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The Effect of Adrenocorticotrophin on Protein Degradation in Rat Adrenal Gland and Liver

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The rate of adrenal protein degradation appears to be slower in rats to which ACTH (adrenocorticotrophin) has been chronically administered. As measured by the exponential decay of radioactively labelled adrenal protein *in vivo*, the mean half-lives of total protein and of mitochondrial, microsomal and 18000*g*-supernatant protein were significantly longer in ACTH-treated animals. Experiments in which either [^{3}H]leucine or $NaH^{14}CO_3$ was used to label proteins showed that of the fractions studied, the effect on mitochondrial protein degradation was most pronounced. The half-lives of the same subcellular fractions in rat liver were not affected by ACTH. The possibility that the results might have been caused by changes in radioisotope reutilization and pool size is discussed.

Evidence has been accumulating that alterations in the rate of protein degradation as well as synthesis may play an important role in cell and organelle growth. In muscle, factors which influence cellular growth have been shown to cause changes in the rate of total protein degradation as well as in the cell's protein-synthetic rate (Goldberg, 1973). In liver, the phenobarbital-induced proliferation of smooth endoplasmic reticulum encompasses both an increase in the rate of synthesis and a decrease in the rate of breakdown of membrane protein (Kuriyama *et al.*, 1969; Arias *et al.*, 1969).

Many trophic hormones are known to stimulate an increase in the concentration of organelles within their target cells as well as overall growth of target tissues. For example, human chorionic gonadotrophin causes a dramatic proliferation of smooth endoplasmic reticulum in Leydig cells of the testis (Murakami & Tonutti, 1966), and ACTH (adrenocorticotrophin) increases the concentration of both mitochondria and smooth endoplasmic reticulum in the adrenal cortex (Nussdorfer *et al.*, 1971). There is some evidence that the mechanism by which ACTH causes growth of adrenal cells and their organelles involves alterations in both the rate of synthesis and the rate of degradation of adrenocortical proteins. Purvis *et al.* (1972, 1973) reported that the increase in the contents of adrenal mitochondrial hydroxylation components after ACTH administration exhibits kinetics consistent with their possible stabilization on the inner mitochondrial membrane. Similar kinetics have been described by Pfeiffer *et al.* (1972).

The present paper reports the effect of ACTH on the degradation of total protein and of the protein within subcellular fractions of rat adrenal and liver.

Experimental

To measure protein degradation *in vivo* in adrenal and liver fractions, labelled substrates were injected into male rats (CD strain; Charles River Breeding Labs., Wilmington, Mass., U.S.A.) weighing 100g. In one experiment, each rat received 220μCi of L-[4,5-^{3}H]leucine (New England Nuclear Corp., Boston, Mass., U.S.A.; sp. radioactivity 33Ci/mmol) intraperitoneally in 0.5ml of 0.9% NaCl. In another experiment, 400μCi of $NaH^{14}CO_3$ (New England Nuclear Corp.; sp. radioactivity 46mCi/mmol) in 0.5ml of 0.9% NaCl was administered intraperitoneally. At 24h after injection of the labelled compound certain rats were randomly selected for chronic ACTH treatment. To maintain circulating ACTH at a constant high concentration, a long-acting ACTH preparation (ACTHar gel; Armour Laboratories, Chicago, Ill., U.S.A.), 5i.u. per rat, subcutaneous, was administered every 12h for the number of days indicated in the Figures. The remaining animals were left untreated until death.

At the times indicated in the figures, groups of rats were killed by decapitation and the liver and adrenals excised. Adrenals were carefully cleaned of all connecting fat and sliced in half. Both adrenals and finely minced liver were homogenized in 0.25M-sucrose by using a Teflon–glass tissue grinder. Liver mitochond-

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ria were prepared by differential centrifugation as described by Greenspan & Purvis (1968). Adrenal fractions were prepared by differential centrifugation as described by Purvis *et al.* (1973), except that the mitochondrial fraction was washed two times with 10–20ml of 0.25M-sucrose and the microsomal fraction was washed once with 10–20ml of 0.15M-KCl.

