protein synthesis in rat liver(16). Inadequate blockade of protein synthesis may account for the differences between the effects of cycloheximide and actinomycin.

Although alternative explanations for these observations are by no means ruled out, it is suggested that actinomycin prevented the appearance of the metabolic changes characteristic of fasting by blocking the synthesis of messenger RNA. A corollary to this hypothesis is that in the conversion to a fasting-type metabolism different genes are expressed and certain new proteins are synthesized. Actinomycin D may have blocked the expression of these "fasting" genes by preventing the synthesis of messenger RNA. Under normal physiological circumstances, it may be that insulin, which is abundant in fed animals and present only in very low concentrations in fasting animals also regulates the expression of "fasting" genes, although activation of "fasting" genes by some extra-pituitary humoral agent is not ruled out.

Summary. In hypophysectomized rats fasting reduced the metabolism of glucose in adipose tissue and diaphragm muscle *in vitro* and increased the mobilization of free fatty acids and glycerol. Hepatic and cardiac glycogen were also reduced. Treatment with actinomycin D reversed these effects, and caused a substantial increase in glucose utilization by adipose tissue. Actinomycin partially prevented the increased lipolysis caused by fasting in normal rats, but failed to reduce the lipolysis evoked by epinephrine or corticotropin.

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1. Goldberg, I. H., Rabinowitz, M., Sci., 1962, v136, 315.

2. Hurwitz, J., Furth, J. J., Malamy, M., Alexander, M., Proc. Nat. Acad. Sci., 1962, v48, 1222.

3. Goodman, H. M., Endocrinol., 1965, v76, 1134.

4. Dole, V. P., J. Clin. Invest., 1956, v35, 150.

5. Goodman, H. M., Endocrinol., 1963, v72, 95.

6. ———, ibid., 1965, v76, 216.

7. ———, Proc. Soc. Exp. Biol. & Med., 1966, v121, 5.

8. ———, Endocrinol., 1967, in press.

9. Goodman, H. M., Knobil, E., ibid., 1957, v65, 451.

10. Russell, J. A., Bloom, W. L., ibid., 1956, v58, 83.

11. Freed, S. C., George, S. St., Proc. Soc. Exp. Biol. & Med., 1965, v119, 773.

12. Fain, J. N., Galton, D. J., Kovacev, V. P., J. Molec. Pharmacol., 1966, v2, 237.

13. Goodman, H. M., Knobil, E., Am. J. Physiol., 1961, v201, 1.

14. Cahill, G. F., Jr., Herrera, M. G., Morgan, A. P., Soeldner, J. S., Steinke, J., Levy, P. L., Reichard, G. A., Jr., Kipnis, D. M., J. Clin. Invest., 1966, v45, 1751.

15. Kerridge, D., J. Gen. Microbiol., 1958, v19, 497.

16. Korner, A., Biochem. J., 1966, v101, 627.

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RNA and Protein Synthesis in the Response of Pigeon Crop-Sac to Prolactin.* (32580)

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The importance of RNA and protein synthesis in hormone-induced tissue responses has been underlined by a number of studies in recent years(1-3). The metabolic inhibitors, actinomycin D (AMD), and puromycin (P), which inhibit RNA and protein syntheses, respectively, have been of great utility in analyzing the action of hormones at the cellular level. AMD has been shown to block the response of several tissues to steroid hormones (4-8) and a similar effect has been observed with P(6,9). Response of tissues to stimulation by protein hormones is also blocked by AMD(10,11) and P(12-17) and they inhibit the action of thyroid hormones(18,19) and block hormone-induction of casein syn-

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thesis(20,21). The importance of RNA synthesis in hormone-induced tissue responses is further illustrated by the demonstration that RNA, extracted from estrogen-treated rat uteri, has an estrogen-mimicking tropic influence on the endometrium of ovariectomized rats(22,23).

