ENDOCRINES AND THEIR RELATION TO INFLUENZA VIRUS INFECTION*

By SEYMOUR S. KALTER, HAROLD J. SMOLIN, JANE M. McELHANEY, AND JAY TEPPERMAN

(From the Departments of Bacteriology and Parasitology, and Pharmacology, State University of New York Medical College, and the Virus Laboratory, Bureau of Laboratories, Syracuse)

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It has recently been demonstrated that with increased host age there is an increase in resistance to the virus of influenza (1). This increase in resistance was thought to be due, in part, to differences in the rates of protein synthesis of mice of different ages. When host growth was inhibited experimentally by reducing protein intake, no detectable change in susceptibility to infection with the PR8 strain of influenza virus could be discerned (2).

Attempts were then made to alter rates of protein anabolism in the host by hormonal means. The first materials employed were pituitary growth hormone and testosterone, compounds which are known to be capable of causing nitrogen retention in the tissues of experimental animals (3, 4). The results of these experiments indicated that increasing protein anabolism of the host was accompanied by an increase in rate of proliferation and amount of influenza virus (5).

The data described in this report are those obtained by altering protein metabolism with ACTH and cortisone, which are generally considered to be protein catabolic (or, possibly, antianabolic) hormones. The rate of virus growth in castrate mice with and without testosterone is also described.

Materials and Methods

Virus.—A mouse-adapted strain (PR8) of influenza virus was used for all the experiments considered here. This strain had been transferred 49 times, through young (3 to 4 weeks old) mice. Following the last passage, approximately 75 mice were inoculated intranasally in the usual manner and the lungs harvested after 72 hours. A 10 per cent lung suspension was made in buffered saline following homogenization in a Waring blendor. This suspension was distributed in glass ampules, sealed, and stored at −60°C. The LD_{50} of this virus suspension was $10^{-7.55}$ when determined in mice 3 to 4 weeks old.

Virus preparations employed in these experiments were made by diluting a vial of stock virus in buffered saline to the desired LD_{50}. The challenge dose used was either approximately 500 LD_{50} or 1000 LD_{50}. During the period of experimentation virulence of the virus did not change significantly.

Mice.—Swiss mice approximately 3 weeks old were obtained from Albino Farms, Red Bank.

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Female mice were used in some experiments while males were used in others. The mice were stored in large cages containing groups of 50 animals. A random sampling of mice from the stock cages was made for each experiment. Water and food (Purina dog chow) were given *ad libitum* throughout the course of the experiments.

The mice were weighed individually 3 times a week. Any animal that appeared ill was eliminated from the experiment.

Hormones.—The ACTH (lot No. 128-105R) was kindly supplied by Mr. Irby Bunding of Armour and Company. The material was diluted in saline so that each animal received either 0.2 mg. or 0.5 mg. daily. The hormone was administered subcutaneously for a period of 10 days, given in divided doses of either 0.1 mg. or 0.25 mg. dissolved in 0.2 ml. of saline.

Cortisone acetate (Merck and Co., Inc.) was given subcutaneously so that each mouse received 0.25 mg. daily for 7 days.

Testosterone cyclopentylpropionate (30 mg./ml.) was supplied by The Upjohn Co. Each mouse received 0.2 ml. (6.0 mg.) subcutaneously once a week for 3 weeks.

Castrations.—Two hundred male mice approximately 6 weeks old were either castrated or sham operated. Testes were removed under ether anesthesia by way of lower abdominal incisions. Bleeding was controlled by hemostat pressure. After removal of the testes, the incision was sutured with surgical silk. No antibiotics were employed. A few fatalities occurred mainly as a result of extensive ether anesthesia. Mice were not inoculated with virus until 3 weeks after operation. Body weight records were kept throughout the postoperative and test periods.

Titration of Virus.—Virus titrations from treated and control animals were made in 11-day-old fertile chick embryos. At the designated time interval at least 5 test and 5 control mice were sacrificed, the lungs removed, pooled according to group, weighed, and quickly frozen in screw-capped tubes. At the conclusion of the experiment, the lungs were removed from the CO_2 ice box and homogenized as 10 per cent suspensions in a Waring blendor. During the 3 minute period of homogenization the blendor was kept cold by means of a water jacket containing circulating cold water. The 10 per cent suspensions were lightly centrifuged and dilutions made in buffered saline. Sufficient penicillin and streptomycin were added to all dilutions in order to insure sterility. Five embryos per dilution were employed; each embryo received 0.2 ml. of virus dilution. After incubation of approximately 36 to 40 hours, the embryos were chilled and the presence or absence of virus determined by the erythrocyte agglutination test. The 50 per cent infectivity end-points were determined by the method of Reed and Muench.

