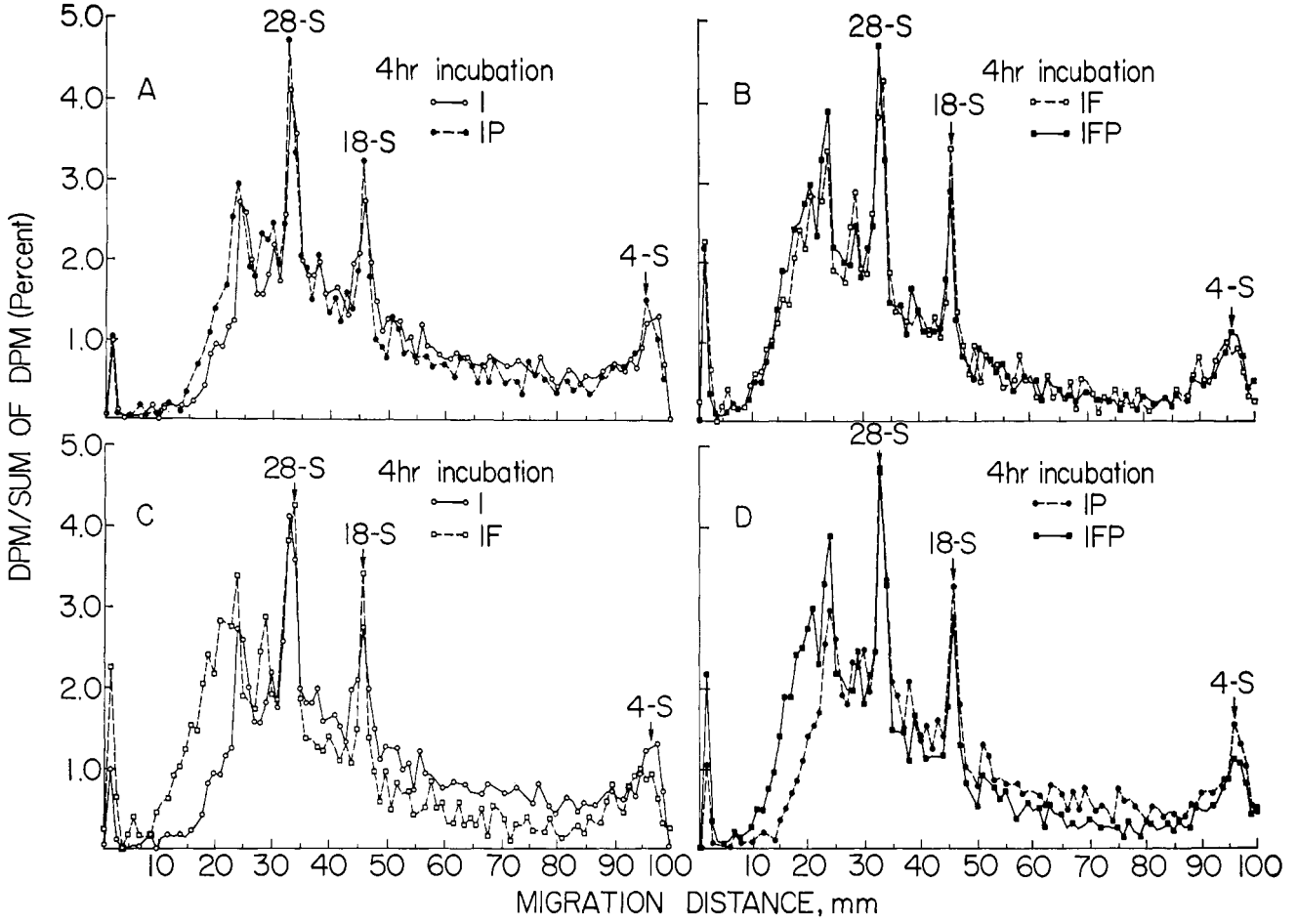

FIGURE 4: Experiment similar to that in Figure 3 except incubations carried out for 4 hr. The comparisons are made in the same way as in Figure 3. A duplicate run showed the same results.

