Effect of ACTH and Insulin on AIB Accumulation in Diaphragm Muscle and Adrenal *in vivo*.*† (26268)

J. EICHHORN, E. SCULLY, I. D. K. HALKERSTON AND O. HECHTER
Worcester Foundation for Experimental Biology, Shrewsbury, Mass.

It has been shown previously that adrenocorticotropin (ACTH) produces changes in sugar permeability in the adrenal which are similar to those produced by insulin in muscle(1,2). In both these tissues of the hypophysectomized rat, the "non-utilizable" pentose, D-xylose, is excluded from a major fraction of the cell water; following ACTH administration the intracellular distribution of D-xylose is markedly increased in adrenal but not in muscle, whereas insulin acts upon sugar transfer in muscle but not adrenal. In addition, insulin also increases amino acid transport in isolated rat diaphragm, as evaluated by use of the "non-utilizable" amino acid α-aminoisobutyrate (AIB)(3). To study further the effects of these hormones on amino acid transport, the present work examines the distribution of AIB in the adrenal and diaphragm of functionally-nephrectomized rats before and after hypophysectomy and of hypophysectomized-nephrectomized rats receiving exogenous ACTH, insulin, corticosterone, or growth hormone.

Methods. To establish appropriate experimental conditions, the distribution of AIB between tissues and plasma was determined at 2 time intervals after administration of AIB, and at a variety of plasma concentrations of AIB. Intact or hypophysectomized (45-50 hr) rats of the Sprague-Dawley strain weighing 120-160 g were used. The renal pedicles were ligated under avertin anesthesia immediately before subcutaneous injection of α-AIB-1-C^{14} (Volk). This route of AIB administration and functional nephrectomy was employed to achieve a relatively constant plasma concentration during the experiment. Qualitatively similar, but more variable results were obtained when the kidneys were not ligated. Dosage and specific activity of AIB was varied to achieve a range of plasma concentrations extending from 0.3 to 850 μg/ml.

AIB determinations were carried out on adrenal, diaphragm, and plasma either 1.5 or 5 hr after AIB administration. After these intervals, the rats were reanesthetized (nembutal), blood was drawn from the aorta into a heparinized syringe. While plasma was being obtained without delay by one of us, others excised the tissues immediately, which were extracted for AIB with hot water in a boiling water bath for 15 min, as employed previously(1). Results obtained by this extraction method were similar to those obtained using the acetic acid-homogenizing method of Noall *et al.*(4). Moreover, it was established that after extracting tissues with hot water, no significant amount of radioactivity could be extracted following homogenization in water or acetic acid; the boiling water procedure extracts much less solid material from tissues than the acetic acid homogenization method, and consequently self-absorption is less in the former method. Tissue extracts and diluted plasma (1:50) were plated and assayed for radioactive content in a windowless flow gas chamber.

To determine whether the radioactivity extracted and measured in this manner is unmodified AIB, pooled extracts from adrenal, diaphragm, and liver were treated as follows. The radioactivity was separated from the bulk of the non-radioactive tissue extractives by applying the aqueous extracts to Whatman #1 paper, and eluting the radioactivity using 95% EtOH as mobile phase. The ethanolic overflow collected for 2-3 days contained all of the radioactivity, and was then chromatographed in the n-butanol: water:acetic acid system (5:5:1) for 8 hours. In all cases, only one radioactive peak of Rf value corresponding to pure AIB was ob-

* Supported in part by Nat. Inst. Health, Nat. Science Foundation, and Commonwealth Fund grants.

† Authors thank Dr. C. H. Li for bovine growth hormone, and Drs. J. D. Fisher and W. F. White of Armour for Corticotropin A.

served. Quantitative elution of the paper revealed that of the total count recovered, 86-90% was present in the AIB zone from adrenal tissue, and 98-100% in those zones from liver, and diaphragm. The data indicate that radioactivity extracted from these tissues primarily represents unchanged AIB, but does not exclude the possibility that a small fraction of the AIB accumulated by adrenal has been modified.