RESULTS

Castration and Testosterone.—Administration of testosterone has been shown to cause an increase in influenza virus proliferation (5). It was pertinent to inquire whether or not virus growth is affected by a deficiency of this hormone. Accordingly, 200 mice were operated on as described above and, 3 weeks after operation, they were divided into the following groups of 50: (*a*) untreated castrates; (*b*) testosterone-treated castrates; (*c*) untreated sham-operated controls; and (*d*) testosterone-treated sham-operated controls.

The mean weights of the castrates and sham-castrates are given in Fig. 1. Three weeks after the operations, the control group of animals weighed approximately 1.5 gm. more than the castrates. At this time 45 of the castrates and 45 of the controls were given testosterone in weekly injections for 3 weeks. The control groups of castrates and sham castrates were given injections of Wesson oil in amounts equal to those given the test groups of animals. The 5 remaining mice in each group were not given any further treatment. These mice

were used in order to determine if any marked difference in time of death or lung lesions would appear after inoculation with influenza virus. There were no differences in these two small groups of animals. Two animals in each group died within 10 days and the survivors all showed approximately the same degree of lobar consolidation.

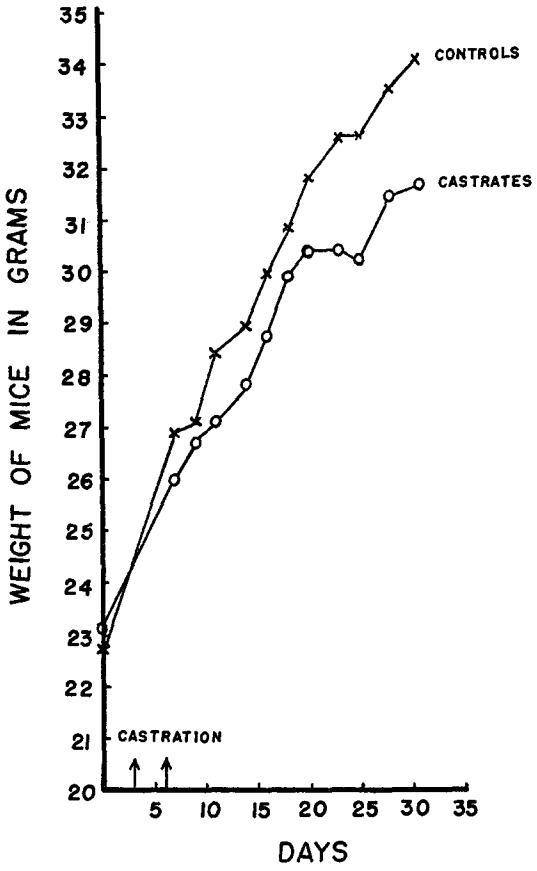

FIG. 1. The mean weights of mice (approximately 3 months old) following castration. Each curve represents the mean growth rate of at least 35 mice.

Table I shows the mean weights of all groups of mice prior to their inoculation with approximately 1000 LD_{50} of influenza virus.

At 3 hour intervals, following the virus inoculations, 5 mice in each group were sacrificed, the lungs removed and weighed and then quickly frozen and stored. Immediately following this, the following tissues were removed and weighed: adrenal, thymus, spleen, and kidney. The accumulated mean weights of all these tissues and the standard errors of the means are listed in Table II.

The figures set in bold face type are those which differ significantly from the corresponding control values. Following castration the adrenals increased markedly in size whereas the kidneys decreased markedly. There was a trend toward decrease in spleen size and toward increase in thymus size.

Determinations of the amount of virus present in the 4 groups of animals were made as described above. During the first 12 hours, there is little apparent

TABLE I

The Mean Weights of Castrated and Control Mice (Approximately 3 Months Old) Following Testosterone Administration

Control	Control given oil	Control given testosterone	Castrates	Castrates given oil	Castrates given testosterone
gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
37.1*	36.6	37.7	34.3	35.4	36.8

* Each value represents the mean of approximately 35 mice.

