References

1. LANE, W. R. *J. sci. Instrum.*, 1947, **24**, 98.
2. LEVVY, G. A. *J. sci. Instrum.*, 1947, **24**, 274.

Preparation of Nonprotein Fractions Possessing Adrenocorticotropic Activity from Fresh Sheep Pituitary Glands[1]

I. I. Geschwind, G. P. Hess, P. G. Condliffe, H. M. Evans, and M. E. Simpson[2, 3]

Institute of Experimental Biology and Department of Biochemistry, University of California, Berkeley

Frequently references have appeared in the literature to substances possessing adrenocorticotropic activity that are not protein in nature. Anselmino, Hoffmann, and Herold (*1*), Tyslowitz (*11*), and C. J. O. R. Morris and colleagues (*2, 8*) have obtained material possessing adrenocorticotropic activity by ultrafiltration of acid, neutral, or alkaline extracts of hog or ox pituitaries. Recently Li and co-workers (*4, 5, 7*) have found that the products of peptic and acid digestion of pure ACTH protein possess adrenocorticotropic activity.

We have recently demonstrated that some sheep ACTH protein preparations can be extracted with trichloracetic acid, with the resulting supernatants possessing increased amounts of activity per unit of nitrogen. These preparations may be dialyzed at certain pH's, and the activity is shown to pass through the dialysis membrane (*3*).

The preparation, from sheep pituitary glands, of nonprotein fractions possessing adrenocorticotropic activity has been undertaken in the hope that good amounts of activity may be so obtained. Five percent trichloracetic acid (TCA) was used as the solvent. In a typical experiment, 250 g of fresh sheep pituitary glands were ground in the cold (5° C) with 175 ml of water. To this mixture 375 ml of 10% TCA was added, and the resulting mixture was stirred for 4 hr in the cold, and then allowed to settle out overnight. The precipitate was removed by centrifugation in the cold, and discarded. The TCA supernatant so obtained was extracted 10 times with 200 ml aliquots of diethyl ether in order to remove the TCA. The solution was then lyophilized, and 2.9 g dry weight of a brownish, hygroscopic powder were obtained. Kjeldahl analyses revealed the powder to contain 10% nitrogen, of which about 10% was ammonia and amide nitrogen, and 43% was amino nitrogen. When an aliquot of the powder was subjected to 2-dimensional paper chromatography in phenol-water followed by lutidine-water (1 : 1), and the paper then sprayed with ninhydrin, it was found that its free amino acid pattern was similar to the patterns obtained from the hydrolysis of casein. In addition, several spots undoubtedly due to other components (peptides?) were obtained.

The powders were then subjected to assay for adrenocorticotropic activity by the ascorbic acid method of Sayers *et al.* (*9*) and by the repair test of Simpson *et al.* (*10*), and were shown to be active. By the ascorbic acid depletion method, a response equivalent to 10 γ of an ACTH protein isolated by Li was obtained from the injection of 0.5 ml/100 g body weight of a solution containing 0.65 mg of the powder per ml. Thus an activity equivalent to 360 mg of an ACTH protein could be obtained from 1 kg of fresh sheep glands.[4]

Previous experiments have shown that peptic hydrolyzates of ACTH, consisting of polypeptides, when chromatographed on paper using phenol-water as solvent, yield among others a fluorescent area, with an R_F value of 0.95, which when eluted and assayed showed considerable adrenal-stimulating activity, whereas the rest of the paper was almost devoid of activity (*6*). This was demonstrated, too, with whole ACTH protein (*3*). TCA supernatants of whole sheep pituitaries, when chromatographed as above, presented a similar fluorescent area at the front (Fig. 1), which, when eluted, was shown to have considerable adrenocorticotropic activity by the ascorbic acid test, whereas the rest of the paper contained minimal amounts of activity. In one such experiment a total of 28 mg of the powder was run on a series of papers, and the fluorescent areas were eluted, combined, and lyophilized, and then dissolved in 4 ml of water. The solution was assayed at a level of 0.5 ml/100 g body weight, and a maximal response was

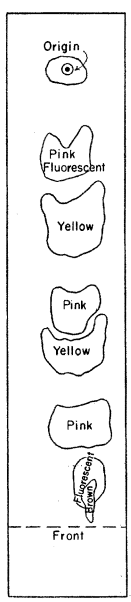

FIG. 1. Ascending chromatogram of 2 mg of the 5% TCA extract of fresh sheep pituitaries run in phenol-H_2O and developed with a 0.2% solution of ninhydrin in ethanol.

[1] Aided by grants from the U. S. Public Health Service RG-409 (C-2).

[2] With the technical assistance of Betsey S. Williams.

[3] We should like to acknowledge the support of C. H. Li in our experimental work, and the advice of John H. Northrop, who was kind enough to read this paper.

[4] In order to demonstrate further the nonprotein nature of the active material, 200 mg of the TCA extract powder was dialyzed for 3 days in the cold through a Visking sausage casing. The dialysate was lyophilized, and the resulting powder was made up to a concentration of 34 mg of the original powder per ml of solution. When assayed at 0.5 ml level, it showed a maximum response.

obtained. In our hands a maximal response corresponds to about 50 γ of our standard ACTH protein. Assuming, therefore, a minimum of an equivalent of 50 γ/0.5 ml, the original 28 mg contained a minimum of 400 γ of activity. Thus, a minimum of an activity equivalent to 170 mg of an ACTH protein can be obtained from 1 kg of fresh sheep glands.

