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GROWTH HORMONE, INSULIN, AND REPLACEMENT OR STORAGE OF NITROGEN[1]

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IN NORMAL DOGS on a constant diet, marked storage of nitrogen occurs regularly after injections of growth hormone (STH). An increase in output of endogenous insulin appears to be required for this response. In depancreatized dogs, with food intake and insulin dosage constant, STH causes loss of nitrogen; storage occurs only if the insulin dose is increased greatly when STH is given. These findings, originally established in experiments with a globulin fraction of bovine anterior pituitary,[1-3] were confirmed with purified bovine STH many years later.[4-6]

In the present experiments, one of our purposes was to compare the effect of STH on replacement of nitrogen lost during a day of fasting, in normal and depancreatized dogs. Another objective was to determine whether the difficulty of inducing nitrogen storage with STH in depancreatized dogs might be due to absence of a product of normal pancreatic digestion. Finally, we attempted to avoid the need for additional insulin, as a condition for induction of nitrogen storage, by supplying energy sources such as fructose or lactose intravenously, in addition to glucose arising from dietary carbohydrate. Glycolytic cycle intermediates, particularly fructose, have often been used in attempts to determine the locus of insulin action in processes that this hormone regulates.[7,8]

ANIMALS, DIETS, METHODS, AND MATERIALS

All of the dogs that we used, whether normal or depancreatized, were adult female mongrels which had been subjects of previous metabolism experiments, and were thus accustomed to the routine involved. A preceding paper[6] includes complete details concerning composition of the basal diet, vitamin supplement for all animals, and supplements of choline and pancreatin, as well as insulin therapy, for depancreatized ones.

Nitrogen was determined by micro-kjeldahl digestion of urine samples containing about 5 mg of this element, steam distillation of ammonia from the alkalinized digest,

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and titration. Reducing substance in urine was determined by Benedict's method,[9] blood "true sugar" by the method of Somogyi,[10] total plasma lipid by Bloor's oxidative method,[11] acetoacetic acid and β-hydroxybutyric acid by the procedures of Behre.[12]

The bovine growth hormone used in this study was a gift of the Endocrine Study Section, National Institutes of Health.

Results

A. *Growth hormone and nitrogen replacement*

Control experiments consisting of a fore-period of 5 or 6 days, a 1-day fast, and an after-period, were carried out on 2 normal dogs (Table I). In parallel experiments, food intake was the same, but 2.5 mg of growth hormone was injected daily for 2 days after the 1-day fast. Average weight loss during the day when food was withheld was 0.25 kg in 4 experiments. Both animals recovered their weight more rapidly in the growth hormone experiment. In dog 64, daily values for urine nitrogen after the fast were obviously not lower in the growth hormone than in the control experiment. All subsequent observations on this animal indicated that it had become refractory to STH, after extensive nitrogen storage had been induced many times. Two experiments in which this animal stored 9.74 gm and 13.95 gm of nitrogen respectively, after injections of STH, were included in Table I of a preceding study.[6] In dog 79, nitrogen storage was definitely induced with STH,

Table I

EFFECT OF GROWTH HORMONE (STH) ON RECOVERY OF WEIGHT AND NITROGEN LOST BY NORMAL DOGS DURING A ONE-DAY FAST

Dog No.	Days	*Control experiments*		*Growth Hormone Experiments*		
		Weight	Urine Nitrogen	STH	Weight	Urine Nitrogen
		kg	gm/day	mg	kg	gm/day
64	1-6	20.35±.04	8.77±.29		20.32±.03	8.82±.30
	7*	20.05	4.41		20.05	3.71
	8	20.05	7.14	2.5	20.16	7.85
	9	20.10	7.82	2.5	20.22	7.89
	10	20.10	8.41		20.30	8.68
	11	20.10	8.51		20.30	8.38
	12	20.16	8.74		20.30	8.55
	13	20.28	8.83		20.41	8.25
79	1-5	17.00±.09	7.77±.32		16.83±.03	7.77±.36
	6*	16.78	3.78		16.61	4.47
	7	16.84	7.04	2.5	16.61	6.85
	8	16.89	7.37	2.5	16.95	5.10
	9	16.95	7.35		17.00	6.11
	10	16.89	7.91		17.06	7.32
	11	16.84	7.67		17.00	7.61

*Food withdrawn for one day in both experiments.
Figures preceded by ± are standard deviations.

but the amount was not large. Nitrogen output during 5 days after the fast was 4.35 gm. lower in the growth hormone experiment than in the control. A series of such experiments would be required to determine whether the effect of STH is reduced during spontaneous replacement of nitrogen, since the amount stored in experiments with STH not preceded by a fast varies widely.

