of *dl*-α,5-dimethylhydantoin-3-acetic acid. The reference compound which was prepared according to Gränacher and Landolt[6] melted at 181–184°; literature m.p. 187–189°.

*Anal.* Calcd. for $C_7H_{10}O_4N_2$: N, 15.0. Found: N, 14.8.

**Diastereomer B.**—Treatment of phenylthiocarbonyl-DL-alanyl-DL-alanine ethyl ester (diastereomer B) (1.2 g.) with lead acetate (0.7 g.) in 70% ethanol (40 ml.) afforded an oily ester; yield 0.74 g. (93%). The compound failed to liberate nitrogen in the Van Slyke determination. Hydrolysis of this ester (0.57 g.)[6] gave a crystalline acid; yield 0.26 g. (52%); m.p. 152–155°. This substance gave no depression when admixed with an authentic sample of the low melting diastereomer of *dl*-α,5-dimethylhydantoin-3-acetic acid. The reference compound which was prepared according to Gränacher and Wolf[7] melted at 153–156°; literature m.p. 158–160°.

*Anal.* Calcd. for $C_7H_{10}O_4N_2$: N, 15.0. Found: N, 15.1.

**Phenylthiocarbonylglycine Carbobenzoxyhydrazide.**—To a solution of phenylthiocarbonylglycine chloride (2.2 g.) in chloroform (50 ml.) was added with cooling a solution of carbobenzoxyhydrazine (3.2 g.) in chloroform (60 ml.) and the mixture kept at room temperature for 16 hours. The compound was isolated in the usual manner and recrystallized from a mixture of ethyl acetate and petroleum ether; yield 2.9 g. (84%); m.p. 132–134°.

*Anal.* Calcd. for $C_{17}H_{17}O_4N_3S$: C, 56.8; H, 4.8; N, 11.7; S, 8.9. Found: C, 56.6; H, 4.6; N, 11.4; S, 8.6.

**2-Carbobenzoxy-3,6-dioxohexahydro-1,2,4-triazine.**—To a warm solution of lead acetate (790 mg.) in 70 per cent. ethanol (60 ml.) was added phenylthiocarbonylglycine carbobenzoxyhydrazide (1.5 g.) and the mixture kept at 80–85° for 6 minutes, when it was cooled to room temperature. The lead phenylmercaptide was removed by filtration, the filtrate evaporated to dryness *in vacuo* and the residue recrystallized from ethanol; yield 820 mg. (78%); m.p. 168–169°.

*Anal.* Calcd. for $C_{11}H_{11}O_4N_3$: C, 53.0; H, 4.4; N, 16.9; Found: C, 53.5; H, 4.4; N, 16.7.

**3,6-Dioxohexahydro-1,2,4-triazine. A. By Hydrogenation of 2-Carbobenzoxy-3,6-dioxohexahydro-1,2,4-triazine.**—A sample of the triazine (500 mg.) was hydrogenated in the usual manner over spongy palladium in methanol (20 ml.) containing three drops of ethanolic hydrogen chloride. The reaction product was recrystallized from dioxane; yield 210 mg. (91%); m.p. 194–195°. The compound failed to liberate nitrogen in the Van Slyke amino-nitrogen determination.

*Anal.* Calcd. for $C_3H_5O_2N_3$: C, 31.3; H, 4.4; N, 36.5. Found: C, 31.2; H, 4.2; N, 36.7.

**B. By Treatment of Phenylthiocarbonylglycine Ethyl Ester with Hydrazine.**—A solution of phenylthiocarbonylglycine ethyl ester (1 g.) and hydrazine hydrate (0.2 ml.) in 70% ethanol (10 ml.) was refluxed for three hours. The mixture was cooled when the reaction product began to crystallize. Crystallization was completed by keeping the mixture at room temperature for 20 hours. The crystals were collected and recrystallized from dioxane; yield 0.2 g. (41%); m.p. 195–196°. No depression of the melting point was observed when this material was admixed with a sample of the same compound prepared according to method A above.

