Enhancement of Adrenocorticotrophic Activity. (20235)

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The effects of adrenocorticotrophin, administered parenterally, in water, are submaximal. The failure to achieve full utilization of the hormone, so given, has been ascribed to destruction of the material at the injection site (1,2). Raben *et al.*(3) found that oxycellulose purified ACTH had twice the effect of the crude material when injected in similar vehicles and have suggested that destructive enzymes, originally present, have been removed during the process of purification of the hormone preparation. Wolfson *et al.*(4) have shown that ACTH administered in heavy gelatin resulted in a twofold increase in clinical effectiveness as compared to that administered in water solution. Solem(5) and Fletcher(6) have prolonged the duration of ACTH action by means of protamine zinc complexes and beeswax in oil respectively. The effectiveness of these methods has been attributed to 1) a slow release from an adsorbed state or a chemical complex and 2) a sequestering effect of the hormone from the tissues with concurrent slow release(4). Recently, Hamburger(7) has shown that polyphloretin phosphate is capable of prolonging the effects of ACTH when administered intramuscularly to rats, and Hogberg *et al.*(8) reported clinical data demonstrating the increased effectiveness of such preparations.

Polyphloretin phosphate has been shown to have antihyaluronidase action(7). This suggested the evaluation of other substances possessing similar properties as a method of prolonging the action of parenterally administered ACTH. In view of the finding by Hamburger(7) that polyphloretin phosphate protected ACTH from *in vitro* inactivation by serum, the substances described below were also examined for antiproteolytic activity.

Material and methods. Sprague-Dawley male rates, 110-115 g body weight, were hypophysectomized by the parapharyngeal approach 24 hours before the experiments. Injections were given subcutaneously in 0.5 ml volume. Purified corticotropin (Princeton), 25 U.S.P. units/mg, was incorporated into the solutions of substances to be tested so that the final solution contained 2 U.S.P. units/ml. Each experimental animal therefore received 1 U.S.P. unit of adrenocorticotrophin. Control animals received injections of equal volumes of the test solutions without ACTH. Adrenal ascorbic acid concentration was determined on the left gland, 3 hours, and on the right gland, 6 hours after injection. The ascorbic acid concentrations were determined by the method of Mindlin and Butler(9). The following substances were tested for their effect on ACTH: 0.2% suramin; 5% phosphorylated hesperidin; 2.5% phosphorylated hesperidin; 5% hesperidin methyl chalcone; 15% gelatin; 15% gelatin plus 20 T.U. Hyaluronidase and 15% gelatin plus 4% phosphorylated hesperidin. All solutions were adjusted to pH 4. Hyaluronidase, when used, was added immediately before injection. For the *in vitro* experiments on anti-proteolytic activity; suramin, phosphorylated hesperidin, and hesperidin methyl chalcone, were tested for possible inhibition of tryptic digestion of casein. In the *first experiment,* trypsin (Princeton), phosphorylated hesperidin solutions of mole ratios 1:2 and 1:4 (0.0006% and 0.001%), were prepared at pH 7.4. Neurath(10) has described a trypsin: diisopropylfluorophosphate complex at mole ratio 1:3 with almost complete loss of enzyme activity. The activities were measured immediately and after incubation at 37°C for 150 minutes. A *second experiment,* to approximate the concentrations of the test compounds used in the ACTH assay, involved the preparation of trypsin solutions in 2.5% and 5% phosphorylated hesperidin and hesperidin methyl chalcone, and 0.2% suramin. Tryptic activity was measured immediately and after 21 hours at 5°C. The method of Anson(11) was used throughout, with appropriate blanks for the materials tested.

TABLE I. Effect of Various Materials Upon ACTH Induced Adrenal Ascorbic Acid Depletion at 3 and 6 Hr Post Injection.

Vehicle %		No. of animals	Dose of ACTH U.S.P. unit	Adrenal ascorbic acid mg/100 g adrenal gland 3 hr	6 hr
			A.		
	H_2O	5	1	279 ± 11	314 ± 6
5	p.h.*	5	0	398 ± 14	383 ± 17
5	p.h.	10	1	180 ± 9	231 ± 13
2.5	p.h.	5	1	175 ± 7	245 ± 14
5	h.	5	0	404 ± 16	353 ± 21
5	h.	5	1	304 ± 16	322 ± 13
0.2	s.	5	1	231 ± 9	315 ± 14
15	g.	10	1	178 ± 10	240 ± 15
15	g + 10 T.U. hy	5	1	212 ± 17	271 ± 10
			B.		
15	g.	5	1	165 ± 8	230 ± 9
15 g + 4%	p.h.	5	1	261 ± 16	152 ± 8

* p.h. = phosphorylated hesperidin; h = hesperidin methyl chalcone; s = suranim; g = gelatin; hy = hyaluronidase. All values are means ± standard error.