It is of interest to note that the only other anterior pituitary hormone that could be demonstrated in these extracts was the follicle-stimulating hormone (FSH), which was apparently present in large quantity. However, it could be separated from the ACTH activity by dialysis, since the FSH did not pass through a dialysis membrane.

References

1. ANSELMINO, K. J., HOFFMANN, F., and HEROLD, L. *Klin. Wochschr.,* 1934, **13**, 209.
2. CROOKE, A. C., HENLY, A. A., and MORRIS, C. J. O. R. *Intern. Physiol. Cong.* (Abstr.), 1947, **16**, 139.
3. GESCHWIND, I. I., *et al. Science,* 1950, **111**, 625.
4. LI, C. H. *Conf. Metab. Aspects Conval.* (Trans. 17th meeting), 1948, **17**, 114.
5. LI, C. H., EVANS, H. M., and SIMPSON, M. E. *J. biol. Chem.,* 1943, **149**, 413.
6. LI, C. H., HESS, G. P., and GREENSPAN, F. S. Unpublished, 1949.
7. LI, C. H., and PEDERSEN, K. O. *Arkiv. Kemi.,* 1950, **1**, 533.
8. MORRIS, P., and MORRIS, C. J. O. R. *Lancet,* 1950, **258**, 117.
9. SAYERS, M. A., SAYERS, G., and WOODBURY, A. *Endocrinology,* 1948, **42**, 379.
10. SIMPSON, M. E., EVANS, H. M., and LI, C. H. *Endocrinology,* 1943, **33**, 261.
11. TYSLOWITZ, R. *Science,* 1943, **98**, 225.

Simple Calibrator for Warburg Respirometers[1]

P. F. Scholander, H. Niemeyer,[2] and C. Lloyd Claff[3]

Department of Biological Chemistry* and *the Laboratory for Surgical Research, Harvard Medical School, Boston, Massachusetts

The standard method of calibrating Warburg respirometers is to determine the volume by weighing with mercury. Less laborious methods have been devised, but most of them lack sufficient accuracy or simplicity. With the present simple calibrator a Warburg respirometer can be calibrated in a few minutes, accurately within 1%.

An accurately known volume of gas is withdrawn from the respirometer with a thermostabilized syringe device. The volume of the apparatus is calculated from the resulting change in manometer reading. The principle is well known and was applied in the Münzer-Neumann method (*1*).

The calibrator (Fig. 1) is essentially a syringe surrounded by a water jacket. The metal or plastic barrel is about 5 cm long and is drilled and reamed for a ⅛-in. drill rod, which serves as the plunger. The upper end of the barrel is provided with a short piece of tygon tubing, which provides an airtight seal for the plunger. A metal spring clip is fastened to the upper end of the barrel and presses lightly against the plunger. Two fine grooves (*C* and *D*) 3 cm apart are machined around the plunger. These serve to arrest the plunger accurately when they engage the metal clip. The apparatus is calibrated by measuring the diameter of the drill rod with a micrometer. The distance between the grooves is measured to within 0.1 mm. A slight film of grease travels with the plunger through the tygon seal, and adds to the volume displaced by the plunger. This amount was found to be negligible ($< 0.1\%$) when a light grease such as vaseline was used. The volume extracted by our calibrator is 234 mm³, known to within ± 0.2 mm³.

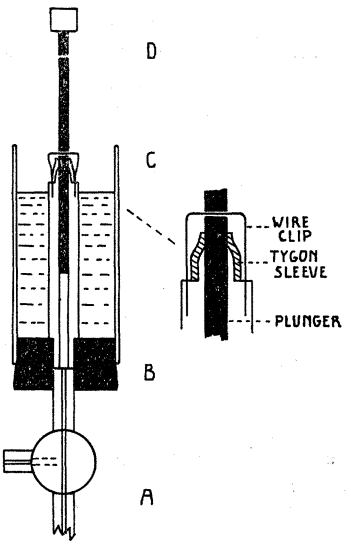

FIG. 1.

The volume of gas determined by the present procedure includes not only the volume of the Warburg apparatus up to the lower opening of the stopcock (Fig. 1, *A*) but, in addition, all air spaces included between *A* and *C*.

The volume *AB* can be determined by filling it with water from a tuberculin syringe. This volume in 10 of our apparatus averaged 80 mm³. The gas volume *B* to *C* can be kept negligible by having a flat connection at *B*, and the plunger in the low position, ending flush with *B*.

Each Warburg vessel is charged with a volume of water from a tuberculin syringe equal to *AB* (in our case 80 mm³). The respirometers are placed in the water bath at room temperature, and the water jacket of the calibrator is filled with water from the water bath.

After a period of thermoequilibration, the calibrator is attached to the respirometer (Fig. 1). A small amount

[1] This work was supported in part by a grant from the William F. Milton Fund.

[2] Fellow of the Guggenheim Foundation. In residence, Instituto de Quimica Fisiologica, Escuela de Medicina, Universidad de Chile, Santiago.

[3] We wish to acknowledge the assistance given us by John Andrews, machinist.