Considering, therefore, that metabolism of AIB does not occur to a significant extent, distribution of AIB between tissues and plasma in these experiments is expressed directly as a ratio, counts per min per g wet tissue/counts per min per ml plasma. AIB distribution in intracellular water (AIB ml cell water/AIB ml plasma) was calculated by measuring inulin space as described previously(1) and by determining total water per g tissue. Total water for the tissues was determined by drying to constant weight at 105°. The intracellular fluid volume was assumed to be the difference between total water and inulin space, assuming that inulin distributes in the extracellular fluid exclusively, and is equilibrated with the plasma.

In those cases where the effect of exogenous hormones was tested, the hormones were administered to hypophysectomized rats just after AIB was administered subcutaneously; in all cases AIB distribution values were obtained 90-100 min after AIB was given in an amount necessary to achieve AIB plasma concentrations ranging from 0.3 to 10 μg/ml. The hormones studied, with the dosage per 100 g body weight, were as follows: 375mU corticotropin A (Armour) given as a single intravenous injection; 30 μg bovine growth hormone (Li), given intravenously; corticosterone, 0.7 mg in a divided dose in 10% alcohol, one-half given intravenously and the remainder subcutaneously; and 1 U insulin (Iletin, Lilly) injected intraperitoneally. In the experiments with insulin, to prevent hypoglycemia, 100 mg glucose per 100 g body weight was injected along with the insulin; controls for this group received 25 mg glucose per 100 g body weight.

Results. Fig. 1 graphically presents the results obtained in adrenal and diaphragm of functionally nephrectomized rats at the 2 time intervals employed, 1.5 and 5 hr. Distribution ratio of AIB in these tissues of both hypophysectomized and pituitary-intact rats is given at plasma concentrations of AIB ranging from about 0.3 to 850 μg/ml. It is clear that hypophysectomy alters distribution of AIB in both tissues in 2 respects. First, level of AIB uptake is reduced in the absence of the pituitary, the extent of the difference being dependent on plasma AIB concentration. Secondly, the tissues of hypophysectomized rats differ from intact in response to increases in plasma AIB concentration. In the hypophysectomized rat, uptake of AIB by these tissues increases in proportion to plasma concentration so that in this case the AIB distribution ratio is relatively constant over the entire range of plasma concentrations studied. In the intact rat, this constancy of distribution appears to be manifest only at the lower levels of plasma AIB concentration, below 10 μg/ml. Above this concentration, in contrast to the findings in the hypoxed rat, AIB distribution declines as plasma levels are progressively increased, so that at plasma concentrations of about 800-850 μg/ml, distribution of AIB in these tissues is reduced to the level observed in the hypophysectomized rats. At the lower plasma concentrations of AIB, to about 10 μg/ml, the decline in distribution after hypophysectomy is nearly identical in both tissues, being 41-44% of the values in the intact.

Table I presents the average of the tissue distribution values obtained in both groups at plasma concentrations of AIB less than 10 μg/ml, where AIB distribution values in both groups were independent of plasma AIB level. Table I also presents the calculated ratio of AIB concentration in intracellular to extracellular water. It is seen that in the adrenal the ratio of AIB_{in}/AIB_{out} is 2.9 at both time intervals in the hypophysectomized rat, while this ratio is 7.0 and 7.3 at 1.5 and 5 hr, respectively, in the pituitary intact rat, indicating that in the adrenal the accumulation of AIB attains a steady state by 1.5 hr. In diaphragm, however, maximal distribution was not achieved in 1.5 hr; intracellular distribution ratio increases from 0.8 at 1.5 hr

