8. Kaplan, S. L., Grumbach, M. M., Nature, 1962, v196, 336.
9. Roos, P., Fevold, H. R., Gemzell, C. A., Biochim. et Biophys. Acta, 1963, v74, 525.
10. Dische, Z., in The Nucleic Acids, Cargaff, E., Davidson, J. N., Eds., Academic Press, New York, 1955, v1, 285.
11. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., J. Biol. Chem., 1951, v193, 265.
12. Greenspan, F. S., Li, C. H., Simpson, M. E., Evans, H. M., Endocrinol., 1949, v45, 455.
13. Kalnitsky, G., Hummel, J. P., Resnick, H., Carter, J. R., Barnett, L. B., Dierks, C., Ann. N. Y. Acad. Sci., 1959, v81, 542.
14. Hilmoe, R. J., J. Biol. Chem., 1960, v235, 2117.
15. Leaver, F. W., Anal. Biochim., 1966, v14.
16. Sober, H. A., Peterson, E. A., in Ion Exchangers in Organic and Biochemistry, Calmon, C., Kressman, T. R. C., eds., Interscience, N. Y., 1957, p318.
17. Li, C. H., in Symposium on Protein Structure, Neuberger, A., ed., Methuen, London, 1958, p302.
18. Fiske, C. H., Subbarow, Y., J. Biol. Chem., 1925, v66, 375.
19. Dixon, J. S., Li, C. H., J. Gen. Physiol., 1962, v45, 176.
20. Meisinger, M. A. P., Cirillo, V. J., Davis, G., Reisfeld, R. A., Nature, 1964, v201, 820.
21. Markham, R., Smith, J. D., Biochem. J., 1952, v52, 565.
22. Adams, E., Smith, E. L., J. Biol. Chem., 1951, v191, 651.

Received January 17, 1966. P.S.E.B.M., 1966, v122.

Separation of Human Growth Hormone into Two Components—GHA and GHB. (31089)

F. W. Leaver (Introduced by S. Katsh)
Research Laboratories, VA Hospital and Department of Pharmacology, University of Colorado School of Medicine, Denver

Korner(1) and other investigators(2,3) have found that growth hormone (GH) stimulates nucleic acid synthesis when given *in vivo*. GH can also stimulate protein synthesis (4,5,6). It has also been reported that GH stimulates protein synthesis *in vitro* without stimulation of nucleic acid synthesis(7). A logical interpretation of these data would be that there are two factors comprising GH: one factor responsible for the direct stimulation of protein synthesis and the other for nucleic acid synthesis. In the absence of evidence relating to this possibility, a study was undertaken to evaluate this interpretation.

The present study provides evidence that the GH is comprised of two components; are necessary for maximum tibia response.

Materials and methods. The alkaline extract (extract #2) of human pituitaries was obtained from the Human Protein Hormone Bank, Veterans Administration Hospital, Denver, Colo. The dried alkaline extract was dissolved in dilute ammonium hydroxide (pH 10-11) or 1 M K_2HPO_4 and neutralized with dilute acid (1 M) to the required pH. If a precipitate formed during the neutralization, it was removed by centrifuging. The precipitate contained little or no GH activity.

Enzymatic digestion of the alkaline extract was performed as follows: For 100 mg of extract, 0.35 ml of 1 M K_2HPO_4 was added with thorough mixing. This was followed by 1.66 ml of 0.3 M KCl and mixed; 1.64 ml of water was added and the pH adjusted to 7.6 with 1 M NaH_2PO_4. Five mg of ribonuclease as a dry powder was added. The mixture was incubated for 1 hr at 37°C. If a further digestion by spleen phosphodiesterase was performed, solid EDTA was added to give 0.01 M solution and the pH lowered to 6.6 with 1 M NaH_2PO_4. Two units of enzyme in 1 ml of water were added. The mixture was digested for 1 hr at 37°C.

Electro-membrane partition was performed by the method previously reported(8), using glycine as a buffer. The solution in the cathode chamber was continuously mixed with the anode. Membranes of known pore size were inserted between the chambers so that a separation by molecular size was obtained.