The possibility that RNA and protein syntheses are prerequisites for the response of the pigeon crop-sac to prolactin is evaluated herein. It is shown that the blocking agents, AMD and P, inhibit the response of this tissue to prolactin. In addition, RNA extracted from prolactin-stimulated crop-sac mucosa duplicates the prolactin response of this tissue, and its effect is specific.

Materials and methods. General. 4 to 6-week old pigeons of the White King strain were used for the studies with AMD, P and the RNA fractions. Pigeons of the same age of the Silver King strain were used in the study with RNase. Crop-sac responses were objectively quantified by the method of Nicoll (24). This consisted of removing a standardized, 4-cm diameter disc of mucosa from the sites of local (intradermal) injection on the treated hemicrops and comparing the dry weight of this tissue to a similar disc removed from the contralateral hemicrops. The mucosal discs were desiccated overnight at 60°C under vacuum. The AMD, P and the RNA preparations were injected locally over one hemicrop of the pigeons. All prolactin injections were given subcutaneously in the flank region in 1 ml of physiologic saline. The AMD and RNA preparations were administered in 0.5 ml injection volumes in a suspension of 7.5% starch. Each injection of P was given in 0.5 ml physiologic saline. Control hemicrops of all pigeons were injected with either 7.5% starch alone or with physiologic saline, corresponding to the vehicle injected in the treated hemicrops. Intracutaneous injection of 7.5% starch solution itself did not affect the crop-sac mucosa. In 6 pigeons the dry weight of the 4-cm disc of mucosa from the untreated hemicrops was 6.6 $\pm$ 0.6 mg while the starch-injected sides weighed 7.6 $\pm$ 0.8 mg. The prolactin was the NIH ovine preparation P-S-7.

AMD Experiment 1. The pigeons received a single injection of 0.1 μg AMD over 1 crop-sac followed 1.5 hours later by a systemic injection of 1 mg of ovine prolactin. The birds were killed about 24 hours later and the crop-sacs were removed for response quantification.

AMD Experiment 2. Each pigeon received 0.5 μg AMD and 250 μg of prolactin daily for four days. The AMD was injected locally 1.5 hours prior to systemic prolactin administration and the birds were killed about 24 hours after the last injection of prolactin.

Puromycin Experiment 1. Pigeons received a local intradermal injection of 100 μg of P over 1 hemicrop followed by subcutaneous injection of 1 mg prolactin 2 hours later. 5 hours after the prolactin administration the birds received a second injection of 100 μg of P at the same injection site. The pigeons were killed 42 hours after the second injection of puromycin.

Puromycin Experiment 2. The same injection schedule was followed each day (2 injections of 100 μg of P) for 4 days except that the birds received 250 μg of prolactin per day. The birds were killed on the fifth day.

RNA extracts. In order to obtain a large quantity of highly developed crop-sac mucosal epithelium, retired breeder pigeons of the White King strain received daily subcutaneous injections of 250 μg of prolactin for 4 days. On the fifth day they were decapitated, the crop-sacs were removed, and the stimulated epithelium was scraped from the underlying submucosa. The mucosal cells were frozen on dry ice and stored thereon. About 1 gram of tissue was harvested from each bird. RNA was extracted from the mucosal epithelial cells by the method used by Segal *et al*(22) except that all centrifugations were done at 9000 $\times$ *g*. RNA, DNA and protein analyses were performed on the RNA precipitate using the orcinol reaction for RNA(25), the diphenylamine reaction for DNA(26), and the method of Lowry *et al*(27) for protein. The RNA preparation did not contain any detectable DNA. The determinations disclosed that each pigeon received approximately 1.5 mg RNA containing 6 μg of protein. Each pigeon received RNA from mucosa derived from approximately 3.5 crop-sacs.