The amount of ^{14}C or ^{3}H present in the protein of the fractions studied was determined by the filter-disc technique as described by Mans & Novelli (1961). Duplicate samples of tissue fractions were placed on Whatman 3MM filter discs, air dried, and placed in cold 10% (w/v) trichloroacetic acid, in which they remained for 1–4 days. After washing in cold 5% (w/v) trichloroacetic acid, 5% (w/v) trichloroacetic acid at 90°C, cold 5% (w/v) trichloroacetic acid, ethanol–ether (1:1, v/v) at 37°C, ethanol–ether (1:1, v/v) at room temperature (24°C), and ether (three times), the filter discs were placed in vials containing 10ml of Aquasol (New England Nuclear Corp.) and counted for radioactivity in a Packard Tri-Carb liquid-scintillation spectrometer.

Radioactivity in adrenal protein was expressed in terms of total radioactivity (c.p.m./adrenal pair), so that untreated and ACTH-treated adrenals, which differed greatly in weight gained after label was injected, could be compared. In this way, all the radioactivity remaining in the protein at various times could be determined irrespective of the quantity of newly synthesized non-radioactive protein added to the fraction after labelling of the protein. The radioactivity of the liver protein was expressed as c.p.m./mg of protein of the fraction examined. The mean half-lives ($t_{\frac{1}{2}}$) of the proteins were determined from semi-logarithmic plots of total radioactivity (for adrenals) or specific radioactivity (for liver) versus time. Regression coefficients (r) and equations for the best straight line were calculated by the least-squares method and are listed in the figure legends. In all decay curves, the day-1 point is common to slopes for both the ACTH-treated and untreated rats.

A two-sided t-test (Armitage, 1971) was run to determine whether treated and untreated slopes in adrenal experiments were significantly different from each other. A computer program for this analysis was prepared by Dr. Eddington Lee of the Laboratory of Human Reproduction and Reproductive Biology, Harvard Medical School. The results are shown in Table 1.

To determine the amount of trichloroacetic acid-soluble radioactivity present in the adrenal and liver, portions of the 18000g supernatants were deproteinized with equal volumes of 20% (w/v) trichloroacetic acid. After pelleting of the precipitate, obtained by centrifugation at 5000g for 10min, 0.5ml samples of the trichloroacetic acid supernatants were counted for radioactivity in Aquasol as described above.

Protein concentrations were determined by the method of Lowry *et al.* (1951) with bovine albumin (fraction V; Sigma Chemical Co., St. Louis, Mo., U.S.A.) as the standard.

Results and Discussion

Growth of the adrenal cortex caused by chronic ACTH treatment is extensive and essentially linear over a 10-day period. Within 10 days, the adrenals from treated animals increased in weight from 27mg to 70mg/adrenal pair, whereas those of the untreated controls increased to 31mg/adrenal pair. Such adrenal hypertrophy is known to involve increases in the synthesis of both RNA and protein (Garren, 1968). The possible role of changes in the rates of protein degradation in the regulation of ACTH-mediated adrenal growth has not been examined previously.