*Anal.* Calcd. for $C_3H_5O_2N_3$: N, 36.5. Found: N, 36.4.

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[CONTRIBUTION FROM THE RESEARCH LABORATORIES, MERCK & CO., INC.]

# The Preparation of Active, Non-antidiuretic Hydrolyzates of ACTH

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Acid hydrolysis of concentrates of ACTH has given products which are active in patients with rheumatoid arthritis. Selected conditions for the hydrolysis retain the hydrolyzate's full adrenocorticotropic activity, but destroy the substance responsible for excessive sodium and water retention in the patient. Such acid hydrolyzates may be purified to yield fractions which are still free of the side effects mentioned above, but which are active clinically at lower dosage levels. The clinical results have been in agreement with the animal assays for both adrenocorticotropic and antidiuretic activity. Pepsin digestion does not destroy the antidiuretic activity of the ACTH preparations studied, but this effect is lost upon subsequent acid treatment.

Acid hydrolyzates of concentrates of the adrenocorticotropic hormone (ACTH) have been found active in rheumatoid arthritis, and appear to possess some therapeutic advantages over unhydrolyzed ACTH, or pepsin digests of ACTH.

ACTH has been described as a protein of molecular weight about 20,000.[1,2] The ability possessed by the hormone to stimulate the adrenal cortex in animals was not destroyed, according to Li,[3] by pepsin digestion or acid hydrolysis under relatively mild conditions. The announcement[4] that ACTH was active in rheumatoid arthritis was followed shortly by reports that fractions of pepsin digests of ACTH freed of materials of high molecular weight by trichloroacetic acid treatment[5] or by dialysis[6] were also active in treating this disease. Other workers have confirmed the activity of pepsin digests of ACTH in rheumatoid arthritis, and have shown that the digests can be fractionated to yield products with many times the activity of the original hormone.[7]

The administration of ACTH preparations to human subjects results in a number of metabolic changes, which include the retention by the subject of sodium and of water.[8] The protein-free peptide mixtures of Li produced metabolic changes including sodium and water retention indistinguishable from those effected by administration of unhydrolyzed ACTH.[5,9] Most recently it has been re-

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(8) *Cf.* H. W. McIntosh, H. T. McAlpine, B. Singer and M. M. Hoffman, "Proceedings of the First Clinical ACTH Conference," The Blakiston Co., Philadelphia-Toronto, 1950, p. 14.

(9) R. Luft, B. Sjögren and C. H. Li, *Acta endocrinol.*, **3**, 299 (1949).

ported[10] that a highly purified polypeptide ACTH preparation having an activity of about one hundred times standard potency by the animal assay was tested in normal human subjects. It produced the same metabolic changes which had been observed with the commercially available clinical ACTH concentrates. There was considerable retention of sodium, although no antidiuretic effect was noted at the dose employed. In general agreement with these observations, animal tests (Table I) have shown that both types of materials possess antidiuretic activity even at dosages well below the therapeutic levels. Purified fractions of pepsin digests of ACTH, in which the adrenocorticotropic activity has been increased many times,[7] still possess antidiuretic activity at low dose levels. Some of these metabolic side effects of concentrates of ACTH or of the pepsin digests are regarded as undesirable. The water retention may lead in some cases to edema and, if not controlled by diuretics, to congestive heart failure.

The purpose of the present work was to study the chemical and biological properties of acid hydrolyzates of ACTH concentrates. A number of such hydrolyzates were prepared under varying conditions, starting with both purified ACTH preparations[1,2] and relatively crude concentrates such as "crude prolactin."[11] These were submitted to Dr. Charles Ragan[12] for clinical investigation, the details of which will be published elsewhere. Dr. Ragan observed that the acid hydrolyzates were active clinically in the arthritic subjects; and further that certain samples showed no significant sodium- or water-retaining effects in the subjects.[10]

In the investigation of the acid hydrolysis of ACTH preparations, conditions were sought which would give products in which the adrenocorticotropic activity was undiminished, but the undesirable side effects discussed above were destroyed. For measurement of ACTH activity, the adrenal ascorbic acid depletion assay employing hypophysectomized rats[13] was used; and the presence or absence of the side effects was followed qualitatively by an antidiuretic assay using adult male rats. Table I contains a summary of the results of antidiuretic tests on some pertinent samples. In all preparations tested clinically, agreement with the results of the animal assays as to both ACTH activity and the presence or absence of water retention was observed.

