Lipemia-Producing Activity of Pituitary Gland: Separation of Lipemia-Producing Component from Other Pituitary Hormones.* (25502)

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(Introduced by David Seegal)
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Our previous reports(1,2,3) described lipemia-producing activity of a crude extract derived from human, sheep, beef or hog pituitary glands. The lipemia-producing activity was present largely in anterior lobe of the gland(2). The 6 recognized anterior lobe hormones, when tested individually, had no effect upon rabbit's serum lipids(2). It was therefore concluded that lipemia-producing activity of crude pituitary extract is caused by the synergistic action of 2 or more of the recognized pituitary hormones, and/or by a pituitary substance which is different from recognized pituitary hormones. Our purpose was to identify the substance or substances in the pituitary gland, responsible for the lipemic effect. This report presents evidence that the pituitary gland contains a lipemia-producing component which is different from the recognized pituitary hormones. Concentration of this component into a fraction ("Fraction H") which is free from recognized pituitary hormones is described. Lipemia-producing activity of Fraction H is potentiated by simultaneous injection of ACTH‡ Production of lipemia in the rabbit by simultaneous injection of ACTH and commercial preparations of TSH, prolactin, or FSH, is also described. A possible relationship between synergism of ACTH with Fraction H, and synergism of ACTH with commercial preparations of TSH, prolactin and FSH, is discussed.

Materials and methods. Lyophilized intact hog pituitary glands were obtained from Armour Labs. Commercial preparations of anterior pituitary hormones, obtained from Armour and Wilson Labs, were derived from pituitary glands of the following species: ACTH, from hog pituitaries; prolactin and FSH, from sheep pituitaries; GH and TSH, from beef pituitaries; ICSH, from horse pituitaries. IRC-50 (CG-50, Type 2) cation exchange resin, furnished by Rohm & Haas Co., was prepared for use by the method of Hirs *et al.*(4). Lipemia-producing activity of various pituitary fractions was assayed, as previously described(2), by measuring change in serum total lipids of the rabbit 18 hours after a single subcutaneous injection. Other pituitary hormonal activities were assayed by methods described as follows: ACTH(5), GH (6), TSH(7), prolactin(8), ICSH(9), FSH (10), oxytocin and vasopressin(11).

Results. Variation in serum lipid level of rabbits treated with a single subcutaneous injection of H_2O was first studied to establish what magnitude of change in serum lipid level of rabbits treated experimentally with pituitary material should be considered statistically significant. The results are shown in Table I. Average change in serum lipid level of 15 rabbits treated with H_2O was +50 mg%. Estimated standard deviation of the change was $\pm$ 142 mg %. In subsequent experiments, the statistical significance of changes in serum lipid level of rabbits treated with pituitary material was evaluated by comparison with findings in the group of rabbits treated with H_2O. Thus, in a group of 4 rabbits injected with experimental material, an increase in the group's average serum lipid level which was less than 380 mg% was considered statistically not significant ($p>0.01$).

The effects upon rabbit's serum lipid level of commercial preparations of the 6 recognized anterior lobe hormones, when tested separately, are described in Table I. Dosages at which ACTH, GH, and TSH were tested

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‡ The following abbreviations are used: ACTH, adrenocorticotropin; GH, growth hormone; TSH, thyroid-stimulating hormone; ICSH, interstitial-cell stimulating (luteinizing) hormone; FSH, follicle-stimulating hormone.

TABLE I. Effect of Various Pituitary Fractions upon Serum Lipid Level of Rabbit, Which Was Measured before, and 18 Hours after, Single Subcutaneous Injection. Following hormone preparations were employed: ACTH, Wilson #104746; GH, Armour #R50109; TSH, Armour #T3308; Prolactin, Armour #U10303; ICSH, Armour #T10812; FSH, Armour #R19911.

Material inj.	No. rabbits	Change in serum total lipid (mg %)
5 ml distilled H_2O	15	+ 50 ± 35*
60 mg lyophilized hog pituitary glands	5	+1450 ± 205
120 units ACTH	5	+ 10 ± 69
12.5 mg GH	5	+ 10 ± 54
25 " "	8	+ 190 ± 64
10 units TSH	7	+ 60 ± 39
25 mg Prolactin	5	+ 110 ± 48
25 " ICSH	6	− 40 ± 64
25 " FSH	8	+ 20 ± 39
60 units ACTH		
+ 6 mg GH	5	+ 270 ± 144
+ 4.6 units TSH	5	+1050 ± 220
+ 12.5 mg Prolactin	4	+ 810 ± 376
+ " " ICSH	4	+ 320 ± 251
+ " " FSH	4	+ 520 ± 233
6 mg GH		
+ 4.6 units TSH	5	+ 130 ± 37
+ 12.5 mg Prolactin	4	+ 100 ± 61
+ " " ICSH	4	+ 70 ± 96
+ " " FSH	4	+ 30 ± 27
4.6 units TSH		
+ 12.5 mg Prolactin	4	+ 100 ± 134
+ " " ICSH	4	+ 130 ± 226
+ " " FSH	4	+ 130 ± 146
12.5 mg Prolactin		
+ 12.5 mg ICSH	4	+ 210 ± 90
+ " " FSH	4	+ 30 ± 52
12.5 mg ICSH + 12.5 mg FSH	4	+ 10 ± 45