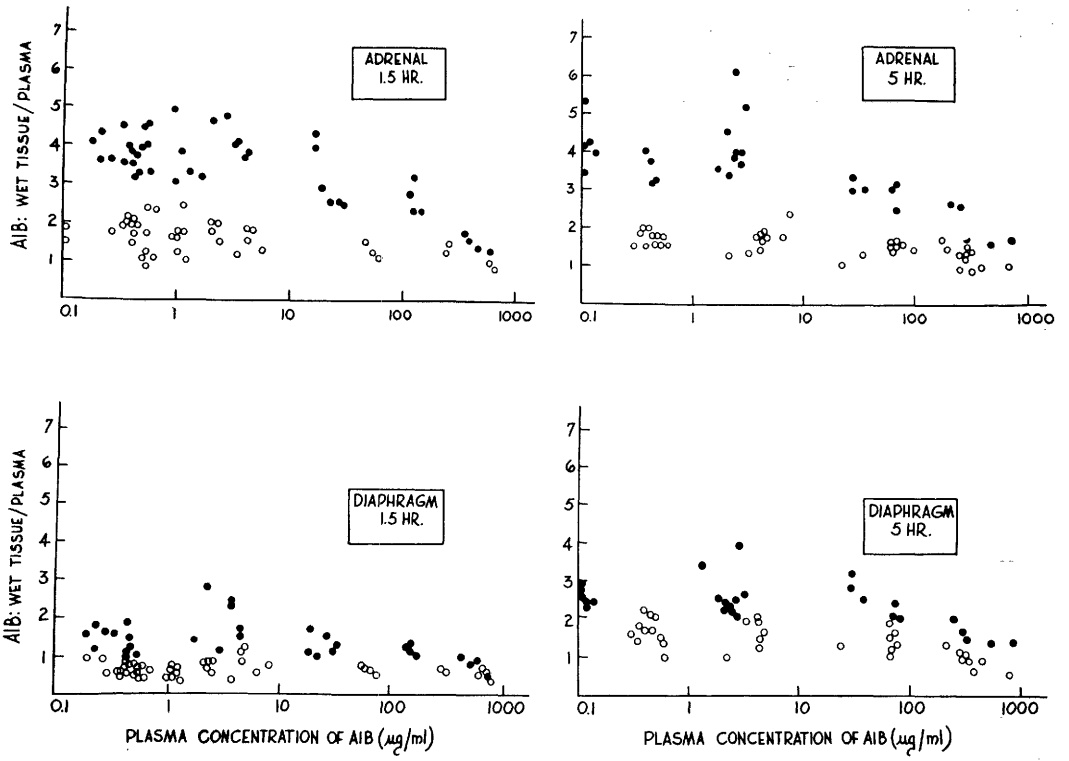

FIG. 1. Distribution ratio of AIB (counts per min. per g wet tissue/counts per min. per ml plasma) for adrenal and diaphragm muscle of hypophysectomized (○) and pituitary intact rats (●) at 1.5 or 5 hr after inj. of AIB, plotted against plasma concentration of AIB (abscissa, logarithmic scale).

to 2.5 at 5 hr in hypophysectomized, and from 2.3 at 1.5 hr to 4.1 at 5 hr in pituitary intact rats. The relationship of the pituitary intact to the hypophysectomized group, and qualitative pattern of AIB distribution, however, are similar at both time intervals.

The effects on AIB transport by various hormones were studied at AIB plasma levels of 10 μg/ml or less because: (a) AIB distribution ratios in adrenal and diaphragm of both hypophysectomized and pituitary-intact rats remain relatively constant, when AIB plasma level is within this range; thus any changes in plasma AIB concentrations associated with a hormonal effect would have minimal influence on AIB distribution ratio and (b) the differences between hypophysectomized and pituitary-intact rats are most clearly revealed in this range, and diminish as AIB plasma level is progressively increased. The effects of various hormones on AIB distribution in the adrenal and dia-

TABLE I. Distribution of AIB between Tissues and Plasma of Intact and Hypophysectomized Rats, 1.5 and 5 Hr after AIB Administration.

Time interval (hr)	Type animal	% distribution of AIB* [(wet tissue/plasma) × 100]		Distribution ratio of AIB (intracellular water/plasma)	
		Adrenal	Diaphragm	Adrenal	Diaphragm
1.5	Intact	400 ± 10 (28)	157 ± 12 (18)	7.0	2.3
	Hypox.	177 ± 8 (34)	65 ± 4 (36)	2.9	.8
5	Intact	417 ± 19 (18)	266 ± 11 (16)	7.3	4.1
	Hypox.	175 ± 6 (21)	166 ± 9 (18)	2.9	2.5

* Mean, stand. error, and No. of observations, obtained at plasma conc. of AIB less than 10 μg/ml.