The hydrolysis is preferably conducted in 0.3 *N* hydrochloric acid for one hour at the reflux temperature. Such treatment produced a sample contaminated by less than one milliunit of antidiuretic hormone activity per milligram, as compared with amounts of the order of 60 to 2500 milliunits per milligram in samples not treated with acid. While these conditions may be varied within reasonable limits, the results of a number of experiments, summarized in Table II below, indicated that substantially milder hydrolytic conditions failed to destroy the antidiuretic activity, and more drastic hydrolysis decreased adrenocorticotropic activity.

The acid hydrolyzates have been fractionated to give products in which the adrenocorticotropic activity has been increased several fold. These upon clinical testing in rheumatoid arthritis at doses as low as 4 mg. daily (as compared to about 50 mg. daily for Armour Standard La-1-A) have been fully active in controlling the disease without producing significant sodium or water retention in the patient. The further purification of the acid hydrolyzates has involved a number of fractionation steps, applied separately or in varied sequences. These steps include conversion of the mixture of hydrochlorides in methanol solution to the free base form, resulting in precipitation of active material; and separations, carried out in either the hydrochloride or free base forms of the material, which depend on the differential partition of active and inactive substances between water and various immiscible organic phases.

It appears most likely that the antidiuretic and possibly the sodium-retaining effects of both unhydrolyzed and pepsin-digested ACTH may be attributed to the presence of other physiologically active substances in the ACTH concentrates, as for example, contaminants from the posterior lobe of the pituitary gland. The loss of antidiuretic activity upon acid treatment of either crude ACTH preparations or considerably purified pepsin digests (Table I) is readily explained by a lesser stability of such contaminants to acidic conditions.

TABLE I

ANTIDIURETIC TESTS

| Sample | ACTH activity[a] | Dosage, mg./kg. | Result |
|---|---|---|---|
| "Pure" whole ACTH, prepared according to Sayers, *et al.*[3] | 1.2x | 1.2 | Positive |
| Acid-hydrolyzed ACTH | 1x | 1.2 | Negative |
| Armour "ACTHAR-A" (Lot 21912) | 1.4x | 1.2 | Positive |
| Armour "ACTHAR-A" (Lot 21912) | 1.4x | 0.6 | Positive |
| Armour "ACTHAR-A" (Lot 21912) | 1.4x | 0.024 | Positive |
| Pepsin digest (TCA-Soluble)[7] of Armour "ACTHAR-A" | 3.5x | 1.2 | Positive |
| Pepsin digest (TCA-Soluble)[7] of Armour "ACTHAR-A" | 3.5x | 0.4 | Positive |
| Purified pepsin digest of ACTH | 6.9x | 0.2 | Positive |
| Purified pepsin digest of ACTH | 80x | 0.024 | Positive |
| Acid-hydrolyzed purified pepsin digest | 10x | 0.12 | Negative |

[a] The ACTH activities are given as multiples of the activity of Armour Standard La-1-A in the adrenal ascorbic acid depletion assay.[13]

The reduction of the antidiuretic effect of a number of sheep ACTH preparations by treatment with acid and heat has been reported by Reinhardt and Li.[14]

## Experimental

For measurement of adrenocorticotropic activity,[14] male rats were used one to three days following hypophysectomy. The animals were divided into groups of three: one group received saline by intravenous injection, a second received 5 μg. of a standard ACTH preparation, and a third group received the ACTH preparation of unknown potency, the dose of which was adjusted by preliminary estimate to match the response of the ACTH control. The response was taken to be the difference between the mean ascorbic

(10) P. H. Forsham, A. Renold and J. B. Lesh, "Proceedings of the Second Clinical ACTH Conference," Vol. I, The Blakiston Co., New York-Philadelphia-Toronto, 1951, pp. 7-19.