[^{3}H]*Leucine as label*

In experiments in which [4,5-^{3}H]leucine was used to label proteins *in vivo*, mean protein degradation in all the adrenal fractions studied appeared to be slower during chronic ACTH administration (Fig. 1). Regression lines drawn according to least-squares analysis revealed that the adrenal mitochondrial fraction (Fig. 1*b*) exhibited the most pronounced difference in the rate of protein degradation, with the mitochondrial protein from treated animals almost completely stabilized over the 10 days of experimental observation ($t_{\frac{1}{2}}$>125 days for treated rats, cf. 8.3 days for untreated). A second experiment in which chronic ACTH treatment was started 2 days after [^{3}H]leucine injection produced a similar though less pronounced effect of ACTH on mitochondrial protein degradation ($t_{\frac{1}{2}}$ = 22 days for treated, cf. 4.6 days for untreated). The homogenate (Fig. 1*a*), which represents total proteins from the tissue, exhibited slower protein degradation in treated ($t_{\frac{1}{2}}$ = 7.0 days) than in untreated animals ($t_{\frac{1}{2}}$ = 4.6 days based on the first four experimental points, or 5.2 days based on all five points), as did the 18000g supernatant (Fig. 1*c*), which is primarily a combination of the microsomal fraction and the soluble cytosol material ($t_{\frac{1}{2}}$ = 6.2 days for treated, cf. 4.3 days for untreated).

In experiments in which liver protein degradation was examined by using [^{3}H]leucine to label the protein, ACTH treatment caused no measurable difference in the degradation of protein in any fraction studied. The half-lives in the presence or absence of administered ACTH were 4.6 days for total homogenate protein, 5.6 days for mitochondrial protein, and 4.4 days for protein of the 18000g supernatant. Presumably ACTH exerts biochemical effects on the liver through enhanced adrenal glucocorticoid secretion. Glucocorticoid concentrations were in fact increased in ACTH-treated animals, as evidenced by a decrease in thymus weight, but such elevated values

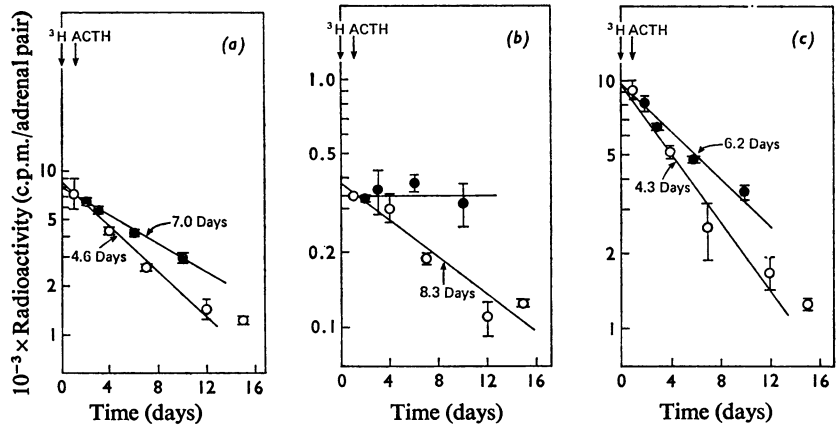

Fig. 1. *Effect of ACTH on the mean half-life of adrenal protein in homogenate (a), mitochondrial fraction (b) and 18000g supernatant (c), determined by protein labelling in vivo*

L-[4,5-^{3}H]Leucine (220μCi) was injected into each of 36 rats at day 0. Then 24h later, ACTH treatment of 16 of the rats was started, the remaining rats receiving no further treatment. Each point represents the average of two separate determinations ±range, two pooled adrenal pairs being used for each determination. Half-lives, which were determined graphically from the decay of total acid-insoluble radioactivity/adrenal pair plotted against time, are specified by the arrows along the slopes. ●, ACTH-treated; ○, untreated. Least-squares analysis. (*a*) ACTH-treated: $r=-0.99$, $\log y=-0.04x+0.90$; untreated: $r=-0.99$, $\log y=-0.06x+0.90$ (based on first four points); $r=-0.98$, $\log y=-0.06x+0.86$ (based on all points). (*b*) ACTH-treated: $r=-0.26$, $\log y=-0.002x+0.55$; untreated: $r=-0.95$, $\log y=-0.04x+0.57$. (*c*) ACTH-treated: $r=-0.98$, $\log y=-0.05x+0.99$; untreated: $r=-0.98$, $\log y=-0.07x+0.99$.

