show a longitudinal (Fig. 1) and nearly transverse (Fig. 2) section through the *nervus ischiadicus* of the adult rabbit fixed in 4% formalin and dehydrated in one case in ethyl alcohol and in the other with pyridine. In the longitudinal section one can see the filamentous fine structure of the central axon and the more "opaque" lamellar texture of the nonlipid portion of the enveloping myelin sheath. The neurilemma outside this and the absence of an axilemma of similar structure between axon and myelin are apparent. The same structures are visible in Fig. 2, which also gives an idea of the relation of a nerve fiber to the connective tissue elements with which it is associated in a complete nerve bundle. This photograph likewise brings out the fact that in many parts of nerve fibers the texture of the lamellae of the myelin is less coarse in the region immediately enveloping the axon. Other photographs we have made both at these and at higher magnifications show the fine details of the structures, the kinds of alteration in these details that result from the use of other fixatives, and the nature of such other optically recognized structures as the clefts of Schmidt-Lantermann and the nodes of Ranvier.

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Studies on Pituitary Adrenocorticotropin

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A considerable portion of the recent work on chemistry of the pituitary adrenocorticotropic hormone (ACTH) has indicated that it might have a relatively small molecular size. This is in contrast to earlier reports (*4*, *8*) of its protein nature and of the isolation of a "pure" protein having a molecular weight of approximately 20,000. Such possibilities are important in view of the limited supply of ACTH from animal sources, since synthesis of the hormone would provide a more adequate solution to the problem of supplying the clinical demand for the material.

Several preliminary reports on the preparation of active peptides from ACTH have appeared. The evidence provided in these preliminary reports does not definitely show that the activity observed is to be identified with a single peptide. Li (*5*) states that the *average* peptide size in a mixture of peptides following hydrolysis with pepsin of ACTH protein is eight amino acid residues.

Cortis-Jones *et al.* (*2*), employing ultrafiltration without partial hydrolysis, showed that biological activity will pass through cellophane membranes impermeable to molecules of 13,700 molecular weight but permeable to those of 8,000. Morris (*7*) indicated that the size of the ACTH molecule varies with pH and that this variation appeared to be a reversible phenomenon.

Brink *et al.* (*1*) reported the clinical activity of dialyzates following peptic hydrolysis.

Li (*6*), employing an acetic acid-butanol-water system on paper, separated six ninhydrin-positive spots, one of which was highly active in the adrenal ascorbic acid assay. Sedimentation and diffusion studies that were made on the peptide mixture led to an average molecular weight of 1200 for the peptide mixture that contained no free amino acids.

Some confusion is added to the picture by the use of ACTH from different species, but there is little evidence thus far to indicate major differences in the hormone from the pituitaries of the three species most widely used, namely, swine, sheep, and cattle.

TABLE 1

FRACTIONATION OF ACTH BY ADJUSTMENT OF pH IN AQUEOUS SOLUTION

Preparation	Initial potency × La-I-A	Fraction insoluble at pH	Fraction soluble at pH	Yield %	Potency × La-I-A
1	2.2	7.3		7.1	8
		6.3		6.2	6
		5.6		5.2	10
			5.2	70	1
2	2	7.3		5	8.6
		6.2		6	2.3
		5.2		5	11
			5.2	67.5	0.5
3	1.9	6.6		10.8	10
		6.0		3.3	6
		5.0		5.4	6
			5.0	74	1.5
4	1.9	7.2		10.5	4
		6.2		6	7
		5.2		6	7
			5.2	75	1.5
5	4	7.6		5.7	22
		6.3		3.5	21
		5.3		4.1	11
			5.3	75	1.6

Our studies on the properties of ACTH carried on over the past two years have been confined largely to that from pork pituitaries, which provide the best yield and upon which clinical experience is based. Although we have been able to concentrate the hormone and have obtained fractions with potencies of the order of 100–150 times standard or 100–150 times that of the previous reported pure ACTH proteins, these fractions appear to have molecular weights of from 2,500 to 10,000 rather than 1,000 or less. The general methods of preparation and characteristics of these fractions are described.