* Avg and stand. error of change in serum total lipid of group.

separately represent amount of each hormone present in 0.5 g or more of desiccated pituitary gland. Prolactin, ICSH and FSH were tested at a dosage of 25 mg§. At these dosage levels, these hormones did not have a statistically significant effect upon rabbit's serum lipid level. Table I also shows marked increase in rabbit's serum lipid level produced by a single injection of an alkaline extract of 60 mg of desiccated hog pituitary gland.

The effect upon rabbit's serum lipid level of injection of various combinations of commercial preparations of the 6 anterior lobe hormones is also shown in Table I. Dosages at which ACTH, GH and TSH were tested in combination, represent amount of each hormone present in 0.2 g of desiccated hog pituitary gland. Prolactin, ICSH and FSH were tested in combination at a level of 12.5 mg§. Table I shows that when ACTH was injected together with TSH, prolactin or FSH, a statistically significant increase was produced in average serum lipid level of treated rabbits. The combinations ACTH + GH, ACTH + ICSH, and the various combinations which did not include ACTH, did not have a statistically significant effect upon rabbit's serum lipid level.

The effect of the combination ACTH + TSH was then reexamined, in experiments which employed 2 different purified preparations of TSH supplied by Dr. R. W. Bates (Nat. Inst. Health). Dosages of ACTH and purified TSH were the same as in experiments described in Table I. The combination ACTH + purified TSH did not have a statistically significant effect upon average serum lipid level of treated rabbits. This lack of effect of the combination ACTH + purified TSH indicates that the lipemic effect of the combination ACTH + commercial TSH shown in Table I, is largely due to synergism of ACTH with some "contaminating" substance in the commercial TSH preparation, rather than to synergism of ACTH with TSH itself. The possibility of a similar basis for the effects of ACTH + commercial prolactin, and ACTH + commercial FSH, has not yet been investigated.

Information was now sought as to whether the synergistic action of ACTH with other pituitary substances is responsible for the entire lipemia-producing activity of crude pituitary extract. In preliminary experiments(12), a crude saline extract of hog pituitary glands was fractionated by the method of Bonsnes and White(13). Lipemia-producing activity was found only in material precipitated between 50% and 90% acetone concentration ("acetone 50-90% fraction"). About one-half of the lipemia-producing activity of pituitary glands used as starting material was re-

§ Concentration of these hormones in desiccated hog pituitary glands is uncertain.

covered in the acetone 50-90% fraction. The major portion of recovered lipemia-producing activity was found in the acetone 75-90% fraction(12). Bonsnes and White demonstrated that the acetone 50-90% fraction is free of ACTH, GH, and prolactin(13). They also showed that the acetone 75-90% fraction is free of TSH as well(13). These hormones are precipitated in earlier fractions in the Bonsnes-White procedure. These observations indicate that the synergistic action of ACTH is responsible for only a part of the total lipemia-producing activity of the pituitary gland, and demonstrate the presence, in acetone 75%-90% fraction, of a lipemia-producing substance which is different from ACTH, GH, TSH and prolactin. A fractionation method, based on Bonsnes-White procedure, was then devised for concentration of this lipemia-producing substance. This method is described below.

All operations are carried out at 0-5°C. Five g of lyophilized intact hog pituitary glands are pulverized and extracted for 4 hours with 150 ml 2% NaCl solution, the extraction mixture being maintained at pH 8.5 by periodic addition of 1 N NaOH. The insoluble material is removed by centrifugation and discarded. The supernatant is adjusted to pH 4.3 by dropwise addition of 0.4 N HCl. The precipitate is removed by centrifugation and discarded. The supernatant solution is adjusted with H_2O to a volume of 100 ml and an equal volume of acetone is added with constant stirring for 5 minutes. The precipitate (acetone 0-50% fraction) is removed by centrifugation and discarded. To the supernatant solution, 200 ml of acetone is added with constant stirring for 5 minutes. The precipitate (acetone 50-75% fraction) is again removed by centrifugation and discarded. To the supernatant solution, 600 ml of acetone is added with constant stirring for 5 minutes. The precipitate (acetone 75-90% fraction) is collected by centrifugation and dissolved in 40 ml H_2O. This solution is adjusted to pH 5.80, dialyzed 8 hours against H_2O and lyophilized. The yield of lyophilized material, called Fraction G, is 15 mg from each gram of desiccated pituitary glands.