The specificity of the crop-sac response to crop-sac RNA was evaluated by extracting

the RNA from a quantity of pigeon liver tissue which was equivalent to the amount of crop-sac mucosa on a wet weight basis. In addition, the possibility that the protein in the crop-sac RNA fraction might have been prolactin was tested by adding 5 mg of ovine prolactin to 17.5 g of liver before extracting the liver RNA. This extract was given to 5 pigeons. 5 mg of ovine prolactin is sufficient to induce maximal local responses in about 100 pigeons and substantial responses in about 1000 birds when injected locally over the crop-sac(24).

RNase Experiment. The specificity of the crop-sac response to crop-sac RNA was further evaluated by digesting the RNA fraction with RNase. Crop-sac RNA was obtained from prolactin-treated retired breeder Silver King pigeons, according to the method described above. This was suspended in Krebs-Ringer buffer containing RNase (Worthington Biochemical Corp., Freehold, N.J.) added in a ratio of 1 part enzyme per 10 parts of substrate. The mixture was incubated for 2 hours at 37°C, then mixed with an equal volume of 15% starch and frozen until used for injections. The control side received a similar amount of RNase in starch carried through the same procedure.

The injection schedule for all experiments with the RNA fractions consisted of 2 intradermal injections per day for 2 days and the birds were killed at 36 to 40 hours after the last injection. The experiments with the crop-sac RNA and the liver RNA were repeated but the crop-sac responses were quantified by histologic measurement of the thickness of the crop-sac mucosa. This was accomplished by preparing hematoxylin and eosin-stained paraffin sections of the hemicrops after they had been stretched on cork boards prior to fixation in Bouin's fluid. The mucosal thickness was measured with an ocular micrometer. In the experiment with the RNase-digested crop-sac RNA, a 5 mm diameter disc of mucosa in the center of the injection site was left on the underlying submucosa and used for histologic determination of mucosal thickness. The remaining mucosal tissue lying within the 4-cm diameter steel plate of the crop-sac holding apparatus(24) was removed from the submucosa for dry-weight determination.

TABLE I. Effects of Actinomycin D and Puromycin on the Response of the Pigeon Crop-Sac to Systemically Injected Prolactin.

Experiment No.[1]	Prolactin dose	Drug dose per bird	No. of pigeons	Mucosal dry wt (mg)			
				Control side	Drug-treated side	Difference	% Difference
AMD-1	1 mg in one injection	0.1 µg in one injection	6	10.3 ± 0.8	8.8 ± 0.9	− 1.5 ± 0.5	−15.4 ± 6.4*
AMD-2	1 mg in four daily injections of 250 µg each	2.0 µg in four daily injections of 0.5 µg each	6	70.3 ± 7.7	51.6 ± 7.7	−18.7 ± 7.0	−17.2 ±10.1*
Puro-1	1 mg in one injection	200 µg in two injections of 100 µg each	5	12.7 ± 1.7	9.3 ± 0.6	− 3.4 ± 1.2	−24.6 ± 4.5†
Puro-2	1 mg in four daily injections of 250 µg each	200 µg in two injections of 100 µg each daily for 4 days (total 800 µg)	8	71.6 ±18.1	47.0 ± 9.7	−24.6 ± 5.9	−28.3 ± 4.8†

[1] AMD = Actinomycin D
Puro = Puromycin

* $P < 0.05$ † $P < 0.001$

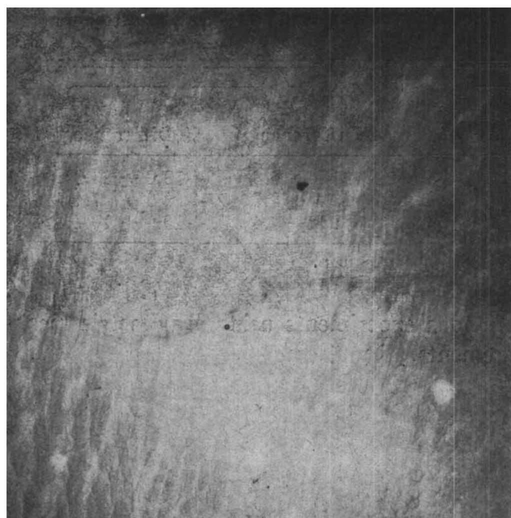

FIG. 1. Transilluminated whole-mount preparation of a hemicrop from a pigeon which received systemic injections of prolactin and local (intradermal) injections of puromycin. The faintly-stained, poorly-stimulated central region is the site of puromycin application. Localized inhibition of the response is apparent. Carmine stain, × 3.