As starting material for preparation of more potent fractions, ACTH prepared for clinical use was employed. These preparations were obtained from fresh, frozen, whole hog pituitary glands by acid-acetone extraction followed by phosphate partition and ammonium sulfate

TABLE 2

RECOVERY OF BIOLOGICAL ACTIVITY OF ACTH FOLLOWING PEPTIC HYDROLYSIS AND TRICHLORACETIC ACID PRECIPITATION

Preparation	Initial Potency × La-I-A	Potency after treatment		Activity recovered in CCl_3COOH-soluble fraction
		CCl_3COOH-soluble × La-I-A	CCl_3COOH-insoluble × La-I-A	
				%
A	2.5	4	1.5	43
B	2.5	7.5	2.4	82
C	10	17	8	53
D	10	16	2.4	42
E*	6	8		100
F*	3	6.4	0.8	45

* Second treatment with pepsin and trichloracetic acid of soluble (E) and insoluble (F) fractions.

precipitation with suitable modifications to reduce posterior pituitary contamination to the low level required for clinical use.

Assay of ACTH was that of Sayers, Sayers and Woodbury (*9*) as modified by Munson and was based upon the depletion of ascorbic acid in the adrenal glands of the hypophysectomized rat. Potencies are reported in terms of Lot La–I–A, an arbitrary standard. One microgram of this standard injected intravenously produces a 20%–30% decrease in adrenal ascorbic acid in the 28-day-old hypophysectomized rat. Representative fractions showing high biological activity by this method have been shown to have comparable potency clinically.

pH effects. Increased potency of ACTH is readily obtained by fractional precipitation of the starting material in aqueous solution by pH adjustment. The removal of insoluble protein at pH 7, 6, and 5 from a 0.5% suspension of starting material in water yields 20% of the preparation showing 3–4 times initial potency. Further adjustment of pH toward higher acidity does not cause precipitation at the concentration employed. Examples of this procedure are shown in Table 1. Study of concentrates from the supernatant fluid shows no indication that a low molecular weight biologically active material is to be found in that fraction.

Peptic hydrolysis. These fractions were then treated with crystallized pepsin to increase further the potency of the materials. A 2% solution of the fraction was treated with 3.7 mg/g of crystallized pepsin for 2 hr at 38° C and pH 2.5. Hydrolysis was stopped by heating the solution to 95° C for 15 min. The protein was then precipitated by addition of trichloracetic acid to 5% and the supernatant fluid was freed of trichloracetic acid by repeated washing with ether. After removal of the ether the supernatant fluid was dried. Approximately twofold increase in potency was obtained by this procedure, as shown in Table 2. Data showing that retreatment of active fractions with pepsin has little or no effect upon the biological activity are also included in this table.

Countercurrent distribution. Studies of the pepsin-treated, trichloracetic acid soluble fraction included use of the countercurrent distribution methods of Craig (*3*). The most satisfactory system found was water-phenol to which varying amounts of ether were added. This system was found to be capable of separating the activity from ninhydrin-positive but biologically inactive fractions. Its chief disadvantage is that emulsions form which require centrifugation before separations can be completed.

Figs. 1 and 2 show a typical fractionation made by countercurrent distribution. In Experiment B-28 (Fig. 1) a 12-plate distribution was made on a pepsin-treated, trichloracetic acid-soluble preparation with a potency of 18 times standard. The most active fractions (5 and 6) from Experiment B-28 were then subjected to a 24-plate run (Fig. 2), using the same system. Based upon total nitrogen, Fraction 12 from the second run had a potency