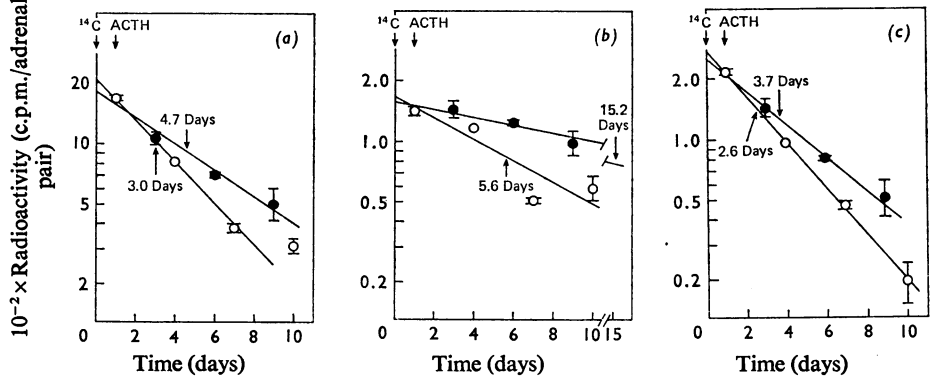

Fig. 2. *Effect of ACTH on the mean half-life of adrenal protein in the homogenate (a), mitochondrial fraction (b) and microsomal fraction (c)*

$NaH^{14}CO_3$ (400μCi) was injected into each of 56 rats at day 0. Then 24h later, ACTH treatment of 24 of the rats was started, the remaining rats receiving no further treatment. Each point represents the average of two separate determinations ±range, four pooled adrenal pairs being used for each determination. Half-lives, which were determined as described in Fig. 1 and the Experimental section, are specified by arrows along the slopes. ●, ACTH-treated; ○, untreated. Least-squares analysis. (*a*) ACTH-treated: $r=-0.97$, $\log y=-0.06x+1.27$; untreated: $r=-0.99$, $\log y=-0.11x+1.33$ (based on the first three points); $r=-0.98$, $\log y=-0.09x+1.27$ (based on all points). (*b*) ACTH-treated: $r=-0.93$, $\log y=-0.02x+0.20$; untreated: $r=-0.89$, $\log y=-0.05x+0.23$. (*c*) ACTH-treated: $r=-0.99$, $\log y=-0.08x+0.41$; untreated: $r=-0.99$, $\log y=-0.12x+0.45$.

apparently have no effect on hepatic protein degradation. Kimberg & Loeb (1972) have tested the effect of administered cortisone on liver mitochondrial protein turnover and have also found no difference in treated and untreated mitochondria.

[^{14}C]Bicarbonate as label

The use of [^{3}H]leucine as a label for protein-degradation studies has been criticized because of the potential problem of radioisotope reutilization (Arias *et al.*, 1969). To circumvent the problems and criticisms inherent in using a reutilizable substrate, many investigators have turned to [^{14}C]guanidino-labelled arginine. In tissues with high arginase activities, the labelled carbon is transferred to urea and is therefore considered nearly non-reutilizable. Although liver and kidney have high arginase activities, the rat adrenal has no measurable arginase activity (J. A. Canick, unpublished work), thus eliminating the possibility of using [*guanidino*-^{14}C]arginine in these studies.

As an alternative label we chose $NaH^{14}CO_3$, shown by Millward (1970) to label muscle protein maximally and to reach very low specific radioactivity in the intracellular amino acid pools by 6h after injection.