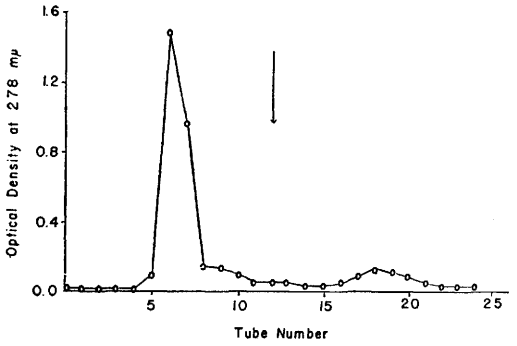

FIG. 1. Chromatogram of 100 mg Fraction G on 2.2 × 30 cm column of IRC-50 resin. Effluent collected in 10 ml fractions. Initial solvent, 0.20 M sodium phosphate, pH 5.80. At arrow, solvent changed to 1.0 M NaCl. Temp. 25°.

TABLE II. Effect of Fraction H upon Serum Lipid Level of Rabbit. Serum total lipid was measured before, and 18 hours after, a single subcutaneous injection.

Material inj.		No. rabbits	Change in serum total lipid (mg %)
Fraction H	1.5 mg	4	+ 240 ± 50*
	2 "	7	+ 510 ± 164
	3 "	4	+1010 ± 142
	5 "	5	+2080 ± 176
0.5 mg Fraction H + 60 units ACTH†		8	+ 500 ± 162
1 mg Fraction H + 60 units ACTH		5	+ 790 ± 241
120 units ACTH		5	+ 10 ± 69

* Avg and stand. error of change in serum total lipid of group.

† ACTH preparation employed was Wilson #104746.

One hundred and twenty mg of Fraction G is dissolved in 15 ml of 0.2 M sodium phosphate buffer, pH 5.80. Insoluble material is removed by centrifugation and discarded. The supernatant solution is placed on 2.2 x 30 cm column of IRC-50 resin, which has been equilibrated to pH 5.80 with 0.2 M sodium phosphate buffer.‖ Operation of the column is described in Fig. 1. The effluent is collected in 10 ml fractions, and protein content of each fraction is estimated from optical density at 278 mμ. Lipemia-producing activity is concentrated in the first fraction (tubes 5 through 10), which contains about 30% of the material placed on the column. These tubes are pooled and $(NH_4)_2SO_4$ added to give a

‖ Before use the column is washed with buffer until pH of effluent is 5.80.

concentration of 42 g % of $(NH_4)_2SO_4$. The resulting precipitate is collected by centrifugation, dissolved in H_2O, dialyzed for 8 hours against H_2O and lyophilized. The yield of lyophilized material, called Fraction H, is 5 mg/g of desiccated pituitary glands.

Fraction H was assayed for lipemia-producing activity and also assayed for other pituitary hormonal activities. The effect of Fraction H upon rabbit serum lipid level is described in Table II. When injected separately at a dose of 3 mg, Fraction H caused an average increase of 200% in rabbit serum lipid level.¶ When injected with 60 units of ACTH, the effect of Fraction H was intensified. As little as 0.5 mg of Fraction H, when injected with 60 units of ACTH, caused a statistically significant increase in average serum lipid level of treated rabbits.

Fraction H was assayed for other pituitary hormonal activities.** The results in Table III show that none of the recognized anterior or posterior pituitary hormones is present in Fraction H in a proportion of more than 0.8% by weight. To determine whether the lipemia-producing activity of Fraction H might be due to the combined effects of these minute amounts of recognized pituitary hormones, a mixture of recognized hormones was made according to maximal proportions in which they can be present in Fraction H. This mixture was injected into rabbits at various dosages, the highest dose containing maximal amount of each pituitary hormone which can be present in 25 mg of Fraction H. No statistically significant effect upon rabbit serum lipid level was observed.

TABLE III. Results of Bioassays of Fraction H. Left-hand column shows hormonal assays performed. Middle column gives result of each assay. Right-hand column gives proportion in which each of pituitary hormones is present in Fraction H. These proportions were calculated by comparing hormonal activities of Fraction H with activities of highly purified preparations of each of the pituitary hormones.