(11) A. White, R. W. Bonsnes and C. N. H. Long, *J. Biol. Chem.*, **143**, 447 (1942).

(12) Columbia University, College of Physicians and Surgeons, New York, N. Y.

(13) M. A. Sayers, G. Sayers and L. A. Woodbury, *Endocrinology*, **42**, 379 (1948).

(14) W. O. Reinhardt and C. H. Li, *Proc. Soc. Exptl. Biol. Med.*, **76**, 836 (1951).

acid content of the "saline control" adrenals and that of the "ACTH control" adrenals, measured one hour after injection. If the response to the unknown sample did not match that of the ACTH standard controls, the sample was reassayed at a different dilution.

The antidiuretic activity of the ACTH samples was tested in male Holtzman rats of about 250 g. weight. Groups of four rats each were fasted overnight, and were given a "priming dose" of 2.5 ml. of water per 100 g. body weight two hours before the start of an assay. At the beginning of an assay period, a "water load" of 5 ml. per 100 g. body weight was administered, and urine collection begun. The time for excretion of 50% of the "water load" was determined for each group. Control runs on 27 groups of rats gave a mean 50%-excretion time of 74.7 minutes, with a standard deviation of 7.45 minutes or about 10%. Injection of 15 milliunits of pitressin gave a mean 50%-excretion time of 117.4 minutes, or about 57% greater time than the controls. A test of a sample of ACTH was arbitrarily considered to be "negative" at the dose employed if the 50% excretion time was within 15% (or about 1.5 standard deviations) of the mean; and "positive" if greater than 30% (or about 3 standard deviations) longer than the mean. With intermediate values, the tests were repeated. In other terms, "positive" samples generally contained the equivalent of 15 milliunits or more of antidiuretic hormone in the administered dose. An exact assay of one "negative" ACTH sample was made by Professor H. Heller of the University of Bristol (personal communication), and found to contain 0.74 ± 0.032 milliunit of antidiuretic activity per milligram.

Table II summarizes the studies done to establish hydrolytic conditions of acid strength, time and temperature for the preparation of a satisfactory product. Products were deemed unsatisfactory either if a substantial loss of ACTH activity occurred, or if the hydrolyzate possessed significant antidiuretic activity in rats. The material used in these experiments was an ACTH concentrate of 0.9–1.1x activity (*i.e.*, 0.9–1.1 times the activity of Armour Standard La-1-A). In the antidiuretic test, the dose of ACTH hydrolyzate given was 1.2 mg./kg., which is approximately the same as the largest ordinary human therapeutic dose.

TABLE II

CONDITIONS OF HYDROLYSIS

| Acid | Time, hour | Temp., °C. | ACTH activity | Antidiuretic test (1.2 mg./kg.) | Product |
|---|---|---|---|---|---|
| 0.1 *N* HCl | 1 | 100 | 1.0x | Positive | Unsatisfactory |
| 0.3 *N* HCl | 1 | 100 | 1.0x | Negative | Satisfactory |
| 0.6 *N* HCl | 1 | 100 | 0.7x | Negative | Satisfactory |
| 1.5 *N* HCl | 1 | 100 | None | ..... | Unsatisfactory |
| 6 *N* HCl | 4 | 37 | 1.0x | Positive | Unsatisfactory |
| 6 *N* HCl | 8 | 37 | 0.5x | ..... | Unsatisfactory |
| 6 *N* HCl | 16 | 37 | None | ..... | Unsatisfactory |
| 0.3 *N* HCl | 1/4 | 100 | 1.3x | Positive | Unsatisfactory |
| 0.3 *N* HCl | 1/2 | 100 | 0.9x | Negative | Satisfactory |
| 0.3 *N* HCl | 2 | 100 | 1.2x | Negative | Satisfactory |
| 0.3 *N* HCl | 4 | 100 | None | ..... | Unsatisfactory |
| 0.3 *N* HCl | 1 | 37 | .. | Positive | Unsatisfactory |
| 0.3 *N* HCl | 1 | 60 | .. | Positive (slight) | Doubtful |
| 0.3 *N* HCl | 1 | 115 | 0.7x | Negative | Satisfactory |
| 0.3 *N* HCl | 1 | 148 | None | ..... | Unsatisfactory |
| 0.3 *N* $H_2SO_4$ | 1 | 100 | 1.1x | Negative | Satisfactory |
| 0.3 *N* HBr | 1 | 100 | 1.1x | Negative | Satisfactory |