Extract #2 was applied to Sephadex G100 chromatogram columns equilibrated with 0.1

TABLE I. Loss of Tibia Activity upon Dialysis of Enzymatically Digested Extract #2.

Exp No.	Dialyzing solution	Initial wt, mg	% Not dialyzable	Tibia activity Initial	Tibia activity Final	% Tibia activity recovered
1	.5 M urea	61.3	36	.71	.58	29.4
2	.03 M ammonium acetate	18.7	62	.43	.39	56
3	.08 M ammonium acetate	18.5	64	.88	.33	24
4	.05 M ammonium acetate	100.1	71.7	1.0	.3	22

The undigested controls did not lose biological activity under the above conditions. Samples which remained in contact with dialyzing solution under same conditions but not dialyzed, did not lose biological activity. All samples given in Table were digested with 5% ribonuclease. The sample in Exp 4 was also digested with phosphodiesterase. Ten ml of the samples were placed in cellulose dialyzing tubing and dialyzed 18 hr, 4°C against 400 ml of dialyzing solution for Exp 1, 2 and 3. For Exp 4, 30 ml of the sample was placed in an electrodialyzer with cellulose dialyzing membranes and dialyzed until the current indicated no ionic migration. Exp 1, 2 and 3 dialyzed at pH 7.6, Exp 4 at 6.6.

M ammonium formate pH 6.3. The eluted fractions were lyophilized directly. The dried material was redissolved in water and relyophilized. This removed most of the ammonium formate. The final traces of ammonium formate were removed by 6 hours dialysis against distilled water at 4°C in a rocking arm dialyzer and again lyophilized.

Growth hormone was assayed according to the method of Greenspan *et al*(10).

Results. Table I shows that 44 to 78% of the total biological activity of the enzymatically digested extract was lost during dialysis against urea or ammonium ion. Control experiments in which the digested extract was in contact with the salts for the same time as the dialyzed experiments indicated no loss of activity; hence, the loss of activity was not due to an instability of the GH. It was also shown that the dialyzable material in the presence of the 0.08 M ammonium acetate was inactive by tibia assay. However, when the dialyzable component obtained in the presence of 0.03 M ammonium acetate was recombined with the non-dialyzable component the activity was 0.53 U/mg indicating a 100% recovery of activity. These results suggested that some component of the enzymatically digested GH was dialyzable (in the presence of ammonium ion or urea) which in itself was not active but which augmented the biological activity of the non-dialyzable component.

It is to be noted that electro-dialysis of the ribonuclease-phosphodiesterase digested extract in 0.5 M ammonium acetate resulted in 78% loss of total activity. Since ammonium acetate can be removed by repeated lyophilization it was possible to obtain all components free of salts under these conditions.

Table II gives the results of another experiment in which all components were recovered. It can be seen by Table II that

TABLE II. Separation of Tibia Activity into Two Fractions by Electrodialysis.

Fraction	Wt, mg	Assay, U/mg	% GH recovered
Centrifuged precipitate	17.7	< .10	0
Cathode	1.8	<(.1)*	0
Anode	.9	<(.1)	0
Dialysis chamber supernatant	2.7	.59	4.3
Precipitate	13.3	.47	16.7
Recovery	36.4		
Initial material	45.0	.83	
% Recovery	80.9		21.0
Recombined all fractions except anode		1.12	109.0
Cathode and center chamber supernatant		.95	

* The tibia assay was not performed on this material. Two other experiments gave <0.1 U/mg of GH in these fractions. The initial activity (0.83) is before the enzyme digest. After the enzyme digest the activity was 1.02. In this experiment the initial material (50 mg) was put into solution with 2 ml of dil. NH_4OH (final pH 9). pH was lowered to 7.6 by 0.5 M acetic acid and digested with 2.5 mg ribonuclease at 37° for 30 min. pH was further lowered to 6.6 by 0.5 M acetic acid and digested another 30 min at 37° with 1 unit (0.7 mg) of spleen phosphodiesterase. Aliquots were taken for assays. The remainder was centrifuged and the precipitate washed 2 times with 2.0 ml of 0.1 M ammonium acetate at pH 6.6. The washings were combined with the supernatant and placed in center chamber of electrodialyzer. The anode and cathode chambers were isolated by a cellulose dialyzing membrane (4.8 mμ). Initial voltage gradient was 2 V/cm. At end of the first hour, the voltage gradient had climbed to 70 V/cm. The run continued 4 more hours.