Hormonal activity assayed	Hormonal activity of Fraction H	Proportion in which each pituitary hormone is present in fraction H (%)
ACTH	<.04 USP units/mg	<.03
GH	<.01 *idem*	<.8
TSH	.01 "	.07
Prolactin	<.0002 I.U./mg	<.001
ICSH	No activity detected at total dose of 4 mg	<.5
FSH	No activity detected at total dose of 2.5 mg	<.06
Oxytocin	.012 unit/mg	.002
Vasopressin	<.010 "	<.004

Discussion. By modification of the Bonsnes-White fractionation method, a pituitary fraction (Fraction H) has been prepared which is highly active in producing lipemia in the rabbit. Three mg of Fraction H has approximately the same effect upon rabbit serum lipid level as an alkaline extract of 60 mg of lyophilized hog pituitary glands (Tables I, II). Results of biological assays show that none of the recognized anterior or posterior pituitary hormones is present in Fraction H in a proportion of more than 0.8% by weight. A mixture containing maximal amounts of the recognized pituitary hormones which can be present in 25 mg of Fraction H had no effect upon rabbit serum lipid level. These observations indicate that Fraction H contains a lipemia-producing substance which is different from the recognized pituitary hormones.

Only a small proportion (about 10%) of lipemia-producing activity of hog pituitary glands is recovered in Fraction H. This low recovery may result either from losses of the lipemia-producing substance in fractions dis-

¶ Chemical analyses of sera obtained from rabbits before and after injection of Fraction H show that the increment of serum lipids following injection is composed largely of triglycerides (85%), together with small proportions of cholesterol (5%) and phospholipids (10%). Serum lipid level begins to increase 6 to 10 hours after the injection and remains elevated for 12 to 24 hours. Serum nonesterified fatty acid level increases markedly within 1 hour after the injection and remains elevated for 12 to 24 hours.

** Assays were performed by the following: ACTH assay by C. N. Mangieri (South Mountain Labs., Maplewood, N. J.); GH assay by A. E. Wilhelmi (Emory Univ.); TSH assay by R. W. Bates (Nat. Inst. Health); prolactin assay by I. Levenstein (Leberco Labs., Roselle Park, N. J.); ICSH assay by S. J. Segal (Rockefeller Inst.); FSH assay by S. L. Steelman (Merck Inst.); oxytocin and vasopressin assays by F. C. Armstrong (Parke, Davis & Co.).

carded during fractionation procedure, or from partial inactivation of the lipemia-producing substance during fractionation. It must also be caused in part by removal of other pituitary hormones, especially ACTH, which may act synergistically with each other or with Fraction H to raise serum lipid level of the rabbit.

Interpretation of the lipemic effect produced by simultaneous injection of ACTH with certain preparations of TSH, prolactin or FSH (Table I), is complicated by the impure nature of commercially available hormone preparations employed. Two possible hypotheses to explain observations in Table I are suggested: ACTH may act in synergism with TSH, prolactin or FSH to increase circulating lipids of the rabbit; or ACTH may act in synergism with some "contaminating" substance present in commercially available preparations of these 3 hormones.

The synergistic action of ACTH upon rabbit serum lipids was greatest when ACTH was combined with the commercial preparation of TSH (Table I). Combination of ACTH with more highly purified TSH preparations did not have a statistically significant effect upon average serum lipid level of treated rabbits. This observation shows that, in the case of the combination ACTH + commercial TSH, ACTH acts in synergism principally with a "contaminating" substance in the TSH preparation, rather than with TSH itself. Similar studies, employing more highly purified preparations of prolactin and FSH, will be required to determine whether the apparent synergism between ACTH and each of these hormones, may also be due to presence of a "contaminating" substance in commercial preparations of these 2 hormones.

The observation that the lipemia-producing activity of Fraction H is enhanced by simultaneous injection of ACTH may provide a clue to the identity of the hypothetical "contaminating" substance in commercial preparations of TSH, prolactin and FSH. It is possible that the synergistic action of ACTH with commercial preparations of TSH, prolactin and FSH may be caused in whole or in part by presence in these preparations of small amounts of the same lipemia-producing substance, concentrated in Fraction H.

Our experiments provide no information concerning the mechanism by which ACTH exerts its synergistic action upon lipid metabolism of the rabbit. This action may be related to other "extra-adrenal" metabolic effects of ACTH(14), or it may depend upon stimulation of adrenal cortical secretion by ACTH.