TABLE II

The Mean Weight of Mouse Tissues Following Castration, Castration Plus Testosterone, and in Appropriate Controls*

	Control weights		Control and testosterone weights		Castrate weights		Castrate and testosterone weights	
	Mg.	Per cent body weight	Mg.	Per cent body weight	Mg.	Per cent body weight	Mg.	Per cent body weight
Lung	225.4 ± 11.2‡	0.63	221.1 ± 12.5	0.59	228.0 ± 7.3	0.65	223.0 ± 13.1	0.62
Adrenal	4.04 ± 0.41	0.0059	4.1 ± 0.39	0.0053	**7.8 ± 0.7**	**0.011**	3.62 ± 0.3	0.0053
Thymus	50.4 ± 3.2	0.13	36.3 ± 1.4	0.097	91.6 ± 19.9	0.256	35.0 ± 3.3	0.097
Spleen	265.7 ± 28.4	0.74	229.6 ± 28.8	0.60	180.9 ± 13.6	0.51	203.0 ± 33.3	0.57
Kidney	622.9 ± 34.8	1.778	726.0 ± 55.3	1.920	**462.0 ± 22.5**	**1.250**	642.9 ± 33.1	1.790

Bold face type indicates a difference from the control value at a probability level of less than 0.01.

* Mice approximately 3 months old.

‡ Standard error of the mean.

difference in amount of virus present in the lungs of these mice. In 24 hours, the normal animals receiving testosterone contain the greatest amount of virus, whereas the castrate group has the least amount. After 48 hours, although there is still a difference between the groups, there appears to be a tendency for maximal virus growth to occur in all groups. The control group and the testosterone-treated castrate group showed similar rates of virus growth (Fig. 2).

ACTH and Cortisone.—The above data confirmed the previous findings that increased protein anabolism is associated with increased rate of influenza virus proliferation. The data also suggest that a relative decrease of protein anabo-

lism as produced in castrated animals is accompanied by decreased virus proliferation.

To observe the effect of increased protein catabolism of the host on virus growth, ACTH and cortisone were studied according to the following plan:—

Approximately 100 mice were separated into two groups: one received 0.2

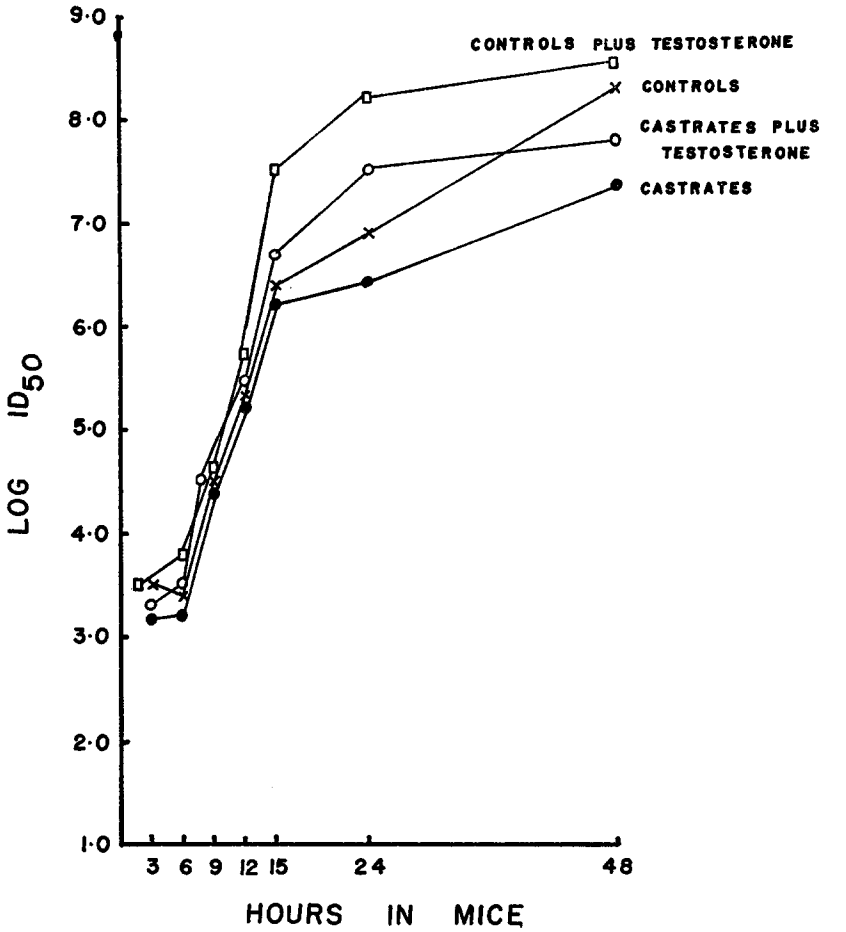

FIG. 2. Influenza virus (strain PR8) proliferation in mice (approximately 3 months old) treated as described in the text.

mg. ACTH (subcutaneously) daily for 12 days, while the other received the same number of injections of physiological saline. The mean weights of these animals are plotted on Fig. 3. The ACTH group tended to be somewhat lighter than the controls. Following influenza virus inoculations, which were made as described for the castration experiments, the lungs were removed and frozen.