Thus, gravimetric and histologic response quantifications were accomplished on the same hemicrops.

Results. The effects of AMD and P on the crop-sac response to systemically injected prolactin are shown in Table I. The average dry weight of the 4-cm diameter discs of the crop-sac mucosa from 6 untreated, 4-6-week-old pigeons was 6.4 ± 0.5 mg. A single subcutaneous injection of 1 mg of ovine prolactin increased the weight between 60% (AMD-1) and 100% (Puro-1). Administering the prolactin in 4 daily injections of 250 μg each resulted in about a 10-fold increase in mucosal dry weight. It is obvious from the results in Table I that both AMD and P can significantly depress the crop-sac response to prolactin, whether the drugs are administered in single or multiple injections. The hemicrops of the pigeons which were locally treated with multiple doses of the drugs, while receiving 4 daily subcutaneous injections of prolactin, showed visually discernible inhibition of mucosal thickening at the site of drug injection. In some cases, the degree of local inhibition was so pronounced that the tissue in the region of the drug administration remained almost transparent while the surrounding mucosa was considerably thickened, rugose and opaque. This is illustrated by a P-treated hemicrop in Fig. 1.

The results obtained with the RNA extracts, as measured gravimetrically, are shown in Table II. Clearly the RNA fraction derived from crop-sac mucosal cells was highly effective in stimulating thickening of the crop-sac mucosa. The dry weight of the mucosal disc was more than doubled as a result of crop-sac RNA injection. This degree of weight increase is about equivalent to that produced by local (intradermal) injection of 25 mU of NIH ovine prolactin in accordance with the same injection schedule(24). It is also evident from Table II that the RNA fractions from pigeon liver, or from liver to which prolactin had been added, were completely devoid of crop-sac stimulating activity. This demonstrates that the response of the crop-sac mucosa to crop-sac RNA is specific. Digestion of the crop-sac RNA with RNase totally abolished the stimulatory influence of the RNA fraction (Table II). Accordingly, the crop-sac stimulating activity of the crop-sac RNA is not the result of non-RNA contaminants.

The effects of the RNA fractions on the thickness of the crop-sac mucosa, as measured

TABLE II. Gravimetric Determination of the Effects of RNA Extracts on Pigeon Crop-Sac Mucosa.

RNA from—	No. of pigeons	Mucosal dry wt (mg) Control side	RNA-treated side	Difference	% Difference
Crop-sac mucosa	7	6.1 ± 0.9	12.6 ± 1.9	+6.5 ± 1.2	+109.5 ± 13.3*
Liver tissue	7	8.6 ± 1.0	7.9 ± 0.9	−0.7 ± 1.0	− 6.2 ± 12.0
Liver tissue + prolactin	5	7.9 ± 0.9	7.5 ± 1.1	−0.4 ± 0.8	− 4.2 ± 9.3
RNase-treated crop-sac RNA	7	8.1 ± 1.0	7.3 ± 1.0	−0.8 ± 1.1	− 10.8 ± 9.4

* P <0.001

TABLE III. Effects of RNA Extracts on the Crop-Sac Mucosa as Determined by Histologic Measurements.