80.9% by weight of the material was recovered, but only 21% of the biological activity remained. However, when all fractions except the anodal fraction were recombined in equivalent proportions to the fraction weight, the recovery of biological activity was 109%. For the recombination experiment, 0.2 mg of cathode, 0.2 mg of dialysis chamber supernatant, 1.5 mg of dialysis chamber precipitate and 1.4 mg of centrifuged precipitate were mixed and assayed. This mixture assayed at 1.12 U of GH/mg, and gave an activity recovery of 109%.

$$\frac{1.12 \times 36.4 \times 100}{37.35} = 109\%$$

Hence, there is no inactivation of the GH during the experiment. Since only 21% of the total activity was recovered in the individual fractions, but 100% was recovered upon recombination of the parts, it implies that at least two components are necessary for full tibia activity. One may well question the accuracy of the tibia assay as to the significance of an assay value of 0.5 as compared to 1.1. However, if the total activity of the recombined material were to reside in the 2 active fractions (*i.e.*, supernatant and precipitate), their average activity would have to be 2.0 U/mg to account for the observed activity of the recombined fractions. It certainly appears unlikely that the tibia assay would have a 4-fold error, and it is even more unlikely that the fractions reported as zero have an actual activity greater than one. If only the supernatant of the center chamber and cathode material were recombined, the activity was 0.95 as compared to an expected 0.35. With modification, this type of experiment has been repeated twice with the same results. Hence, it is evident that dialysis, electrodialysis or electropartition do not destroy the activity. Instead it appears that two factors are necessary for the tibia growth and one of the factors is dialyzable in the presence of ammonium ion. The dialyzable component was termed GH-B. The non-dialyzable component was designed as GH-A.

In the above experiment all ribonuclease and spleen phosphodiesterase were present in the center chamber. It was found that these enzymes could be separated from GH-A if a glycine buffer and a 100 mμ membrane were used(8). An enzyme digest of extract #2 was subjected to electropartition in 0.1 M glycine buffer adjusted to a pH of 3.8 with acetic acid. Under these conditions the dialyzable component GH-B readily passed through Visking dialyzing membrane and GH-A readily passed through a 100 mμ membrane. Since GH-A passed through a 100 mμ membrane the molecular weight appears to be less than 13,000. Before enzymatic digestion as much as 70% of the GH of some preparations could not pass through a 1,000 mμ membrane indicating a molecular weight greater than 250,000(8). This confirms the observations that enzymatic digestion brings about a marked decrease in the molecular weight.

The enzymes are unable to pass through the 100 mμ membrane. This was determined by the observation that full enzymatic activity was recovered in the first chamber and no activity could be detected in the second chamber. The GH-B was isolated from the buffer by 8 hours dialysis against distilled water in a rocking arm dialyzer. The material was lyophilized. The GH-A obtained from the second chamber indicated 0.60 U/mg at 45 μg level in tibia assay. No tibia activity was noted in the buffer at 45 μg level. The total recovery of the activity from the individual fractions was 42%. When combined, 20 μg GH-A and 12 μg GH-B gave a tibia activity of 1.3 U/mg. This indicated a 95% recovery of activity. The starting material had an activity of 0.7 U/mg. In this set of tibia assays the material to be tested was dissolved in saline containing 0.01% bovine serum albumin to prevent adsorption of GH on glass(9).

To test the possibility that GH-A could be separated from GH-B chromatographically, a Sephadex G100 column was prepared using 0.1 M ammonium formate as the eluant. Fig. 1 gives the results of such an experiment. It is to be noted that fraction 3 was cut well before its minimum and pooled into fraction 4.