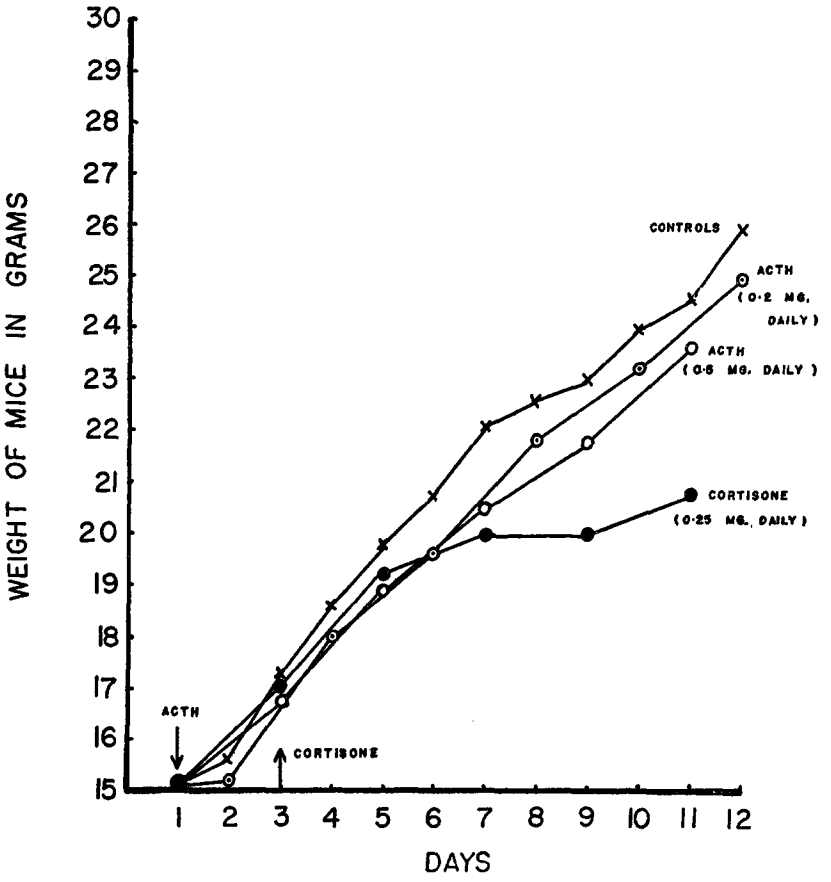

FIG. 3. The effect of ACTH and cortisone on growth of mice (3 to 4 weeks old). Arrows indicate the date of administration of hormone. Each curve represents the mean growth rate of at least 40 mice.

TABLE III

The Mean Weight of Mouse Tissues Following Treatment with ACTH (0.2 Mg. Daily)*

	Control weights		ACTH (0.2 mg. daily) weights	
	Mg.	Per cent body weight	Mg.	Per cent body weight
Lung	171.5 ± 5.5‡	0.66	159.0 ± 4.6	0.64
Adrenal	3.42 ± 0.85	0.0061	**4.08 ± 0.73**	**0.0081**
Thymus	91.2 ± 2.0	0.35	**72.11 ± 3.7**	**0.274**
Spleen	113.6 ± 6.4	0.435	91.7 ± 4.3	0.364

Bold face type indicates a difference from the control value at a probability level of less than 0.01.

* Mice approximately 5 to 6 weeks old.

‡ Standard error of the mean.

The adrenals, thymus, and spleen were also removed and weighed (Table III). Significant weight changes were obtained with this dosage in the adrenals, thymus, and spleen ($P < 0.02$).