TABLE II. Influence of Various Hormones on Distribution of AIB in Adrenal and Diaphragm of Functionally-Nephrectomized, Hypophysectomized Rats.

Treatment (dosage/100 g body wt)	% distribution of AIB (total wet tissue)*	
	Adrenal	Diaphragm
Saline (0.9%)	177 ± 8 (33)	65 ± 4 (36)
Corticotropin A, Armour (375 mU)	298 ± 23 (10)	90 ± 8 (8)
Growth hormone, Li (30 μg)	176 ± 8 (5)	54 ± 5 (5)
Alcohol (10%)	178 ± 8 (5)	57 ± 7 (5)
Corticosterone (0.7 mg in 10% alcohol)	114 ± 14 (5)	40 ± 6 (5)
Glucose (25 mg)	192 ± 14 (5)	72 ± 7 (5)
Insulin (1 U), glucose (100 mg)	291 ± 11 (16)	420 ±110 (16)
Insulin (1 U) + Corti A (375 mU)	429 ± 18 (5)	510 ± 69 (5)

* Values are mean, stand. error, and No. of observations, obtained 1.5 hr after administration of AIB and hormones, at plasma concentration of AIB less than 10 μg/ml.

phragm of functionally-nephrectomized, hypophysectomized rats obtained under these conditions are shown in Table II. Intravenous administration of corticotropin A significantly increased distribution in both tissues, giving values 68% above hypophysectomized in the adrenal ($p = <.001$) and 38% higher in diaphragm ($p = <.01$. Growth hormone, in the dosage used (30 μg/100 g) had no effect on AIB distribution in these tissues of the hypophysectomized rat. Corticosterone reduced distribution in adrenal 36% below that of the alcohol control ($p = <.01$), and tended to reduce values in diaphragm. With insulin, adrenal distribution increased 52%, and diaphragm increased 480% ($p = <.001$, in both cases). When corticotropin A was given together with insulin, a further significant increase in adrenal occurred ($p = <.001$) giving an average value equivalent to that in the intact. This combined treatment also tended to elevate distribution in diaphragm above that with insulin alone, but this change is not statistically significant.

Discussion. The present findings describing the effects of insulin and ACTH on transport of AIB in adrenal and diaphragm muscle of hypophysectomized rats contrast sharply in 2 respects with previous studies on sugar permeability as evaluated with D-xylose(1). First, D-xylose was excluded from a major fraction of the cell water of both adrenal and diaphragm, whereas AIB is accumulated against an apparent concentration gradient in both tissues. Secondly, the hormones specifically increased D-xylose transfer in their target organs; ACTH influenced adrenal but not muscle, while insulin affected muscle but not adrenal. With AIB, on the other hand, either ACTH or insulin produce increased accumulation of AIB in both adrenal and diaphragm muscle. The responses in adrenal to ACTH and insulin are equivalent, but the increase in muscle AIB accumulation caused by insulin is greater than that produced by ACTH. The mechanisms of sugar transfer and amino acid transport are obscure, and accordingly the basis of the observed differences in hormone specificity cannot be evaluated. Part of the difficulty may relate to the fact that the adrenal represents 2 tissues and the effects observed may involve either cortex or medulla, or both. While it is reasonable to expect that ACTH acts on the adrenal cortex, the locus of insulin action in the present adrenal studies remains unresolved. However, it may be significant that insulin is reported to increase adrenal gland weight of hypophysectomized rats(5) and pigeons(6); in the latter case, the changes caused by insulin were more pronounced in the cortex and were cytologically identical to those produced by ACTH.