Experiments in which proteins were labelled by using $NaH^{14}CO_3$ as precursor confirmed the [^{3}H]-leucine experiments in demonstrating a difference between the mean degradation rates of adrenal protein in ACTH-treated and untreated rats (Fig. 2). Again, the effect of ACTH on protein degradation was most pronounced in the mitochondrial fraction ($t_{\frac{1}{2}} = 15.2$ days in treated rats, cf. 5.6 days in untreated; Fig. 3*b*). The half-life of total adrenal protein (Fig. 3*a*) was 4.7 days in treated animals and 3.0 days in untreated animals ($t_{\frac{1}{2}} = 3.0$ days based on the first three experimental points; $t_{\frac{1}{2}} = 3.6$ days based on all four points). Adrenal microsomal fractions from treated animals also exhibited slower protein degradation than those from normal ($t_{\frac{1}{2}} = 3.7$ and 2.6 days respectively; Fig. 3*c*). Because $NaH^{14}CO_3$ is reutilized to a lesser extent than [^{3}H]leucine, the mean half-lives calculated for the proteins in the comparable fractions were shorter than in those based on experiments with [^{3}H]leucine (Table 1).

The problem of radioisotope reutilization

Results such as we have obtained could have been caused if the label were reutilized more effectively by rapidly growing ACTH-treated tissue than by slowly growing control tissue. Such increased reutilization can stem from either a higher specific radioactivity of the precursor pool or a higher rate of reincorporation into protein, or both.

We have not yet measured directly the precursor pool specific radioactivity. Supporting data on the disappearance of acid-soluble radioactivity lends indirect support to the conclusion that ACTH causes a real change in the rate of degradation of adrenal protein. After [^{3}H]leucine administration, acid-soluble radioactivity in both the adrenal and liver decreased exponentially, at the same rate in ACTH-treated and untreated rats. The half-life of acid-soluble ^{3}H-labelled material, measured between 1 and 15 days, was approx. 5 days in all cases. In rats which received $NaH^{14}CO_3$, the decrease of acid-soluble radioactivity to low values was very rapid (Fig. 3). Although the profile of labelled molecules within the cell after administration of $NaH^{14}CO_3$ was not determined, it is unlikely that all of the acid-soluble ^{14}C is in amino acids available for reutilization in protein synthesis.

Control of adrenal growth

As pointed out by Berlin & Schimke (1965), the steady-state concentration of a protein (P) is deter-

Table 1. *Calculated mean half-lives of adrenal proteins based on experiments with [^{3}H]leucine and $NaH^{14}CO_3$*

Half-lives were determined graphically and were taken from Figs. 1 and 2. *P* is the probability of a significant difference between slopes of curves for the ACTH-treated and untreated rats (see the Experimental section). n.d., not determined.

Fraction	Label	Half-life (days)		*P*
		ACTH-treated	Untreated	
Homogenate	[^{3}H]Leucine	7.0	4.6	<0.02*
	$NaH^{14}CO_3$	4.7	3.0	<0.01†
Mitochondria	[^{3}H]Leucine	>125.0	8.3	<0.01
	$NaH^{14}CO_3$	15.2	5.6	<0.06
18000*g* Supernatant	[^{3}H]Leucine	6.2	4.3	<0.14
	$NaH^{14}CO_3$	n.d.	n.d.	—
Microsomal fraction	[^{3}H]Leucine	n.d.	n.d.	—
	$NaH^{14}CO_3$	3.7	2.6	<0.01

* 15-day point on 'untreated' curve was not included in this calculation. If included, $0.1 > P > 0.05$.
† 10-day point on 'untreated' curve was not included in this calculation. If included, $0.1 > P > 0.05$.

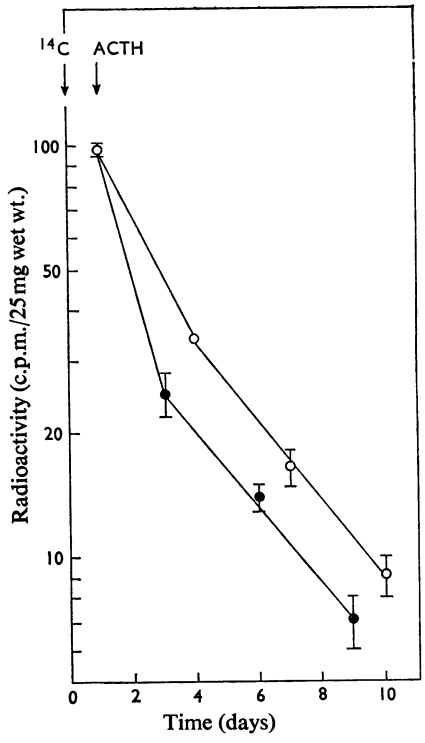

Fig. 3. *Effect of ACTH on the concentration of acid-soluble radioactivity in the adrenal*