more difficult an estimate of the distribution of the several labeled species of RNA. For this reason, the distribution of radioactivity as a function of migration distance was made on a percentage basis, minimizing the effect of variations in the amount of radioactivity involved. Using such plots, it was possible to make comparisons between pairs as follows: I, IP; IF, IFP; I, IF; IP, IFP. We have made such comparisons by superimposing plots in pairs. Figures 3 and 4 show such comparisons for RNAs labeled during 2- and 4-hr incubation periods, respectively. The following observations are common to both sets of data. Pairs from the I incubation and pairs from the IF incubation are generally very similar in the distribution of mobilities (Figures 3 and 4, panels A and B). The same four distributions are used in an alternate comparison in panels C and D of these two figures, from which it is apparent that those explants which have been cultured in the presence of hydrocortisone (F) differ from those explants cultured in its absence in that a larger percentage of the radioactivity in the F systems is found in a slowly migrating RNA. This comparison is particularly evident on comparison of the percentage migrating at distances between 10 and 20 mm. Many of these experiments were repeated several times during the course of a year. In addition, some experiments were performed in duplicate. In each case the characteristic similarities and differences shown in Figure 3 and Figure 4 were observed. Figures 3 and 4 also indicate that there are differences in the 18S–4S region (approximately 45–100 mm). In this area the percentage values for the F specimens are lower in general than those observed for the non-F specimens. The difference between the levels of labeled slow-moving RNAs in the F and non-F systems was not due to differential degradation of this fraction during the isolation procedure. This conclusion is based on the observation that the amount and percentage distribution were not altered when labeled IFP explants were processed in the presence of an equal weight of unlabeled IP or IFP explants. Figures 3 and 4 also indicate that labeling of the 28S and 18S RNAs became distinct in this tissue only after about 4 hr but that there is no important distinction to be made among the hormone supplementation schedules on the basis of the timed rate of appearance of rRNA. The differences between supplementing or not supplementing with F (slowly migrating RNA on F supplementation) are as evident in the 2-hr incubation as they are in the 4-hr incubation (see panels C and D).

There were large and consistent differences in incorporation of radioactive precursor into RNA, depending on the hormone supplementation schedule, as shown in Table II. These effects are most notably (a) a very substantially reduced incorporation in those tissues incubated in the presence of hydrocortisone (IF) as compared to those incubated in its absence and (b) a stimulation of incorporation in the IF system on the addition of prolactin. Incorporation by systems incubated in the absence of hydrocortisone was less affected by prolactin, as may be seen from Table II, although a stimulatory effect was observed in most cases. In agreement with previous findings, the stimulation by P of an I system is not maintained

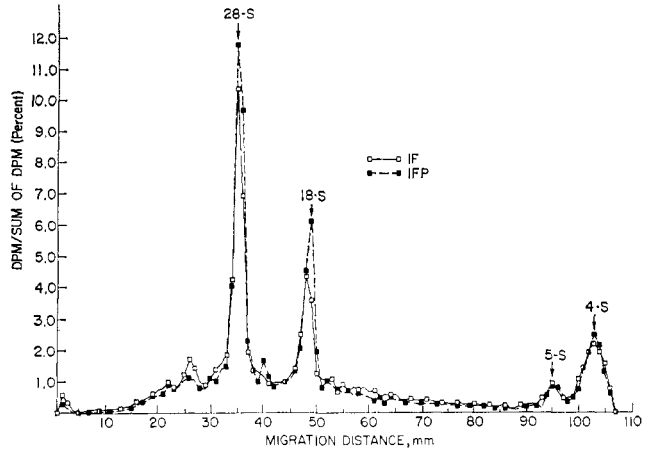

FIGURE 5: Distribution of radioactivity in explants cultured for 5 days in insulin plus hydrocortisone or insulin plus hydrocortisone plus prolactin and labeled for 24 hr with uridine. The distributions are very similar.

with time, relative to the stimulation by P of an IF system, and it appears that, due to the different time scale of this experiment, the observed stimulation by P of an I system is less than reported earlier (Green and Topper, 1970).

In another experiment the effect of a longer incubation with prolactin was investigated. In this study the schedule in Table I was not used. Instead, the insulin-containing medium was supplemented with hydrocortisone or hydrocortisone plus prolactin for a 5-day period commencing at time zero. Labeled uridine was added at the beginning of the fifth day to both cultures and incubation continued for 24 hr. The distribution of radioactivity in the two samples is compared in Figure 5, from which it is evident that the distribution of radioactivity is very similar in both cases. By the end of 24 hr the amount of radioactive precursor which had been incorporated into the stable forms, that is, 28S, 18S, 5S, and 4S RNAs, was much greater than that which accumulates in the more labile forms migrating more slowly. Thus, the distribution of radioactivity resembles very closely the distribution of RNA observed in the stained preparations. In these experiments, where prolactin was included for 5 days, the prolactin-supplemented explants incorporated 2.7 times as much radioactivity as those explants cultured in the presence of insulin and hydrocortisone only.

