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Comparative *in vitro* Corticoidogenic Effects of Rat Adenohypophysial Extract and U.S.P. Reference Standard Corticotropin.* (24441)

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Consistently higher values for pituitary ACTH concentration, in the intact rat, have been obtained with Saffran and Schally's *in vitro* corticoidogenic assay(1) than with Sayers' *in vivo* adrenal ascorbic acid depletion method(2). Due allowances being made for the different strains of rats and extraction procedures utilized by various groups, the values recorded, in terms of milliunits (mu) of ACTH/mg of fresh pituitary tissue, range from 10 to 18(3-6) with Sayers', and from 52 to 241(7,8) with Saffran and Schally's assay. A mean concentration of 64.6 ± 1.8 mu/mg was recorded, in this laboratory, from 100 *in vitro* assays (average λ : 0.106 ± 0.004) of 0.1N HCl-extracted pituitaries from intact male Sprague-Dawley rats (Fortier, unpublished). To account for the discrepancy in results yielded by the 2 assaying procedures, the possibility was raised by Birmingham *et al.*(7) that *steroidogenic/ascorbic acid depleting* (S/AA) activity ratio might be higher in native (pituitary extract) than in more purified (Reference Standard) corticotropin. This view was not supported by results of concurrent 4-point-2-dose assays of the same pituitary extract against the same standard, respectively based on *in vitro* corticoidogenesis, and on adrenal ascorbic acid depletion and rise of adrenal and plasma corticosteroids in hypophysectomized rats (Guillemin, Fortier and Lipscomb, unpublished). Nearly identical values (20-24 mu/mg) were obtained on the basis of *in vivo* corticoidogenesis and ascorbic acid depletion, whereas a markedly higher concentration (62 mu/mg) was again recorded on the basis of *in vitro* corticoidogenesis. The discrepancy would thus appear to be related to differences in the nature (*in vivo* or *in vitro*) rather than in the end-points (adrenal ascorbic acid depletion or corticoidogenesis) of the assays. The following possibilities were accordingly considered in the present study: a) Conditions prevailing in the *in vitro* assay might favor activation of a structurally related, hypothetical precursor of ACTH (*protocorticotropin*), contained in rat pituitary extract; b) Differences in the fates of, or responses to, the presumably distinct molecules corresponding to rat and hog (U.S.P. Reference Standard) corticotropins might be selectively enhanced by conditions prevailing in one of the 2 assaying systems under consideration. These possibilities were assessed by comparing rat pituitary extract and U.S.P. Reference Standard Corticotropin, in Saffran and Schally's *in vitro* system, with respect to inactivation, corticoidogenic effects, and extent of corticotrophic activation conferred to the incubated glands, as a result of a limited period of contact.

Materials and methods. Pituitary extracts. Adenohypophyses from adult male Sprague-