$NaH^{14}CO_3$ was administered as described in Fig. 2. ^{14}C contents were measured in deproteinized 18000*g* supernatants and expressed graphically as c.p.m./25mg wet wt. of adrenal tissue plotted against time. The points represent the average of two separate determinations±range, two adrenal pairs being used for each determination. ●, ACTH-treated; ○, untreated.

mined both by its rate of synthesis (k_s) and rate of degradation (k_d):

$$P = k_s/k_d = k_s \cdot t_{\frac{1}{2}}/\ln 2$$

Therefore without considering the increase in the rate of protein synthesis, which has been shown to take place in the presence of administered ACTH, the experimentally determined changes in the rates of degradation alone can account for appreciable increases in protein content (Table 2). The theoretical increase in the amount of mitochondrial protein is most dramatic (171% or more), since it is the result of the largest change in degradation rate. A comparison of these with the actual increases observed in the fractions after 10 days of ACTH treatment (Table 2) suggests that a decrease in protein breakdown must have been accompanied by an increase in protein synthesis.

Since protein and phospholipid are the major components of biological membranes, it is interesting to note in conjunction with our findings that adrenal phospholipid turnover has been shown to be altered by chronic ACTH administration (Ichii *et al.*, 1971). Although these authors describe a complete cessation of phospholipid breakdown in the presence of ACTH, it is probable that an isotope reutilization or exchange problem has caused an overestimation of the effect. The actual extent of phospholipid stabilization is therefore questionable, but the notion of a simultaneous stabilization of adrenal protein and phospholipid is quite appealing.

Both Purvis *et al.* (1972, 1973) and Pfeiffer *et al.* (1972) have shown that during chronic ACTH administration to hypophysectomized rats the increases in the various membrane-bound components associated with adrenal steroid hydroxylation, including cytochrome *P*-450 and adrenodoxin, follow linear rather

Table 2. *Increase in adrenal protein expected solely from the change in half-life in the presence or absence of ACTH compared with the increase observed after* 10 *days*

Percentage increases in adrenal protein were calculated from the difference in the half-lives of the various adrenal fractions in the presence and absence of administered ACTH. The graphically determined half-lives from Figs. 1 and 2 were used. The increases were computed solely from $t_{\frac{1}{2}}$ as follows: $[t_{\frac{1}{2}}$ (treated)$-t_{\frac{1}{2}}$ (untreated)$]/t_{\frac{1}{2}}$ (untreated), since $t_{\frac{1}{2}}$ is directly proportional to protein concentration (P) by $P = k_s \cdot t_{\frac{1}{2}}/\ln 2$, and k_s (rate of synthesis) is held constant. Observed percentage increases were determined from plots of the protein yields of the fractions. The protein yields at 10 days were compared with those at zero time.

	Increase in protein (%)		
	Calculated from half-life data		
Fraction	[^{3}H]Leucine	$NaH^{14}CO_3$	Observed after 10 days
Homogenate	63	57	118
Mitochondria	No plateau	171	217
18000*g* supernatant	31	—	—
Microsomal fraction	—	42	171

than hyperbolic induction kinetics. Linear induction kinetics are consistent with an increase in these components due at least in part to a decrease in their breakdown. Similarly, Purvis has found that luteinizing hormone lowers the rate of degradation of cytochrome *P*-450 in the rat testis (J. L. Purvis, personal communication).

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