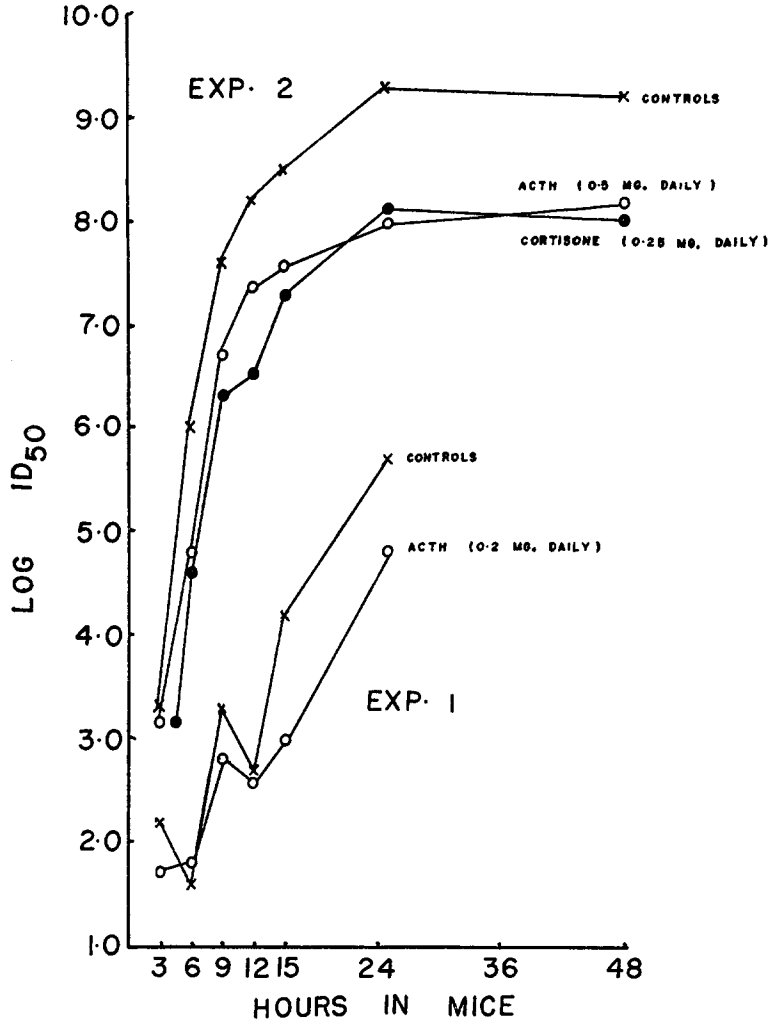

FIG. 4. Influenza virus (strain PR8) proliferation in mice (5 to 6 weeks old) following treatment with ACTH or cortisone for 10 and 7 days respectively.

Influenza virus growth was also determined in this experiment. These results are indicated on Fig. 4 as Experiment 1 and are very similar to those obtained in the castration experiment. Less virus is found in the ACTH-treated group of animals.

This experiment was repeated with some slight modifications. A larger dose of ACTH was given (0.5 mg. daily) and more virus was used as a challenge dose (1000 LD_{50}). In addition, cortisone was also included in the study. The experimental procedure was the same as in the other experiments. Again, the changes in rate of virus growth were of the same order of magnitude as in Experiment 1. Following treatment with ACTH or cortisone, less virus growth was obtained. These results are also shown in Fig. 4 as Experiment 2.

The changes in the tissues deserve comment inasmuch as the ACTH and cortisone caused marked effects. With ACTH weights of the thymus and spleen were significantly decreased. The adrenal increased significantly in weight. After

TABLE IV

The Mean Weight of Mouse Tissues Following Treatment with ACTH (0.5 Mg. Daily) and Cortisone (0.25 Mg. Daily)*

	Control weights		ACTH weights		Cortisone weights	
	Mg.	Per cent body weight	Mg.	Per cent body weight	Mg.	Per cent body weight
Lung	184.9 ± 8.2‡	0.75	179.9 ± 6.2	0.762	159.7 ± 7.8	0.76
Adrenal	3.9 ± 0.38	0.00811	**5.2 ± 0.62**	**0.011**	**1.4 ± 0.02**	**0.0033**
Thymus	68.3 ± 5.8	0.278	**51.3 ± 2.9**	**0.218**	**26.8 ± 2.1**	**0.108**
Spleen	195.9 ± 10.8	0.800	**86.8 ± 2.2**	**0.368**	**51.3 ± 7.4**	**0.244**
Kidney	366.8 ± 16.2	1.490	356.5 ± 6.1	1.516	**409.8 ± 1.2**	**1.938**

Bold face type indicates a difference from the control value at a probability level of less than 0.02.