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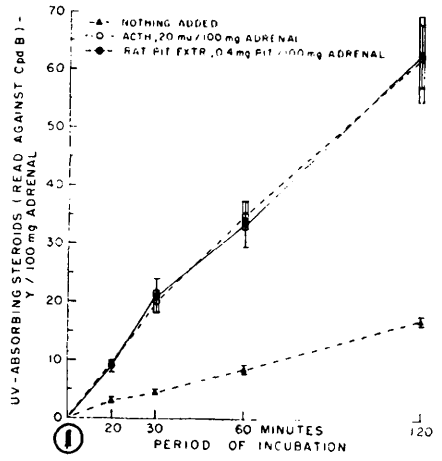
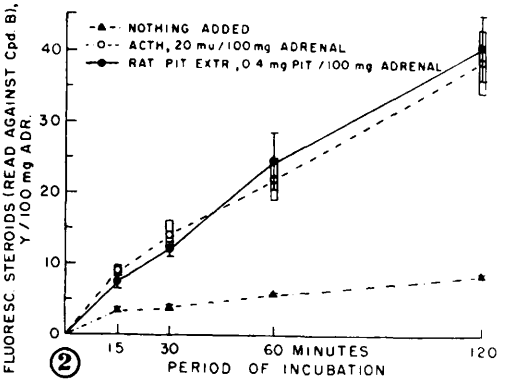
Dawley rats, killed by decapitation, were quickly removed from the skull, separated from neural lobe, wiped clean of blood, and weighed to nearest 0.05 mg. Pooled glands from 3 rats were ground with a few grains of sand in 15 ml conical centrifuge tube containing 0.6 ml of 0.1N HCl. The rod was rinsed into tube with 0.3 ml of 0.1N HCl. After 30 minutes at room temperature, the stoppered extract was stored in deep-freeze. A weighed sample of U.S.P. Reference Standard Corticotropin was dissolved in equinormal HCl, and stored for same period as the extract. On day of assay, both were thawed, and adjusted to 0.01 normality. *Comparative effects of incubation on in vitro corticoidogenic activity of U.S.P. Reference Standard Corticotropin and rat pituitary extract.* Aliquots of U.S.P. Reference Standard Corticotropin, corresponding to 10 (U^1) and to 30 (U^2) mu of ACTH, in 0.3 ml of solvent, /100 mg adrenal, were equally distributed among 4 of a set (A) of 8 beakers containing 1.4 ml of Krebs-Ringer-Bicarbonate-Glucose medium. These beakers were incubated for 2 hours in Dubnoff Metabolic Shaking Incubator, at 38°C and in water-saturated atmosphere of 95% O_2, 5% CO_2. At end of this incubation period, corresponding to end of concurrent one-hour preincubation of another set (B) of 8 beakers containing quartered adrenal glands in the same volume of medium, similar aliquots (S^1 and S^2) of fresh ACTH were added to the 4 remaining beakers of set A, and the glands rapidly transferred from beakers of set B to those of set A. Incubation was resumed for additional 2 hours, and potency ratio of incubated to fresh material assessed by means of Bliss' methods for factorial analysis and analysis of variance(9), on the basis of their respective effects on *in vitro* production of UV-absorbing steroids(1). In analogous experiments, aliquots of fresh and incubated rat pituitary extract, corresponding to one (S^1 and U^1) and to 3 (S^2 and U^2) fiftieths of a gland, in a volume of 0.3 ml, /100 mg adrenal, were substituted to those of Reference Standard Corticotropin. In both instances, actual weights of glands with which they would eventually be incubated being unknown by the time the U aliquots were added, the doses/100 mg adrenal were determined on basis of average adrenal weight of 2 rats of the same B.W. as those to be killed for assay one hour later. The S doses of fresh material were adjusted for each beaker, by multiplying true weight of glands therein contained by the ratio: *assumed adrenal weight/true adrenal weight,* in corresponding U beaker. *Comparative in vitro corticoidogenic effects of U.S.P. Reference Standard Corticotropin and Rat Pituitary Extract.* Quartered adrenal glands from 48 female Sprague-Dawley rats (120-130 g, B.W.) were distributed, in separate experiments, among 6 homogeneous sets of 8 units, each made up of 8 quarters from same donor population. These were weighed to nearest 0.05 mg, and allotted to beakers containing 1.5 ml of freshly gassed medium. Following preincubation for one hour, the fluid was aspirated off and replaced with 1.4 ml of fresh medium. In 4 of the 6 sets, 20 mu of Reference Standard Corticotropin, in 0.3 ml of solvent, /100 mg adrenal was added to one-half of the beakers; a similar volume of extract, corresponding to 0.4 mg of fresh pituitary tissue,† /100 mg adrenal, to the other half. In the remaining 2 sets, only the solvent (0.01N HCl) was added. Incubation was resumed for periods of 15, 30, 60 and 120 minutes (for 2 units of each set), at the end of which 1 and 0.1 ml of the media were respectively used for determination of the UV-absorbing compounds by spectrophotometric method of Saffran and Schally(1), and of the corticosterone-like steroids by the method of Silber, recently described by Guillemin *et al.* (10, 11), and based on the sulfuric acid-induced fluorescence of these steroids(12). *Effect of time of contact with incubated glands on relative potency of rat pituitary extract to U.S.P. Reference Standard Corticotropin.* Following concurrent preincubation, under usual conditions, of 2 sets (A and B) of 8 beakers containing 8 adrenal quarters from the same donor population, the fluid was aspirated off and replaced with 1.4 ml of fresh medium. Aliquots of 10 (S^1) and 30 (S^2) mu/100 mg adrenal of the reference standard,

† In preliminary assay of extract, this amount of pituitary tissue was observed to correspond to 20 mu of ACTH.