Six experiments of the same type were carried out on 2 depancreatized dogs (Table II). During corresponding control and growth hormone experiments, food intake and insulin dosage were both uniform, the latter being reduced 50% or more during the 1-day fast. Loss of weight during the day without food was about twice as large as in normal dogs; it averaged 0.45 kg in 6 experiments. In the first growth hormone experiment on dog 73, with insulin dosage of 20 units per day, weight was recovered more rapidly than in the control experiment; urine nitrogen was reduced, and glucosuria not marked. Although insulin dosage was only 2 units per day lower in the second pair of experiments on this animal, and glucosuria was negligible during the entire control experiment and during the fore-period of the one with STH, injection

Table II

EFFECT OF GROWTH HORMONE (STH) ON RECOVERY OF WEIGHT AND NITROGEN LOST BY DEPANCREATIZED DOGS DURING A ONE-DAY FAST

Dog No.	Days	Insulin	*Control experiments*			*Growth Hormone Experiments*			
			Weight	Urine Nitrogen	Urine Sugar	STH	Weight	Urine Nitrogen	Urine Sugar
		U/day	kg	gm/day	gm/day	mg	kg	gm/day	gm/day
73	1-5	20	18.53±.12	11.40±.52	< 1		18.88±.10	10.80±.10	< 1
	6*	10	18.23	5.44	< 1		18.57	4.93	< 1
	7	20	18.35	10.57	< 1	2.5	18.80	8.85	< 1
	8	20	18.35	10.50	< 2	2.5	18.63	9.83	18.0
	9	20	18.40	9.92	< 1		18.80	8.87	2.4
	10	20	18.35	11.18	< 1		18.97	9.74	< 1
	11	20	18.63	10.78	< 1		19.03	9.54	< 1
73	1-5	18	19.35±.03	10.96±.40	< 1		19.73±.05	11.38±.57	< 1
	6*	8	18.85	5.70	< 1		19.22	5.23	< 1
	7	18	19.20	9.43	< 1	2.5	19.10	10.89	39.0
	8	18	19.20	10.45	< 1	2.5	18.76	16.58	81.8
	9	18	19.31	10.06	< 1		18.93	13.01	23.7
	10	18	19.36	11.10	< 1		19.38	10.66	38.3
	11	18	19.27	11.59	< 1		19.27	10.58	22.6
63	1-5	30	17.53±.07	11.72±.27	7.3		17.51±.05	12.70±.35	3.2
	6*	14	17.06	4.12	3.0		16.89	4.44	3.2
	7	30	17.37	9.73	2.2	2.5	17.18	10.86	< 1
	8	30	17.46	10.76	< 2	2.5	17.29	12.63	7.9
	9	30	17.46	11.00	< 2		17.34	12.32	3.0
	10	30	17.18	9.28	4.7		17.40	11.05	< 1
	11	30	17.46	11.72	6.9		17.40	11.61	< 2

*Food withdrawn for one day, both experiments.
Insulin dosages were identical in control and growth hormone experiments.
Figures preceded by ± are standard deviations.

of the hormone caused loss of weight and nitrogen, accompanied by profound glucosuria. Dog 63 had been depancreatized five and one-half years before the experiments recorded in Table II were begun, and was in excellent condition. This animal always had the high insulin requirement that is recorded. Recovery of weight after the fast was not improved by STH, neither was nitrogen output reduced, although glucosuria was negligible.

Results of these experiments resemble those of previous ones in which food intake was not interrupted. Nitrogen storage, in depancreatized dogs with insulin dosage constant, is only occasionally observed when growth hormone is given; as a rule, nitrogen loss occurs. Storage fails to occur whether or not there is glucosuria. Also, as stated elsewhere,[6] marked storage of nitrogen occurs despite enormous glucosuria, when dosages of insulin and growth hormone are both large.