Results and discussion. The data presented in Table IA indicate that a subcutaneous dose of ACTH can be more effectively utilized in the rat if administered in a medium which delays absorption of the hormone. Phosphorylated hesperidin and heavy gelatin seem to be the most effective single agents, of those tested, in producing such a delay. However, a combination of these materials results in an augmentation of effectiveness, with the maximum response delayed from the third hour to the sixth hour post injection. Further studies extending the period of examination to 12 and 24 hours are now in progress. These substances conceivably act by different mechanisms to produce the prolongation of activity.

The phosphorylated hesperidin probably acts by inhibiting tissue hyaluronidase. The correlation of the efficacy of phosphorylated hesperidin in prolonging the action of ACTH, with the anti-hyaluronidase properties of the compound(12), indicates that the tissue spreading factor is involved. The methyl chalcone of hesperidin, which has no significant anti-hyaluronidase properties(12) has no enhancing effect on ACTH, despite its inhibition of proteolytic activity, *in vitro,* equivalent at the concentration used, to that of phosphorylated hesperidin.

In vitro studies with suramin and the hesperidins showed that both the methyl chalcone and phosphorylated hesperidin, as well as suramin are capable of inhibiting the proteolytic activity of trypsin (Table II). Inasmuch as both of these hesperidins exhibit similar degrees of anti-proteolytic effect, whereas only the phosphorylated hesperidin shows enhancing properties when combined with ACTH, it is concluded that the mechanism involved in the latter phenomenon is probably an action on the spreading factor. Unfortunately, suramin could not be used in larger doses *in vivo* due to its toxicity, and therefore its effect in prolonging the action of ACTH could not be measured at a concentration similar to that

TABLE II. Inhibition of Tryptic Digestion of Casein.

Material	% conc.	Hr of contact of trypsin with test substance before digestion with casein	°C	% inhibition†
P.h.*	.0006	.0		0
"	.0006	2.5	37	29
"	.0012	.0		0
"	.0012	2.5	37	71
	5.0	.0		50
"	2.5	.0		49
"	2.5	21	5	68
H	5.0	.0		58
"	2.5	.0		23
"	2.5	21	5	25
S	.2	.0		0
"	.2	21	37	83

* P.h. = Phosphorylated hesperidin; H = hesperidin methyl chalcone; S = Suramin.

† As compared to activity of trypsin solutions of equal concentration, under same conditions, in acetate buffer without test substances.

used for phosphorylated hesperidin. In the data obtained with the corticotrophin in gelatin plus phosphorylated hesperidin (Table IB) a summation effect is evident. There is the possibility that here the anti-proteolytic effect of the phosphorylated hesperidin (Table II) may also be a factor in that the gelatin is not acted upon by tissue proteinases and thus the gelatin retains its depot effect for a longer period of time. Thus the overall action of the gelatin-phosphorylated hesperidin combination may be the result of an increased depot effect of the gelatin due to the anti-proteolytic action of the phosphorylated hesperidin as well as the anti-hyaluronidase effect of this compound. The findings discussed above point to the use of a depot agent—anti-hyaluronidase vehicle for extending the duration of effect of other parenterally administered substances.

Preliminary clinical studies confirm the animal data in that the effectiveness of ACTH administered in phosphorylated hesperidin is as effective if not superior to gelatin preparations.

Summary. 1. Subcutaneous administration of purified corticotrophin in gelatin; in phosphorylated hesperidin; and in gelatin plus phosphorylated hesperidin results in an enhanced effect of the hormone upon the adrenal cortex as measured by adrenal ascorbic acid. The combination of gelatin plus phosphorylated hesperidin has been shown to be most effective in extending the duration of effect of the corticotrophin. 2. The mechanism of action is attributed to the antihyaluronidase as well as the anti-proteolytic properties of the phosphorylated hesperidin.

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Age Susceptibility Pattern of the Rat to Epidemic Keratoconjunctivitis Virus. (20236)

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Recent investigations(1,2) indicate that the viruses of epidemic keratoconjunctivitis (EK) and St. Louis encephalitis (SLE) are closely related. The two viruses would appear to be so closely related that it is not possible to differentiate them by serological methods. By studying the host range of the two viruses it was noted, however, that the viruses are not identical. It was observed(3) that while both viruses induce a fatal encephalitis in mice only the EK virus was capable of initiating a fatal encephalitis in guinea pigs and rabbits. It was also observed that EK virus, like SLE, does not produce any clinical evidence of infection in young adult rats following intracerebral inoculation of the virus(3). Since it has already been demonstrated that 7- or 8-day-old rats are highly susceptible to SLE virus,

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