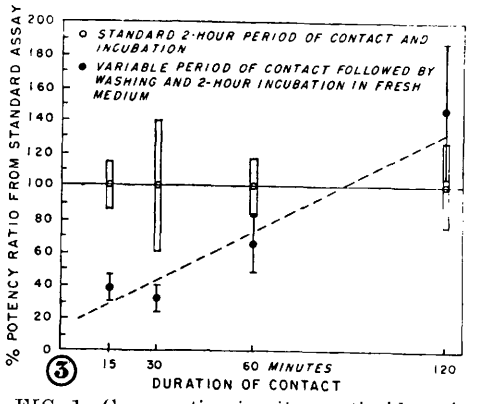


FIG. 1. Comparative *in vitro* corticoidogenic effects, in terms of UV-absorbing steroid production, of U. S. P. Reference Standard Corticotropin and rat pituitary extract. In this, as in following figures, stand. errors are indicated by T-shaped bars and columns.

FIG. 2. Comparative *in vitro* corticoidogenic effects, in terms of fluorescent steroid production, of U. S. P. Reference Standard Corticotropin and rat pituitary extract.

TABLE I. Effect of Incubation for 2 Hours in a Cell-Free Medium on *In Vitro* Corticoidogenic Activity of U.S.P. Reference Standard Corticotropin and Rat Pituitary Extract.

Sample	Potency ratio* (incubated /fresh)	95% confidence limits†	Index of precision (λ)
ACTH, U.S.P. ref. std.	.959	.780-1.175	.044
Rat pituitary extr.	.974	.294-3.039	.174
	.813	.660- .986	.043

* Antilog M. † Antilog $C^2M \pm CtSm(9)$.

in 0.3 ml of solvent, were added to 4 of the beakers of each set; doses of pituitary extract, corresponding to one (U^1) and to 3 (U^2) fiftieths of a gland, to the other 4. In 4 successive experiments of this type, incubation was resumed for a period of 2 hours for the A sets, of 15, 30, 60 and 120 minutes respectively, for the B sets. The media from the latter were aspirated off and discarded. The glands were twice washed with fresh medium, 1.5 ml of which was added, prior to a final 2 hour-incubation. Potency ratios from standard (A) and modified (B) assays were determined as usual, and the effect of variable periods of contact, ascertained from their differences.

Results. Little alteration of the *in vitro* corticoidogenic activity of U.S.P. Reference Standard Corticotropin or rat pituitary extract resulted from their respective incubations for 2 hours in a cell-free medium‡ (Table I). The closeness of the potency ra-

FIG. 3. Effect of duration of contact with the incubated glands on potency ratio of rat pituitary extract to U. S. P. Reference Standard Corticotropin, determined on basis of residual corticotrophic activation conferred to the glands. Of the 2 concurrent assays run for each time ordinate, one was carried out according to standard procedure; the corticotrophic principles remaining in contact with the glands throughout the 2-hr period of incubation, the second involved a limited period of contact, followed by washing of the glands, prior to resumption of incubation for 2 hr in fresh medium. Results are expressed as percentages of potency ratios from the corresponding standard assays. Stand. errors were derived from Sm's of the assays.

‡ Partial (44%) loss of activity of the reference standard, previously reported(13) as a result of incubation, is imputed to neutralization of the sample, prior to incubation.

tios and overlap of their confidence limits would seem to preclude, furthermore, a selective effect of incubation on 1 of the 2 corticotropins. Both corticotropins markedly enhanced the basal production of UV-absorbing (Fig. 1) and fluorescent (Fig. 2) steroids by the incubated glands. From the parallelism of the slopes, contiguity of the means, and overlap of the standard errors, the *in vitro* corticoidogenic effects of reference standard and pituitary extract appeared to be qualitatively and quantitatively identical, at their respective dosage levels. When, however, relative potency of extract to standard was ascertained in terms of corticotrophic activation conferred to the glands, as a result of a limited period of contact (Fig. 3), it was shown to increase linearly with duration of contact.

Discussion. *In vitro* activation of a hypothetical *protocorticotropin* in the pituitary extract would have been expected to result in increasing divergence, with time, of the corticoidogenic responses to the extract and to the reference standard. This possibility is excluded by the observed parallelism of the responses. Differences in rates of *in vitro* inactivation of the 2 principles, as a result of incubation in a cell-free medium or in the presence of the glands, are likewise precluded by the similarity of their *incubated/fresh* potency ratios, and the lack of divergence of their time-response slopes throughout incubation.