Discussion

The action of prolactin in promoting milk protein formation by IF cells requires RNA snythesis (Stockdale *et al.*, 1966; Turkington, 1968). Our data confirm previous findings (Green and Topper, 1970) that prolactin increases uridine incorporation in explants previously cultured on an insulin or insulin plus hydrocortisone medium (Table II). Based on the electrophoretic data shown in Figures 3 and 4, it appears that, in spite of changes in the level of incorporation, there is no change in the distribution of radioactivity in RNA as a result of prolactin treatment.

Previous results (Green and Topper, 1970; Palmiter, 1969) indicate that the increase in uridine incorporation of an IF system is followed by an increase in the net amount of total RNA present as well as an increase in the ratio of trichloroacetic acid insoluble to trichloracetic acid soluble radioactivity, particularly after prolactin has been present for about 6 hr.

TABLE II: Radioactivity in RNA.

Expt	[^{3}H]Uridine Pulse (hr)	Total DPM ($\times 10^{-2}$) I	IP	IF	IFP
Figure 3	2	190	189	93	154
	4[a]	248	338	173	352
Figure 4	4	247	263	125	258
	4[a]	228	275	138	211

[a] Not shown in the figure.

Recent work on the effect of estradiol administration on the uterus indicates that increased incorporation of radioactive precursor into RNA during the first 5 hr of hormone stimulation was due mainly to increased specific activity of the precursor pool due to an increased transport of radioactive precursors into the uterus (Billing *et al.*, 1969a). A substantial increase in rate of synthesis of rRNA and tRNA commenced after about 6 hr (Billing *et al.*, 1969b). The pattern of distribution of high molecular weight, rapidly labeled RNA (10- to 40-min pulses of [5-^{3}H]uridine) on sucrose gradients was not affected by intraperitoneal estradiol administration to immature rats 2–12 hr before sacrifice (Joel and Hagerman, 1969).

In the present study no attempt was made to enrich, by cell fractionation, any particular species of RNA which may be present because we were interested primarily in determining patterns which could be demonstrated when RNA was extracted under the mildest and most rapid conditions possible. It is conceivable, however, that such enrichment procedures would bring out differences not observed by the methods we employed.

IF and IFP explants have a greater percentage of the slowly migrating RNA species after 2- and 4-hr incubations with tritiated uridine than I and IP explants. The observed increase in percentage of slowly migrating RNAs did not depend on the amount of RNA synthesized. Hydrocortisone depressed the amount of tritiated uridine incorporated into RNA of intact and deepithelialized explants (Green and Topper, 1970), and insulin plus corticosterone incubated explants contained less RNA than explants cultured on insulin alone (Mayne *et al.*, 1968). Further studies are necessary to define these effects as a function of the length of time of exposure to hydrocortisone.

The present experiments do not distinguish between effects on the adipose or epithelial cell components of the mammary gland directly, but the following considerations suggest that the increase in the slowly migrating RNA comes from the epithelial cells. Prolactin does not affect the accumulation of RNA in fat cells but increases the accumulation of RNA in epithelial cells (Green and Topper, 1970). The amount of slowly migrating RNA is much greater in the IFP system than in the IF system. Because the amount of slowly migrating RNA (as well as the amounts of the other RNA species present) was increased by prolactin treatment, it is likely that these species of RNA are produced by epithelial cells. The increased percentage of this slowly migrating RNA is primarily dependent on the presence of hydrocortisone, but prolactin enables the tissue to increase the amount synthesized.

Another system in which the effect of hydrocortisone on protein and RNA synthesis has been studied extensively *in*

vitro is that of the HCT cell line (Tomkins *et al.*, 1969). Hydrocortisone increases tyrosine transaminase synthesis, but significant changes in RNA distribution were not observed (Gelehrter and Tomkins, 1967).