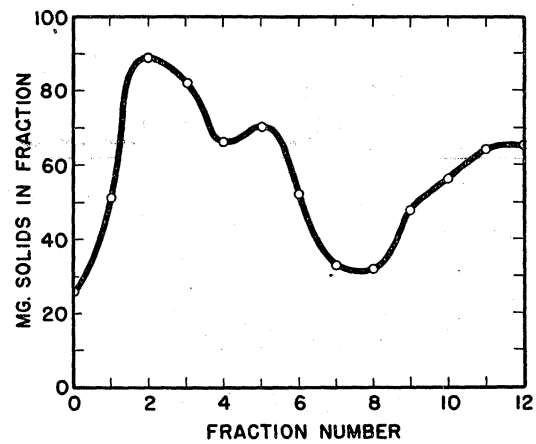

FIG. 1. Experiment B-28. A 12-plate distribution of peptic hydrolyzate material having a potency that is 18 times standard.

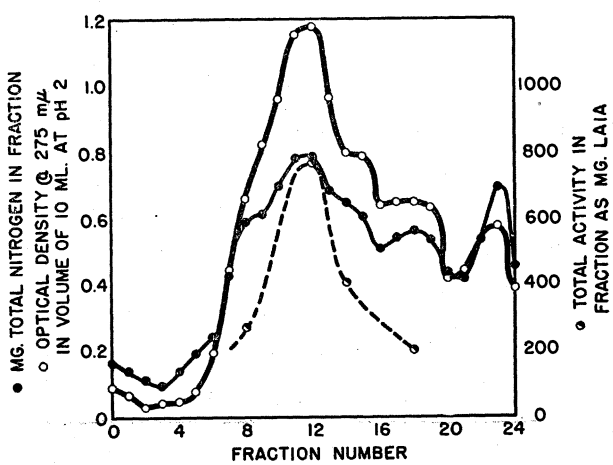

FIG. 2. Experiment C1. A 24-plate distribution made on Fractions 5 and 6 from Experiment B-28.

of 120 times standard, giving an over-all sixfold increase in biological potency.

The ultraviolet absorption spectrum of the most active fraction from Experiment C1 closely resembles those of proteins containing tyrosine and/or tryptophan and the parallelism between the absorption curve and the activity curve (Fig. 2) suggests that either or both of these amino acids is present in the active moiety.

Acid hydrolyzates of fractions from Experiment C1 were tested for amino acids by paper chromatography.[1] As shown in Fig. 3, 12 clearly defined ninhydrin-positive spots were obtained from Fraction 11, indicating at least 12 different amino acids. As shown in the legend below Fig. 3, it is not possible to resolve the pairs valine-methionine and leucine-isoleucine in the system used. Also, because of the proximity of phenylalanine to the leucine-isoleucine spot, it is possible that at least traces of phenylalanine were present. Finally, because an acid hydrolyzate was used, tryptophan was not identified in this experiment.

It is interesting to note that this highly potent fraction exhibits essentially the same amino acid composition as the pure protein of Li (*6*).

Chromatography. Studies employing paper in strips, columns, and chromatopiles; and starch columns, as well as adsorbing agents and ion exchange resins, have been carried out both for fractionation purposes and as analytical methods. In some of these procedures it has been possible to separate the major part of the biological activity from ninhydrin-positive material. Several of the highly potent preparations show negative ninhydrin reaction under the conditions employed.

Ultracentrifugal experiments. Through the courtesy of D. F. Waugh[2] a number of pepsin-treated, trichloracetic acid-soluble ACTH preparations were studied in the ultracentrifuge to determine the approximate molecular size of the active materials. ACTH fractions were dissolved in 0.05 *N* HCl, 0.5% NaCl, and run at 28.5° C for 12 hr at 60,000 rpm. Samples from the upper, middle, and lower portions of the cell were then assayed. As shown in Table 3, approximately 80% of the activity was found in the lower portion of the cell. Similar treatment of known proteins indicates that particles of molecular size of the order of 1,000 molecular weight would not sediment under these conditions, but that particles having molecular weight between 2,500 and 10,000 would behave in such a manner.