Fractions 2 and 3 were rechromatogrammed. Fraction 3 again was cut where indi-

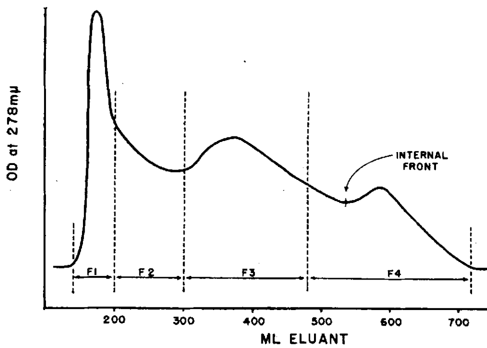

FIG. 1. Separation of GH-B and GH-A by Sephadex Chromatography. Bed volume of Sephadex G100 was 40 x 390 mm. Two hundred mg extract #2 was eluted with 0.1 M ammonium formate pH 6.3 in 2 runs. Fraction 2 was pooled and rechromatogrammed. Fraction 3 was also pooled and rechromatogrammed.

cated in Fig. 1 and pooled into fraction 4. Table III gives the analytical results, which clearly demonstrate that recombination resulted in a marked increase in GH activity. By summation of individual fraction activities, 45.1% of the activity was recovered; by recombination, 97.5% was recovered. To obtain a 97% recovery of activity from the individual parts, fractions 2 and 3 would have to have an average activity of 1.7 U/mg instead of the observed 0.8 U/mg. This is a 2-fold error which is unlikely to be encountered in both assays. Nor is it likely that fractions 1 and 4 contain any appreciable activity. This experiment corroborates the finding that more than one component is necessary for tibia activity.

Discussion. The observation that crude growth hormone is composed of 2 components

TABLE III. Analysis of Fractions from Sephadex G100 Column Illustrated in Fig. 1.

	Weight		Tibia assay		
Fraction	mg	%	Specific activity, U/mg	Total U	% Recovered
1	29.1	23.9	0	0	0
2	25.9	21.2	.57	14.8	15.2
3	29.2	23.9	1.00	29.2	29.9
4	27.6	22.6	0	0	0
Sum	111.8	91.6		44.0	45.1
Initial	122.0		.80		
% Recovered		91.6			45.1
Recombined			.85*	97.6	97.5

* Recombined 1 mg each of fractions 1, 2, 3 and 4. The arithmetic average activity is .39.

greatly complicates its purification. It is evident that an assay must be developed which responds to only one component. If the tibia assay is to be employed, one component must be present in excess in order to accurately assay the other component. The component present in excess must be relatively free of the component being assayed in order for the assayed component to be the limiting factor. The latter requirement appears to be met as GH-B has no significant activity. If GH-B were contaminated with GH-A, some tibia activity would be expected.

It appears that only the digestion of the alkaline extract by ribonuclease is necessary in order to separate GH-B from GH-A. This is evident from the experiments given in Tables I and III. However, a more complete separation is obtained if a phosphodiesterase digestion is also performed as indicated by the lesser recovery of biological activity before recombination as shown in Tables I and II.

It is evident that GH-B is bound rather tightly to GH-A or a similar molecule, for we have not been able to separate the two components in the absence of ammonium ions or an amine. Dialysis of the digested extracts in the presence of K^+, Na^+, HCO_3^-, $HPO_4^{=}$ or acetate did not result in any detectable loss of activity.

These observations may explain in part the variable tibia assays obtained from one preparation to another. If one component is low in a particular preparation, then the observed recovery of biological activity would be low. The method of isolation of GH in most preparations is such that much, if not all, of the nucleic acids would be removed. Therefore, those procedures where the preparation is dialyzed in the presence of amines or ammonium ion would likely result in some loss of GH-B resulting in a variable loss of tibia activity. This is particularly noticeable in the low recoveries of activity obtained when the GH was precipitated by ammonium sulfate and subsequently dialyzed(11,12).