The study with growth hormone indicates that it is unlikely the observed effects of ACTH were due to trace amounts of growth hormone in the preparation employed, since growth hormone alone, injected under conditions identical to ACTH, had no effect on AIB levels in adrenal or diaphragm muscle of hypophysectomized rats.‡ Similarly, the results with corticosterone suggest that the stimulatory effect of ACTH was not due to

‡ The conditions employed in this study are not necessarily optimal for studying the effect of growth hormone on AIB transport, since effects might have been obtained had different conditions been employed. Thus, it has been reported that growth hormone added *in vitro* increases AIB uptake by diaphragm muscle excised from rats hypophysectomized 14 days or longer(7).

a secondary effect of corticoid released under ACTH stimulation, since corticosterone significantly inhibited AIB uptake in adrenal and tended to lower AIB in diaphragm. Nor does it seem probable, in view of the results with corticosterone, that the effects of ACTH are due to stimulation of the release of endogenous insulin by corticoids released following ACTH administration. This is supported by the fact that ACTH given under similar conditions had no effect on D-xylose transfer into diaphragm muscle(1).

It should be noted that a single large intravenous dose of corticotropin A does not restore adrenal AIB distribution to that found in pituitary-intact rats. This might be due to the particular mode of administration of ACTH employed in this study, to a decreased responsivity of the adrenal to ACTH following hypophysectomy, or to lack of an additional factor, which could be either pituitary or extra-pituitary in origin. The latter possibility should be considered since it was found that when insulin was given together with ACTH to hypophysectomized rats, AIB distribution was raised to the pituitary-intact level.

The significance of studies with AIB is related to the assumption that the behavior of AIB serves as an index of the transport of natural amino acids, and further that a change in the availability of amino acid to the interior of cells may influence protein synthesis and/or amino acid metabolism. On this basis, the present studies suggest that ACTH increases the uptake of amino acids by adrenal cells, and that this effect may be important in the increased protein synthesis and growth caused by ACTH in adrenal cortex. Insulin also appears to be involved in amino acid transport in adrenal as well as muscle, thus providing additional evidence that this hormone may have a role in adrenal maintenance and growth.

Summary. Hypophysectomy causes a decline in accumulation of the amino acid analogue, α-aminoisobutyrate (AIB), in adrenal and diaphragm muscle of functionally nephrectomized rats. Either Corticotropin A or insulin increase AIB accumulation to an equivalent extent in adrenal of hypophysectomized rats; both hormones also increase AIB uptake in diaphragm muscle, but insulin is more effective in this tissue. The combined influence of these hormones on both tissues is greater than either alone. The significance of these observations for protein synthesis and growth in the adrenal are discussed.

1. Eichhorn, J., Halkerston, I. D. K., Feinstein, M., Hechter, O., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 515.

2. Levine, R., *Survey Biol. Progr.*, 1957, v3, 185.

3. Kipnis, D. M., Noall, M. W., *Biochim. et Biophys. Acta*, 1958, v28, 226.

4. Noall, M. W., Riggs, T. R., Walker, L. M., Christensen, H. N., *Science*, 1957, v126, 1002.

5. Salter, J. M., Davidson, I. W. F., Best, C. H., *Canad. J. Biochem. Physiol.*, 1957, v35, 913.

6. Miller, R. A., Riddle, O., PROC. SOC. EXP. BIOL. AND MED., 1941, v47, 449.

7. Kostyo, J. L., Hotchkiss, J., Knobil, E., *Science*, 1959, v130, 1653.

Received November 9, 1960. P.S.E.B.M., 1961, v106.

Observations on Effect of 3-Methylcholanthrene in Scorbutic Guinea Pigs. (26269)

G. R. MARTIN,* HAROLD M. FULLMER AND J. J. BURNS†

National Institute of Dental Research and National Heart Institute, N.I.H., P.H.S., U. S. Dept. of H.E.W., Bethesda, Md., and N. Y. University Research Service, Goldwater Memorial Hosp., N. Y.

Previous studies have demonstrated a marked increase in synthesis of L-ascorbic acid following administration of 3-methylcholanthrene (3-MC) to rats(1,2). Since guinea pigs lack the ability to synthesize L-

* Research Associate, Am. Dental Assn.

† Present address: Wellcome Research Laboratory, 1 Searsdale Road, Tuckahoe, N. Y.