* Mice approximately 5 to 6 weeks old.

‡ Standard error of the mean.

cortisone, the adrenal, thymus, and spleen all decreased in weight. Table IV is a tabulation of these data. Determinations of the differences in death rate under these conditions were also made. Essentially no differences in either time of death or extent of lung involvement could be demonstrated in any of these experiments.

DISCUSSION

The data obtained in the above experiments appear to be in keeping with the previous statement that influenza virus growth is, to some extent, related to the metabolic activities of the host cell. More impressive than the subtle differences in rates of virus growth reported here is the consistency with which these changes were observed. Moreover, the pattern of virus growth closely paralleled the experimental manipulation of the host's protein metabolism. These results do not define a relationship between rate of virus proliferation and the level of protein anabolic activity in the host cell. They merely indicate that such a relationship exists under the conditions of our experiments. Further,

they do not indicate that this relationship is the only, or, indeed, an important determinant of the rate of virus growth. Until more is known of the mechanisms involved in protein synthesis in the cell and of the oxidative systems which power those synthetic reactions, the role of the virus in the complete system must remain a matter for speculation.

There is evidence that protein changes occur in the host following influenza infection. Kilbourne and Horsfall demonstrated that the total protein of the allantoic fluids is higher in infected chick embryos as compared with controls (6). Specific amino acid changes were also shown to occur in the host after infection with influenza viruses (7). These findings indicate that some alteration in protein metabolism occurs after infection.

Certain related studies of the effects of hormone treatment and hormone deficiencies on virus growth have been reported. For example, Aycock demonstrated in a group of 10 female monkeys that 4 of 5 castrates not receiving estrin succumbed to poliomyelitis. Of the treated group only one of the 5 succumbed. A non-castrate control group showed an incidence of 3 of 5 monkeys (8). Sprunt and his group studied the effect of sex hormones on vaccinia virus. Their findings indicate that estrogenic hormone-treated and pseudopregnant animals are more resistant to infection with the virus of vaccinia (9, 10). It is suggested by the authors that this increased resistance is dependent upon some sort of a "neutralization" of the virus particle before it enters the host cell.

The increased resistance of mice to the virus of poliomyelitis at low temperatures is considered to be a manifestation of increased metabolism according to Holtman (11). The effect of thiouracil and thyroxine was also studied by Holtman, as these hormones are considered to cause changes in metabolic rate. His findings, on a small group of mice, indicate that thyroid extract causes a prolongation of the incubation period. Thiouracil treatment resulted in a decreased incubation period (12). Gollan, in studies with another neurotropic virus (polioencephalitis), was unable to alter host susceptibility by treatment with various doses of crystalline thyroxine (13). More recently, Shwartzman determined the effect of very large doses of ACTH and cortisone on poliomyelitis virus proliferation (14). The results of this experiment demonstrated that these hormones enhance the virulence of the virus for mice and hamsters in the doses used.

In attempting to evaluate these data in relation to each other, several pertinent facts emerge. The viruses considered in these reports are different. It is highly probable that each virus follows a specific growth pattern after invasion of the host cell. Studies on neurotropic viruses and metabolic activity of host cells are limited by the metabolic stability of nervous tissue. Brophy and McEachern have recently demonstrated that brain tissue does not show any increase in oxygen consumption after thyroxine administration (15). This may be due to the failure of brain tissue to take up thyroxine (16). There are probably other factors involved in studies of thyroxine and metabolism. The effect of

thyroxine on the pituitary and adrenals is not clearly understood. However, it has been demonstrated that thyroxine may raise the level of adrenal activity by stimulating an increased output of pituitary ACTH (17, 18). Another consideration in the evaluation of these data is that these reports concern themselves with gross infectivity and not the virus growth occurring in the early periods of infection.

The data reported here record the rate of growth of the virus at the beginning of the infection. An attempt is made to correlate host metabolism with rate of virus proliferation. These findings would appear to indicate that further studies on many different viruses are necessary in order to evaluate (1) individual differences among viruses, (2) the biochemical site of virus activity within the host cell, and (3) the relationship between dosage of hormone and final effect on both host metabolism and virus growth.

SUMMARY

Treatment with testosterone increases proliferation of influenza virus as well as protein anabolism. A relative lack of testosterone caused by castration is associated with a diminished rate of virus growth. When protein catabolism is increased by ACTH or cortisone, the rate of virus proliferation decreases. These results suggest the existence of a correlation between alterations of protein metabolism and virus proliferation.

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