RNA from—	No. of pigeons	Mucosal thickness (μ) Control side, μ	RNA-treated side, μ	Difference	% Difference
Crop-sac mucosa	6	32.5 ± 2.1	54.4 ± 4.9	+21.9 ± 6.8	+73.8 ± 24.2*
Liver tissue	6	45.4 ± 9.4	46.0 ± 8.0	+ 2.4 ± 2.7	+ 6.0 ± 7.4
Liver tissue + prolactin	5	38.2 ± 6.5	40.5 ± 5.3	+ 2.3 ± 3.4	+10.6 ± 10.7

* $P < 0.025$

histologically, are shown in Table III. The RNA fractions extracted from pigeon liver and from liver to which prolactin was added showed no capacity to increase the thickness of the crop-sac mucosa. In contrast, the crop-sac RNA fraction was highly effective. This is in agreement with the results obtained in the previous experiments using gravimetric measurements. Representative histologic sections of crop tissues, which were treated with starch and with crop-sac RNA, are shown in Figure 2.

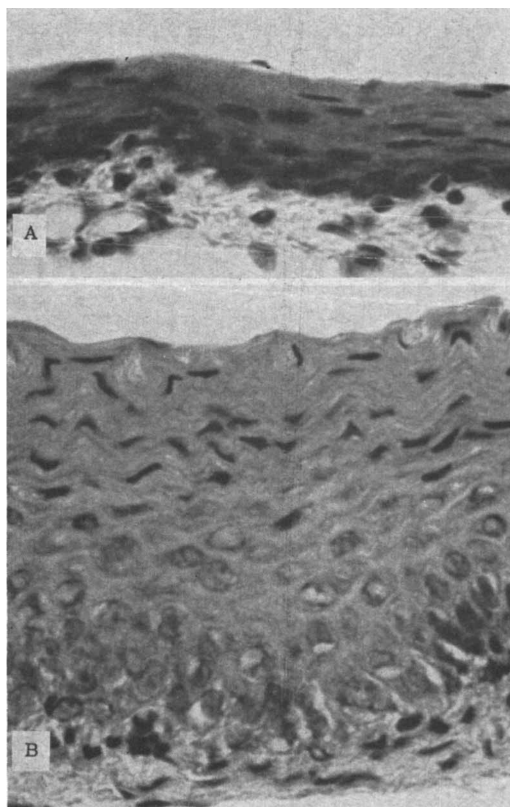

FIG. 2 A. Photomicrograph of a hemicrop which received four intradermal injections of 7.5% starch in Krebs-Ringer buffer. B. Photomicrograph of the hemicrop contralateral to A. This tissue received four local injections of 7.5% starch in buffer containing crop-sac mucosal RNA. Stimulation of the mucosa is obvious. Both tissues taken from the injection sites. Hematoxylin and eosin-stained 8 μ paraffin sections, × 1130.

Discussion. The results of these studies clearly demonstrate that the response of the pigeon crop-sac to systemically injected prolactin is inhibited by local application of either AMD or P over one hemicrop. These results are consistent with the hypothesis that stimulation of RNA and protein synthesis is prerequisite for the manifestation of hormone-induced tissue responses. Inasmuch as these metabolic blocking agents were applied to a localized region of the crop-sac in very small doses, and their inhibitory influence was confined to an area at the site of injection, their efficacy in depressing the crop-sac response to prolactin cannot be attributed to generalized (systemic) toxic effects. However, it has been emphasized that caution must be exercised in interpreting results obtained with these drugs(2,28,29) because of possible effects unrelated to their presumed primary action of inhibiting DNA-dependent messenger RNA synthesis (AMD) or of blocking protein synthesis at the ribosomal level(P). In this test system we have observed that local injections of doses of AMD of 0.5 μg or more were highly inflammatory.

The specificity of the crop-sac stimulating activity of the crop-sac mucosal RNA, and the destruction of this activity by RNase hydrolysis, provide additional support to the hypothesis that many hormone-induced tissue responses are mediated *via* RNA. It has now been shown that homologous RNA fractions can duplicate the effects of steroid hormones on the uterine endometrium(22,23) and seminal vesicles(30) of rats. Indeed, Villee(31,32) has reported that RNA extracted from steroid-

secreting glands can alter the pattern of hormone secretion by other steroidogenic organs *in vitro*. Our results extend these observations of RNA-induced, endocrine-like responses to include a target organ which is responsive to a protein hormone.