All of the results described indicate that the active moiety of the ACTH protein is of molecular size considerably greater than the ACTH peptides recently reported. It is not unlikely that the activity of the mixtures of peptides previously reported was due to the presence of a few percent of a larger molecule having a very high biological potency. Our results do not eliminate the possibility that adsorption of ACTH to larger molecules has occurred. If such is the case, the complex is an extremely stable one that is resistant to pepsin and to acid hydrolysis, and no evidence for dissociation under any of the conditions described above has been observed. This is further substantiated by its behavior during electrophoresis, in the ultracentrifuge, in countercurrent distribution, and during starch and paper chromatography. On the other hand, presence of peptide linkages in the biologically active material is demonstrated by the fact that it is destroyed by trypsin, carboxypeptidase, and by complete acid hydrolysis. Efforts are continuing toward the isolation of a pure adrenocorticotropic hormone.

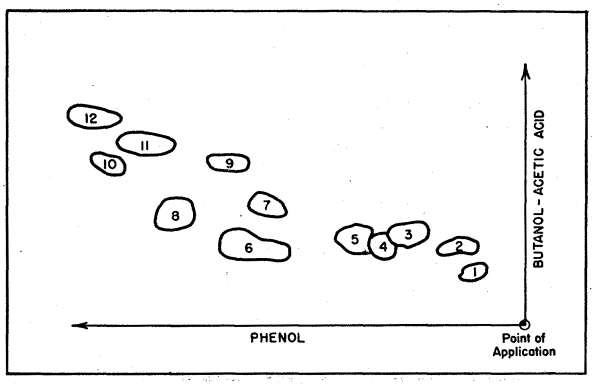

FIG. 3. Amino acid pattern for acid hydrolyzate of Fraction 11 from Experiment C1. Identification of spots is as follows: (1) cystine, (2) aspartic acid, (3) glutamic acid, (4) serine, (5) glycine, (6) lysine, (7) alanine, (8) arginine, (9) tyrosine, (10) proline, (11) valine (methionine), (12) leucine (isoleucine).

TABLE 3

SEPARATION OF ACTH ACTIVITY IN THE ULTRACENTRIFUGE

Sample	Initial potency × La-I-A	Fractions				
			Initial		Final	
		Position in cell	Proportion of activity %	Activity mg La-I-A	Activity mg La-I-A	Proportion of activity %
CA-1	15	Upper	26	25.4	13	11
		Middle	23	22.4	11	9
		Lower	51	49.7	100	80
		Total	100	97.5	124	
PC-2	35	Upper	18	41.5	11	15
		Middle	18	41.5	4	5
		Lower	63	142	56	80
		Total	100	225	70	

[1] Conducted by J. F. Roland and A. Gross of this laboratory.

[2] Department of Biology, MIT. A detailed report of the technique and results will be published shortly.

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Sanitary Seal for Infrared Microcells

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When biological extracts are analyzed through infrared absorption studies, cells having sample volumes of the order of 25 mm³ are often required. The sample is usually contained between two sodium chloride windows for infrared transparency and in a metal cell body for rigidity. Cells suitable for such work should have: (a) sample volumes consistent with adequate absorption thicknesses; (b) a construction which permits ease of assembly, filling, emptying, and cleaning; and (c) a seal between the windows and cell body which does not contaminate the sample or its solvent. Cells which have met requirements (a) and (b) have been reported (*2–5*), and seals based upon cemented (*5*), gasketed (*1, 2*), or amalgam-sealed joints (*3, 4*) have been described. However, because of the excellent solvent action of the commonly used diluents, CS_2 and $CHCl_3$, difficulties resulting from contamination, assembly, or accurate determination of absorption thickness have been experienced. The last-mentioned difficulties can be removed if the use of the intermediary material between the windows and cell body is eliminated and a seal resulting from adhesive attraction is used. A successful technique for such seals is described as it is used in a typical cell employed in ketosteroid absorption studies (Figs. 1 and 2).[3]