In a typical hydrolysis, one gram of porcine "crude prolactin fraction"[10] was treated with 20 ml. of boiling 0.3 *N* hydrochloric acid and the mixture was boiled under reflux for one hour, cooled and after the addition of two drops of tributyl citrate to prevent foaming, it was concentrated to dryness *in vacuo*. The product, a grayish-purple solid, was triturated with three 5-ml. portions of methanol, and the methanol-insoluble residue was discarded. The combined clear methanolic extracts were treated with 150 ml. of ether, giving a precipitate which was powdery and light in color. The precipitate was collected by centrifugation and dried *in vacuo*. The product weighed 547 mg., and showed an ACTH assay value of 1.2x. It was tested in animals and found to be devoid of antidiuretic activity at a level of 1.2 mg./kg.

The sample was tested clinically in a patient with rheumatoid arthritis. It was given for four days, 81 mg. the first day, and between 37 and 48 mg. daily for the next three days. It produced an excellent remission of the arthritis, with no significant retention of sodium or water. The patient's sedimentation rate dropped satisfactorily.

An eight-gram portion of a hydrolyzate prepared in the manner described above was extracted with 100 ml. of methanol. A small insoluble residue was removed, and the solution was treated with 20 ml. of a mixture of five volumes of methanol to one volume of triethylamine. The mixture was chilled in an ice-bath, centrifuged and the supernatant discarded. The precipitate, which consisted of a mixture of free bases and containing most of the ACTH activity, was washed with three 10-ml. portions of cold methanol and dried *in vacuo* for two hours. It was then dissolved in 50 ml. of the aqueous phase resulting from the mixture of three volumes of butanol, two volumes of liquefied phenol, and five volumes of water, and a small insoluble residue was again discarded. The aqueous solution was then washed with four 50-ml. portions of the corresponding organic phase, during which process a large amount of relatively inert material was extracted from the aqueous phase. The aqueous phase was washed with four 50-ml. portions of ether, acidified with hydrochloric acid and concentrated to dryness. The residue was dissolved in 15 ml. of methanol and the solution added to 50 ml. of ether. The precipitated product weighed 0.2 g., gave an ACTH assay value of 5.4x, and showed no antidiuretic activity at a dose of 1.2 mg./kg.

Upon clinical trial in two cases of rheumatoid arthritis, the material appeared on a weight basis to be from five to eight times as active as Armour Standard ACTH. It produced no sodium or water retention, and brought about only a small eosinophile drop.

In another experiment, the hydrolyzate was carried through the same purification steps as those described above. However, near the end of the process and after the aqueous solution of the free base material had been washed with 3:2 butanol–phenol, the aqueous solution before acidification was shaken with an equal volume of liquefied phenol, thus extracting active material selectively into the organic phase. The product was recovered from the phenol solution by addition of several volumes of ether and extraction of the organic mixture with dilute (*ca.* 0.1 *N*) aqueous hydrochloric acid. The aqueous extract was washed with ether and lyophilized. The resulting material showed in the ACTH assay an activity of 8x, and contained no detectable antidiuretic activity at a dose of 1.2 mg./kg. In a clinical test, it was reported to be more active in rheumatoid arthritis at 4 mg./day than was Armour Standard at 50 mg./day. No sodium or water retention was observed.

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