The observation that the rat tibia responds equally well to the aggregate GH as to the digested hormone is probably due to the fact that in the animal the crude hormone aggre-

gate comes in contact with enzymes which break down the aggregate to produce a hormone molecule which is active at the cellular level.

Conclusion. Human growth hormone has been shown to be composed of two components as indicated by tibia assay. One of the components at neutral pH will pass through cellulose dialyzing tubing in the presence of ammonium ion, urea or glycine.

The author is grateful to Dr. H. Elrick for his generosity in supplying adequate amounts of Extract #2 used in this study and to V. Yearwood-Drayton for his preparation of Extract #2. The technical assistance of Y. Arai and E. Martinez for the tibia assays is gratefully acknowledged. Finally, the many discussions with Dr. Teh Lee and Dr. S. Katsh were very helpful.

1. Korner, A., Biochem. J., 1964, v92, 449.

2. Talwar, G. P., Gupta, S. L., Gros, F., ibid., 1964, v91, 565.

3. Bransom, E. D., Reddy, W. J., Endocrinology, 1961, v69, 997.

4. Kostyo, J. L., ibid., 1964, v75, 113.

5. Herrera, M. G., Renold, A. E., ibid., 1964, v75, 222.

6. Goodman, H. M., ibid., 1963, v73, 421.

7. Kostyo, J. L., Porcaro, M. A., 1965, Abst. 33, Endocrine Soc. Meeting, 1965.

8. Leaver, F. W., Anal. Biochem., 1966, v14.

9. Greenwood, F. C., Hunter, W. B., Biochem. J., 1963, v89, 114.

10. Greenspan, F. S., Li, C. H., Simpson, M. E., Evans, H. M., Endocrinology, 1949, v45, 455.

11. Elrick, H., Yearwood-Drayton, V., Arai, Y., Leaver, F., Morris, H. G., J. Clin. Endorin. & Met., 1963, v23, 694.

12. Wilhelmi, A. E., Canad. J. Biochem., 1961, v39, 1660.

Received January 17, 1966. P.S.E.B.M., 1966, v122.

Myocardial Depressant Factor in Plasma from Cats in Irreversible Post-Oligemic Shock.* (31090)

EUGENE D. BRAND AND ALLAN M. LEFER (Introduced by D. R. H. Gourley)
Departments of Pharmacology and Physiology, University of Virginia School of Medicine, Charlottesville

Post-oligemic shock, irreversible to blood transfusion therapy, has been reversed by therapy with large volumes of water and solutes in the dog(1) and in the cat(2). Because in the studies of Brand *et al*(2), blood or low molecular weight dextran did not reverse post-oligemic shock, it was reasoned that simple cardiovascular support did not wholly account for this effect. The positive correlation of a large urine volume with survival incidence and duration suggested that removal of a toxic factor from the plasma into the urine might be a major mechanism in the observed reversal of shock. Several authors (3,4,5) have found myocardial weakness in irreversible post-oligemic shock. Other workers have postulated the presence of a toxic factor in the blood of rabbits and dogs in post-oligemic shock(4,6,7,8,9) which might act by depressing the heart. In apparent contrast to these findings *in vivo, in vitro* studies of papillary muscles removed from rats and cats early in shock and bathed in a buffer solution show them to function normally (10,11). Taken together, these observations suggest that a myocardial depressant factor is present in the plasma during shock. This substance may diffuse out of the muscle into the bath solution, so that the muscle then functions normally.

In the present investigation we used isolated papillary muscles to test this hypothesis.

Methods. Shock was induced by the method of Brand *et al*(2) in healthy adult cats of either sex. The cats were anesthetized with pentobarbital (35 mg/kg i.p.), heparinized (2250 units/kg), and bled from the femoral artery into a reservoir in which the pressure was maintained between 40 and 45 mm Hg.

* This work was supported by U.S.P.H.S. Grants HE-01942 and HE-09924 and a grant from Virginia Heart Assn.