Both the sodium chloride windows and the cell body must be finished flat enough for adhesion to result. For the metal body, this is accomplished through a finishing technique of grinding and lapping before the cell fittings are added, but after all other machining operations have been completed. Superfinish can be substituted for lapping, but it is to be noted that flatness rather than smoothness alone is required. This limits the choice of material for the cell body, which should be easily machinable and have proper surface characteristics to permit finishing to optical flatness. Both these requirements can be met through the use of brass or mild steel for cell body machining and a hard, chemically inert plating for surface finishing. The cell body shown in the figures was made from mild steel and plated with hard chromium 0.001 in. thick. To prevent the slight increase in the plating thickness and a porous surface along the periphery of the cell body during plating, which was anticipated from the electrical gradient concentration and increased current density along the outer edge, the cell body was surrounded by a close-fitting ring ground with it to the same thickness. That similar precautions with regard to the fitting holes and cell openings were not required probably resulted from the small size of these openings relative to the body surface. The resulting surface is hard, a fact which permits finishing to optical flatness with cast iron laps and silicon carbide flour pastes.

The sodium chloride crystals were finished through the commonly used rouge lapping technique. Large soft

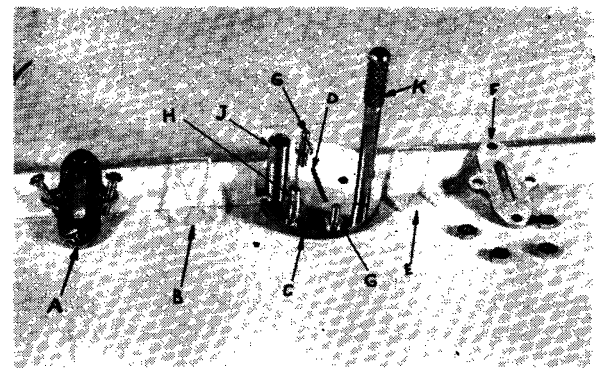

FIG. 1. Disassembled absorption cell. *A*, front cover plate; *B*, front sodium chloride window; *C*, cell body; *D*, front aperture; *E*, rear window; *F*, rear cover plate; *G*, cover plate studs (internally and externally threaded); *H*, hypodermic filling needle; *J*, syringe fitting for emptying and cleaning; *K*, handle. Holes in *C* connect *H* and *J* to the sample container.

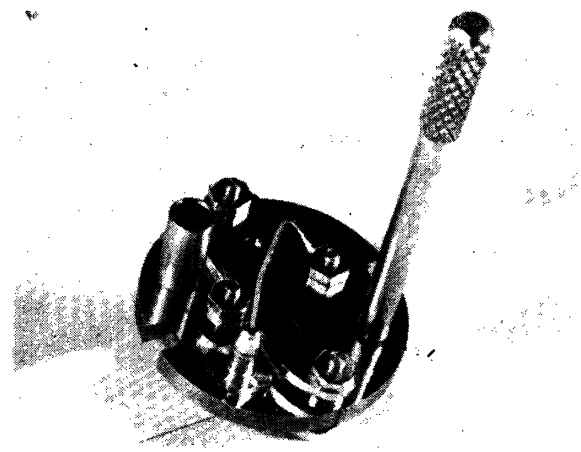

FIG. 2. Assembled absorption cell. Cell body dimensions: thickness 3.00 mm: diam 32.25 mm; front aperture 1.00 mm wide by 12.0 mm long; rear aperture 0.50 mm wide by 12.0 mm long. Window dimensions: 10 mm by 20 mm by 1 mm thick. Sample volume 25 mm³.

[1] At present at the National Cancer Institute, on assignment to MIT, Cambridge, Mass.

[2] The author is indebted to Drs. K. Dobriner and J. D. Hardy for presenting the problem and having the seal tested.

[3] The first cells of this type were due to the excellent efforts of Mr. R. J. Zabelicky, of the Sloan-Kettering Institute Instrumentation Shop.