B. *The problem of alimentation*

In all experiments in which the complete diet described in a previous publication[6] was used, fecal nitrogen of depancreatized dogs was low in comparison with values found in the literature. For example, in dog 63, which was in excellent condition for 6.5 years after pancreatectomy, average fecal nitrogen during 20 periods of 3 to 7 days each was 1.19 gm per day, with standard deviation of $\pm$ 0.27 gm. A liberal pancreatin supplement — 7.5 gm with each of the two daily feedings — probably was an important factor in this result. In clinical cases of cystic fibrosis of the pancreas the importance of using adequate amounts of pancreatin was long overlooked. In depancreatized dogs, the offensive character of the stools disappears when the dose of pancreatin exceeds 5 gm per day. Whether the use of more expensive preparations than U.S.P. pancreatin is justifiable cannot be determined in one experiment. It is, however, of interest that fecal nitrogen of dog 63 averaged 1.12 gm per day during a 7-day period in which the usual 15 gm. of U.S.P. pancreatin included in the daily ration was replaced by an equal weight of "pancreatic granules". Thus the low nitrogen output observed with this dose of pancreatin could not be reduced further with the other preparation.

An experiment on dog 63 was carried out in which the diet was first supplemented by adding 10 gm of a proprietary protein digest, Amigen, to each of the two meals per day. After a preliminary period of 38 days, each meal was further supplemented with 2.5 gm of methionine. During a 5-day control period on this diet, containing both Amigen and methionine, urine nitrogen averaged 13.38 gm per day. The diet then remained constant for 3 more days, during which 5 mg of growth hormone were injected daily. Insulin dosage during the control period and first day of STH was 32 units; it was increased to 46 and 56 units respectively on the second and third days. Urine nitrogen during the three days when STH was given averaged 12.85 gm per day, and the lowest value was not below the lowest one of the control period. Failure to induce significant nitrogen storage in this experiment can hardly be ascribed to deficient alimentation.

C. *Experiments with glucose intermediates as energy sources*

Since fructose, lactate, and other compounds in the glucolytic cycle are largely converted to glycogen in the liver, if given by mouth, it was necessary to give them intravenously. Moreover, dogs do not tolerate very large single injections of sodium lactate or other glucolysis products that must be given as salts, and continuous intravenous infusion necessitates either anaesthesia or prolonged training of the animals. Consequently, we designed a type of experiment with observation periods of 3 hours, so that a single injection of 15 gm of fructose or sodium lactate could supply a large fraction of the total energy required during the period.

An experimental day consisting of four 3-hour periods and one of 12 hours was preceded and followed by control periods of 3 days each, during which 24-hour urines were collected, and analyzed for nitrogen and reducing substance. Body weight was also recorded daily after catheterization. Seven such experiments were carried out on Dog 73, from May 21 to Sept. 1, 1960. Food intake and insulin dosage were the same throughout this period. The relevant data are presented in Table III. Results for a given experiment are found in vertical columns, while corresponding values in different experiments appear on the same horizontal lines.

Two control experiments established the effects of food intake and insulin injections. The hours for feeding and giving insulin were 9 a.m. (0 hour) and 3 p.m. (6 hour). Increased nitrogen output during the 3 to 6 and 9 to 12 hour periods is due to the two meals, given at 0 and 6 hours. Values for blood sugar represent "true sugar", and reflect the effect of the two insulin injections.

Urine nitrogen values during the four 3-hour periods of the experimental day were below the control level during the fructose experiment, lower still during the one with fructose and STH, above the control values in the experiment with growth hormone alone, and unaffected by lactate or lactate and STH. Unfortunately, the results for nitrogen in the experiment with fructose and STH are difficult to interpret. The low value of 0.91 gm during the period from 0 to 3 hours was not due to fructose, since this sugar was given at the end of that period. It could have been due to growth hormone, which was given at the start of the last control day before the experiment, and at 0 hour of the experimental day. However, in the experiment with STH alone, which is identical with the one with fructose and STH throughout the fore period and during the first 3-hour period of the experimental day, no similar decrease in nitrogen output occurred. An adequate interval of 42 days was allowed between these two experiments. Since the nitrogen output of 12.83 gm on the first control day in the experiment with fructose and STH is high, it is also possible that spontaneous replacement of nitrogen took place later, thus complicating the experiment.