The UV-absorbing and fluorescent properties of the steroids, used in this study as indices of *in vitro* corticoidogenic activity, have been respectively ascribed to the Δ 4-3-ketone group(14,15) and, on the basis of indirect evidence(16), α-ketolic side chains.§ The strict comparability of the corticoidogenic effects of extract and standard, ascertained in terms of either type of steroids, provides further evidence of their similarity with respect to fate and mode of action in the *in vitro* system. However, the increase, with time of contact, of the corticotrophic activation conferred to the glands by the extract, relatively to the standard, points to a difference. If the residual activation of the corticosteroidogenic process, which persists after withdrawal of ACTH from the medium, is related, as appears likely, to the amount of hormone already fixed or adsorbed by the incubated adrenals(17), this finding could be explained in terms of different rates of adsorption, by the glands, of 2 distinct molecules of corticotropin. Such a difference would be masked by the negligible rate of ACTH inactivation observed in the *in vitro* system, but would have a definite bearing on relative potency of the 2 corticotropins under conditions of rapid inactivation or disappearance from the medium, such as those prevailing *in vivo*(18). Assuming identical rates of inactivation or disappearance of the 2 molecules, under these conditions, their potency ratio would be conditioned, at least partly, by their respective rates of adsorption. There may lie the explanation for the divergent estimates of rat pituitary ACTH concentration yielded by *in vivo* and *in vitro* assays.

Summary. Rat pituitary extract and U.S.P. Reference Standard Corticotropin were compared, in Saffran and Schally's *in vitro* system, with respect to inactivation, corticoidogenic effects, and corticotrophic activation conferred to the incubated glands, as a result of a limited period of contact. The only difference noted pertained to the extent of this residual activation. The relative potency of pituitary extract to reference standard, determined on that basis, was observed to increase linearly with time of contact. This is ascribed to different rates of adsorption, by the glands, of the 2 corticotropins. Taken in conjunction with the more rapid inactivation and disappearance of ACTH *in vivo* than *in vitro*, differential adsorption is suggested as the basis of the discrepancy in the estimates of rat pituitary ACTH concentration, yielded by *in vivo* and *in vitro* assays.

§ Other characteristics are, no doubt, involved in the fluorescence reaction(12). However, α-ketolic side chains admittedly accounting for blue tetrazolium reduction, production ratios of fluorescent to UV-absorbing steroids by adrenal glands, incubated in presence or absence of ACTH, were observed to correspond to the previously recorded(14) *blue tetrazolium reducing/UV-absorbing* ratios under comparable conditions.

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Effect of Pantothenic Acid on the Longevity of Mice. (24442)

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The disparity between the life span of worker and queen bees suggested the possibility that pantothenic acid which is the most conspicuous nutritional constituent of royal jelly(1,2) might have the effect of prolonging life in other species. An additional suggestion came from the finding of Gardner(3) that this vitamin acts as an anti-ageing factor in drosophila.

Method. Thirty-four male and 40 female, C-57 black mice, age 4-5 weeks, were procured from the Jackson Memorial Laboratory. The mice were grouped with either 6 or 7 animals of the same sex in each cage. Thirteen male and 20 female mice were given supplementary pantothenic acid and 21 male and 20 female mice were used for the control group. All mice were fed Purina laboratory pellets throughout the experiment. It was desired to give each of the 33 mice in the experimental group a supplement of approximately 300 μg of calcium pantothenate per day throughout the experiment. The vitamin was added to the drinking water and the amount added was based on the previous week's water consumption, *i.e.,* if average daily water consumption for the group in one cage was 4 ml per mouse, calcium pantothenate was added in the amount of 75 μg/ml. If the consumption was 5 ml then 60 μg of the vitamin were added per ml of water. The age of each mouse was

TABLE I. Summary and Statistical Data for Mice Which Received Pantothenic Acid Supplement and Controls.

	Mean life span in days							
	Mice with pantothenic acid supplement			Controls			t test(4)	U test(5)
	No.	Days	S.D.	No.	Days	S.D.	P =	P =
Both sexes	33	653.1	201	41	549.8	233	.05	.01
Male mice	13	660.4	220	21	559.0	167	.15	.04
Female mice	20	648.4	187	20	540.2	285	.18	.05