Earlier studies have shown that there is an inverse relation between the molecular weight of RNA and its migration in gel electrophoresis (Peacock and Dingman, 1968). This relation seems particularly to hold for rRNA but is probably subject to perturbations caused by variations in secondary structure (Loening, 1969). In the present instance, we cannot be sure that the slowly migrating RNA which is found after hydrocortisone stimulation is actually of higher molecular weight than the bulk of that formed in its absence, but this seems to us to be the most reasonable interpretation.

According to this view, we may calculate a few representative molecular weights as applied to Figure 4, for example. The two rRNAs have molecular weights of 1.8 and 0.73 million, respectively, as determined by comparison to HeLa rRNA as a standard (McConkey and Hopkins, 1969). Fractions at 25 mm are approximately 2.5 million, and fractions migrating at 17 mm are 5 million. Thus, the slowly migrating RNAs found after exposure to hydrocortisone may have molecular weights of 5–10 million.

We cannot tell whether the slowly migrating RNA found in the presence of hydrocortisone is qualitatively different from that found without the hormonal stimulation, or whether the increase results from a selective change in rates of synthesis or degradation of the same set of RNAs. Some of the ways in which hydrocortisone might affect the distribution of RNAs produced in this tissue are (1) the rate of synthesis (initiation, elongation, etc.) would be altered by the hormone; (2) the rate of degradation could be affected, as by strengthening of lysosomes, thereby preventing the release of their nucleases (Lwoff, 1969); or (3) the relative contribution of the epithelial and fat cells could be altered, without any change in the type of RNA produced by each cell type. Whatever the mechanism that produces the new RNA distribution, it is highly replicable and results in a change large enough to be easily measurable.

Acknowledgments

We thank Mr. Louis Grosman of the Division of Computer Research and Technology, National Institutes of Health, for computer programs used in the preparation of these data and Mrs. Gail Arnold for assistance in processing of data and preparation of the manuscript. We thank Dr. Yale J. Topper for the use of his laboratory during the early course of this work and for his continuing interest.

References

Billing, R. J., Barbiroli, B., and Smellie, R. M. S. (1969a), *Biochim. Biophys. Acta 190*, 52.

Billing, R. J., Barbiroli, B., and Smellie, R. M. S. (1969b), *Biochim. Biophys. Acta 190*, 60.

Chen, J. M. (1954), *Exp. Cell Res. 7*, 518.

Dahlberg, A. E., Dingman, C. W., and Peacock, A. C. (1969), *J. Mol. Biol. 41*, 139.

Dingman, C. W., and Peacock, A. C. (1968), *Biochemistry 7*, 659.

Elias, J. J. (1957), *Science 126*, 842.

Gelehrter, T. D., and Tomkins, G. M. (1967), *J. Mol. Biol. 29*, 59.

Green, M. R., and Topper, Y. J. (1970), *Biochim. Biophys. Acta 204*, 441.

Joel, P. B., and Hagerman, D. D. (1969), *Biochim. Biophys. Acta 195*, 328.

Lockwood, D. H., Stockdale, F. E., and Topper, Y. J. (1967), *Science 156*, 945.

Loening, U. E. (1969), *Biochem. J. 113*, 131.

Lwoff, A. (1969), *Bacteriol. Rev. 33*, 390.

Mayne, R., Forsyth, I. A., and Barry, J. M. (1968), *J. Endocrinol. 41*, 247.

McConkey, E. H., and Hopkins, J. W. (1969), *J. Mol. Biol. 39*, 545.

Mills, E. S., and Topper, Y. J. (1970), *J. Cell Biol. 44*, 310.

Palmiter, R. D. (1969), *Biochem. J. 113*, 409.

Peacock, A. C., and Dingman, C. W. (1967), *Biochemistry 6*, 1818.

Peacock, A. C., and Dingman, C. W. (1968), *Biochemistry 7*, 668.

Stockdale, F. E., Juergens, W. G., and Topper, Y. J. (1966), *Develop. Biol. 13*, 266.

Tomkins, G. M., Gelehrter, T. D., Granner, D., Martin, D., Jr., Samuels, H. H., and Thompson, E. B. (1969), *Science 166*, 1474.

Turkington, R. W. (1968), *Endocrinology 82*, 575.

Turkington, R. W., Brew, K., Vanaman, T. C., and Hill, R. L. (1968), *J. Biol. Chem. 243*, 3382.

Turkington, R. W., Lockwood, D. H., and Topper, Y. J. (1967), *Biochim. Biophys. Acta 148*, 475.