On the other hand, fructose was utilized in both of the experiments in which it was given. Reducing sugar in the urine was negligible in these experiments. Blood sugar, and output of acetoacetic and β-hydroxybutyric acids, were lower in the one

Table III

EFFECTS OF FRUCTOSE, GROWTH HORMONE (STH), LACTATE, and STH PLUS EITHER FRUCTOSE OR LACTATE, IN DEPANCREATIZED DOG 73

Time before or after start of experiment	Experiment						
	Control	Fructose	Fructose and STH	STH	Control	Lactate	Lactate and STH
days			*Urine nitrogen in grams*				
3	11.57	10.68	12.83	10.61	10.69	12.48	10.61
2	10.83	10.56	10.55	11.42	11.49	11.27	10.70
1	11.24	10.35	9.28	12.27	11.40	10.20	12.84
hours							
0 - 3	1.58	1.22	0.91	1.63	1.23	1.19	1.00
3*- 6	2.04	1.89	1.81	2.35	1.95	2.03	2.25
6 - 9	1.93	1.71	1.57	2.64	1.91	1.83	1.86
9*- 12	2.33	2.01	1.90	2.72	2.32	2.15	2.26
12 - 24	3.96	3.81	3.99	6.96	5.34	3.59	4.86
hours			*Urine sugar in grams*				
0 - 3	< 1	< 1	< 1	7.5	< 1	< 1	5.2
3*- 6	< 1	2.7	3.9	1.3	< 1	< 1	6.5
6 - 9	< 1	< 1	3.3	4.5	< 1	< 1	8.8
9*- 12	< 1	2.5	3.2	5.5	< 1	< 1	7.3
12 - 24	< 1	< 1	3.7	21.9	< 1	< 1	21.8
hours			*Blood sugar in mg%*				
0	29	72	136	361	38	105	318
3	21	55	146	208	37	98	264
6	63	38	236	314	28	98	333
9	41	49	217	261	29	98	291
12	34	109	223	354	173	82	310
24	46	46	241	309	27	193	
hours			*Plasma lipids in mg%*				
0	928	827	980	1059	925	694	982
3	917	896	1077	1024	872	768	905
6	1024	1474	1011	851	943	917	832
9	908	1141	1080	743	736	671	710
12	821	841	1632	871	691	811	761
24	1027	1529	1275	752	919	823	
Average	937	1118	1176	883	848	781	838
S.D.	± 78	± 318	± 246	± 133	± 107	± 91	± 109
hours			*Acetoacetic acid in urine, mg. per period (as acetone)*				
0 - 3	1.33	0.68	0.42	2.16	0.52	0.44	1.05
3*- 6	1.20	1.08	1.12	1.92	0.78	1.54	4.95
6 - 9	1.04	1.18	1.24	1.73	0.75	0.78	1.37
9*- 12	2.31	2.50	3.20	2.04	0.84	1.92	5.25
12 - 24	2.04	1.48	4.41	13.44	1.44	0.98	7.47
hours			*β-hydroxybutyric acid in urine, mg. per period (as acetone)*				
0 - 3	1.48	2.00	1.92	6.39	1.04	1.54	4.26
3*- 6	2.76	1.80	1.68	5.94	1.14	6.65	21.51
6 - 9	2.30	1.60	4.56	8.93	1.44	2.28	8.33
9*- 12	2.31	2.25	6.10	10.20	1.93	3.84	28.91
12 - 24	3.32	3.68	10.85	41.58	3.68	3.22	34.86
Average			*Weight in kilograms*				
for 7 days	21.33	21.73	21.99	22.46	22.63	22.51	22.70
S.D.	± 0.10	± 0.16	± 0.12	± 0.14	± 0.06	± 0.13	± 0.37

*Asterisk indicates hour when fructose or lactate was given intravenously.