Summary. Intradermal injection of either actinomycin D or puromycin locally over 1 side of the pigeon crop-sac inhibited the tissue's response to systemically administered prolactin. RNA extracted from prolactin-stimulated crop-sac mucosal tissue had a high degree of crop-sac stimulating activity when injected intradermally over the crop-sac. In contrast, similar RNA fractions extracted from pigeon liver tissue or from liver to which prolactin was added prior to extraction were devoid of this activity. Hydrolysis of the crop-sac RNA by RNase completely eliminated its crop-sac stimulating capacity. These results indicate that RNA and protein synthesis is prerequisite for the expression of the crop-sac response to prolactin.

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1. Litwack, G., Kritchevsky, D., Actions of Hormones on Molecular Processes. John Wiley & Sons, N. Y., 1964.

2. Hechter, O., Halkerston, I. D. K., Ann. Rev. Physiol., 1965, v27, 133.

3. Karlson, P., Sekeris, C. E., Acta Endocrinol., 1966, v53, 505.

4. Talwar, G. P., Segal, S. J., Proc. Nat. Acad. Sci., 1963, v50, 226.

5. Ui, H., Mueller, G. C., ibid., 1963, v50, 256.

6. Edelman, I. S., Bogoroch, R., Porter, G. A., ibid., 1963, v50, 1169.

7. Bialy, G., Pincus, G., Endocrinol., 1966, v78, 286.

8. Nicolette, J. A., Mueller, G. C., ibid., 1966, v79, 1162.

9. Mueller, G. C., Gorski, J., Aizawa, Y., Proc. Nat. Acad. Sci., 1961, v47, 164.

10. Chambers, J., Bass, A. D., Fed. Proc., 1967, v26, 258.

11. Rasmussen, H., Arnaud, C., Hawker, C., Sci., 1964, v144, 1019.

12. Ferguson, J. A., J. Biol. Chem., 1963, v238, 2754.

13. Fain, J. N., Biochim. Biophys. Acta, 1964, v84, 636.

14. Tong, W., Endocrinol., 1965, v76, 163.

15. Kunin, A. S., Krane, S. M., ibid., 1965, v76, 343.

16. Straw, J. A., Hart, M. M., Fed. Proc., 1967, v26, 729.

17. Elsas, L. J., Albrecht, I., Rosenberg, L. E., ibid., 1967, v26, 258.

18. Tata, J. R., Nature, 1963, v197, 1167.

19. ———, Dev. Biol., 1966, v13, 77.

20. Stockdale, F. E., Juergens, G., Topper, Y. J., ibid., 1966, v13, 266.

21. Turkington, R. W., Topper, Y. J., Biochim. Biophys. Acta, 1966, v127, 366.

22. Segal, S. J., Davidson, O. W., Wada, K., Proc. Nat. Acad. Sci., 1965, v54, 782.

23. Mansour, M., Niu, M., ibid., 1965, v53, 764.

24. Nicoll, C. S., Endocrinol., 1967, v80, 641.

25. Mejbaum, W., Z. physiol. Chem., 1939, v258, 117.

26. Dische, Z., Microchemie, 1930, v8, 4.

27. Lowry, O. M., Rosebrough, N. J., Farr, A. L., Randall, R. J., J. Biol. Chem., 1951, v193, 265.

28. Laszlo, J., Miller, D. S., McCarty, K. S., Hochstein, P., Sci., 1966, v151, 1007.

29. Bransome, E. D., Endocrine Soc. Abstracts, 1967, 49th meeting, 63.

30. Fujii, T., Villee, C., Fed. Proc., 1967, v26, 757.

31. Villee, D., ibid., 1967, v26, 604.

32. ———, Endocrine Soc. Abstracts, 1967, 49th meeting, 67.

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