In the 3 experiments in which growth hormone was used, two 5mg doses were given 24 hours apart — at the start of day 1 in the fore-period, and at 0 hour of the day of experiment. The diet, and insulin dose (18 units daily), were constant in all experiments.

with fructose and STH than in the one with STH alone. In general, we were left with the impression that experiments similar to the first three in Table III, performed on a number of depancreatized dogs, might be worth attempting.

Lactic acid output in the urine was determined, but is not recorded in Table III. Growth hormone increased lactic acid output before lactate was injected, in the experiment with lactate and STH, and also increased the loss of injected lactate. During the 3-6 and 9-12 hour periods, lactic acid output exceeded that of preceding control periods by 1.72 and 1.58 gm respectively, in the experiment with lactate alone; and by 2.77 and 2.32 gm in the one with lactate and STH. Since 15 gm of sodium lactate injected at the start of each of these periods is equivalent to 12.05 gm of lactic acid, a large percentage was utilized in both experiments. In the experiments with STH alone and with lactate plus STH, values for blood sugar, and output of acetoacetic and β-hydroxybutyric acids, were quite similar.

Discussion

Since our principal purpose was to determine whether extensive nitrogen storage, such as occurs in normal dogs treated with STH, can be induced in depancreatized ones receiving a constant dose of insulin, a few experiments sufficed to convince us that fasting the animals for a day, or correcting a possible defect in alimentation, were not promising approaches by which this goal might be reached. The experiment with fructose and STH (Table III) was also a negative one, in the sense that extensive storage of nitrogen was certainly not induced. There was, however, a suggestive decrease in nitrogen output that should be studied further.

Important ancillary findings must not be lost sight of as a result of preoccupation with the problem of nitrogen storage. Lower blood sugar values in the experiment with STH and fructose (Table III) than in the one with STH alone; evident utilization of fructose in the depancreatized dog receiving a constant dose of insulin, with or without STH; reduction of ketosis incident to giving STH, by injection of fructose; and effects of STH on utilization of injected lactate, are all findings of interest that merit further investigation.

The relationship between growth hormone and insulin is a very intriguing one, discussed in numerous reviews[13-17] and books. In some respects, the two hormones can be regarded as antagonists. Hyperglycemia, glucosuria, and ketosis of diabetes are abolished by insulin and intensified by STH. Similarly, insulin promotes synthesis and deposition of fat, while STH stimulates formation of tissue abnormally low in fat. On the other hand, both hormones favorably affect nitrogen balance, under certain conditions. Lukens and McCann[13] pointed out that "growth hormone does not act entirely by stimulating the secretion of insulin, because insulin alone does not reproduce all of the effects of growth hormone". The relationship of the two hormones to nitrogen retention is of sufficient interest to justify further discussion. It was of interest in the earliest studies, and still is, in the most recent ones.

Soon after insulin was discovered, the high nitrogen output characteristic of diabetes was reduced with this hormone in depancreatized dogs[18] and in a human diabetic.[19] Some reduction of nitrogen output, much smaller than that obtainable with STH, also occurred in intact dogs kept on constant diets, if they were given sufficient insulin to produce preconvulsive symptoms.[20,21] A direct relationship between insulin dosage and nitrogen balance was demonstrated later, in depancreatized dogs.[22] Anterior pituitary extract reduced the rate of NPN accumulation in nephrectomized dogs, but increased it if the animals were also depancreatized.[23] In depancreatized hypophysectomized cats, treated with STH in the absence of insulin, nitrogen storage did not occur.[24] In depancreatized dogs, STH induced nitrogen storage if the insulin dose was increased sufficiently.[3,6]

While these findings show that insulin is an essential requirement for induction of maximal nitrogen storage with STH, one cannot ascribe this effect of STH entirely to insulin. In fasting rats,[25] dogs,[26] and human subjects[27] insulin increased urea output, and correspondingly reduced tissue amino acids.[25] Anterior pituitary extract, however, reduced nitrogen output and blood NPN in fasting rats.[28] Some storage of nitrogen was induced with impure growth hormone in depancreatized adrenalectomized dogs, with diet and insulin constant.[3] In a human male maintained on a constant diet, even 60 units of insulin per day left nitrogen output unchanged.[29] On the other hand, human growth hormone induces nitrogen storage both in normal subjects and in hypopituitarism.[30] Detectable storage in the absence of insulin has also been reported[31] in depancreatized-hypophysectomized dogs given very small doses (0.05 mg/kg/day) of STH. However, in intact dogs treated with STH nitrogen storage is many times greater.[4-6] Thus an optimal response occurs only when STH and insulin are both present in adequate amounts.

Some recent studies suggest that STH and insulin may regulate different steps in the process of protein biosynthesis. This process consists of a series of reactions,[32,33] and the reacting substances appear in different fractions when cells are disrupted and subjected to differential ultracentrifugation. Cell-free systems which incorporate amino acids into protein can be reconstructed from the isolated components. Korner[34] prepared such systems from microsomal and supernatant fractions of livers from normal and hypophysectomized rats. Incorporation of amino acids into protein was reduced in systems containing microsomes from hypophysectomized rats, whether the supernatant was obtained from normal rats or hypophysectomized ones; incorporation was normal in systems containing microsomes from normal rats, whether supernatant from normal or hypophysectomized animals was used. Adding STH to a defective cell-free system did not improve its amino acid incorporating activity, but giving the hormone to hypophysectomized rats corrected the microsomal defect. Further studies[35] indicated that STH increased production of messenger RNA, and thus the yield of polysomes.

Since protein synthesis is an endergonic process, the conjecture that insulin might be required to assure availability of energy seems logical. Some evidence has been

presented[13] that the effect of insulin on nitrogen retention in the whole animal can be equated with its action on carbohydrate utilization. However, Wool and Krahl[36] found that insulin stimulates amino acid incorporation into muscle of isolated rat diaphragm in the absence of glucose. DeSchepper *et al*[37] agreed that glucose *entry* does not influence this process, but they found that inhibiting endogenous carbohydrate metabolism with 2-deoxy-D-glucose decreased incorporation of C^{14}-labeled leucine into protein of isolated diaphragm and abolished the stimulating action of insulin. Bessman[38] suggested that insulin may facilitate delivery of ATP from its generation site to the amino acid activating system. Thus more ATP could be made available for protein synthesis, without increasing either glucolysis or oxidation of glucose.

Wool[39] postulated that insulin initiates transcription — a term applied to formation of messenger RNA reflecting the structure of a given gene, and subsequent "translation" of the message into primary structure of a protein. Wool and Munro had shown that insulin, under some conditions, stimulated formation of messenger RNA. However, Wool and Moyer[40] found that actinomycin, which blocks DNA-directed RNA synthesis, did not prevent insulin from stimulating amino acid incorporation into muscle of isolated rat diaphragm. They made the additional suggestions that insulin might be involved in "translation" of the message, or that the hormone may affect the stability, or life span, of messenger RNA.

Although administration of STH or insulin may result in *net* synthesis of protein, neither hormone stimulates indiscriminate synthesis of all proteins. In experiments with STH, the writer and associates found that some enzyme activities increased, others decreased, and still others remained unaltered.[41] MacLean and Gurney[42] found that STH reduced the activity of urea cycle enzymes, particularly argininosuccinate synthetase. Insulin restores some activities that are reduced in diabetes,[43] and suppresses others that are increased.[44] Even if insulin and STH both stimulate production of messenger RNA, their role could be quite different, since many types of messenger RNA must be required to direct synthesis of body proteins.

Summary

1. In a few experiments on normal and insulin-treated depancreatized dogs, no definite effect of growth hormone (STH) on replacement of nitrogen lost during a single day of fasting was apparent.
2. Failure to induce nitrogen storage with STH in depancreatized dogs receiving a constant amount of food and insulin is probably not due to deficient alimentation.
3. In experiments with and without STH, fructose or sodium lactate injected intravenously were largely utilized by a depancreatized dog receiving constant amounts of food and insulin. STH reduced lactate utilization somewhat. Diminished nitrogen output, in the experiment with fructose and STH, was suggestive but not convincing.
4. The relationship of insulin and STH to nitrogen storage is discussed in the light of old and recent experiments.

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