These observations show that lipemia-producing activity of crude hog pituitary extract is caused by the combined actions of (a) lipemia-producing component in Fraction H; (b) ACTH, and (c) substances present in commercial preparations of TSH, prolactin and FSH. Further studies will be required before it will be possible to compare the contributions of each of these pituitary components to total lipemia-producing activity of the pituitary gland.

Summary. A method for fractionation of hog pituitary glands by saline extraction, fractional precipitation with acetone at pH 4.3, and ion-exchange chromatography, is described. The final fraction has high lipemia-producing activity in the rabbit and is free of recognized pituitary hormones. Lipemia-producing activity of this fraction is enhanced by simultaneous injection of ACTH. Production of lipemia in the rabbit by injection of ACTH together with commercial preparations of TSH, prolactin or FSH is also described. In the combination ACTH + commercial TSH, evidence is presented that ACTH acts in synergism with some "contaminating" substance in commercial TSH preparation, rather than with TSH itself.

1. Rudman, D., Seidman, F., *Circulation,* 1958, v18, 486.

2. ———, PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 146.

3. Rudman, D., Seidman, F., Reid, M. B., *J. Clin. Invest.,* 1959, v38, 1038.

4. Hirs, C. H. W., Moore, S., Stein, W. H., *J. Biol. Chem.,* 1953, v200, 493.

5. U. S. Pharmacopeia, 1955, XV revision, 176.

6. Marx, W., Simpson, M. E., Evans, H. M., *Endocrinol.,* 1942, v30, 1.

7. Bates, R. W., Cornfield, J., *ibid.,* 1957, v60, 225.

8. Lyons, W. R., Page, E., PROC. SOC. EXP. BIOL. AND MED., 1935, v32, 1049.
9. Segal, S. J., *Science,* 1957, v126, 1242.
10. Steelman, S. L., Pohley, F. M., *Endocrinol.,* 1953, v53, 604.
11. U. S. Pharmacopeia, 1955, XV revision, 776.
12. Rudman, D., Seidman, F., Reid, M. B., Abst. 13th Annual Meet., Am. Soc. for Study of Arteriosclerosis, 1959.
13. Bonsnes, R. W., White, A., *Endocrinol.,* 1940, v26, 990.
14. Engel, F. L., *Yale J. Biol. Med.,* 1957, v30, 201.

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Interpretation of the Master "2-Step" Exercise Electrocardiogram by Quantitative Analysis of RS-T Segment. (25503)

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This study was undertaken to formulate additional criteria for interpretation of the Master "2-Step" exercise test. While the significance of a negative test has been established(1), "false positive" responses occur not infrequently in healthy individuals. Our experiment was based on the electrophysiologic principle that *duration* of RS-T depression in a given cycle is more important than *extent* of its depression. Thus, RS-T segment depression due to current of injury produced by subendocardial ischemia should persist for a greater portion of ventricular systole than the depression associated with simple tachycardia, or benign changes in ventricular gradient(2).

Material and method. 234 consecutive "2-Step" tests in which there was RS-T segment depression after exercise, were analyzed. In each case, the resting 12 lead tracing was entirely normal. There were 163 men and 71 women, all followed for 2 to 8 years, average 4 years. On the basis of comprehensive clinical evaluation and follow-up, these were divided in 2 groups: (a) 100 patients with definite organic ischemic heart disease (77 males and 23 females) and (b) 134 without organic heart disease (86 men and 48 women). Standard lead II and leads V_2 through V_6 were recorded in all patients immediately after exercise, as well as in 2 minutes and 6 minutes. Duration of RS-T segment depression in electrical systole, independent of heart rate, was measured according to the technic of Lepeschkin and Surawicz(3). In Fig. 1 the RS-T segment is deviated below the baseline. X is the point of return of depressed segment to isoelectricity. The time from beginning of ventricular activation to point X (QX interval) is expressed as percentile of QT (electrical systole) interval for that complex. (QX/QT ratio). The QX/QT ratio was recorded in every lead and in each time interval, wherever RS-T segment depression, however minimal, was present. Intervals were measured by calipers. In each case, the greatest QX/QT ratio was recorded, independent of the extent of RS-T depression in mm.

Results. The mean QX/QT ratio among 100 organic patients, both male and female, was 57.4%, exceeding 50% in all but one case.

The mean ratio among individuals without heart disease, was 43.9%, all but 12% failing to attain values of 50% or greater. These findings are illustrated in cumulative frequency distribution curves (Figs. 2a, 2b). Analysis by sex indicates that only 4 of 86 functional males had ratios of QX/QT greater than 50% (4.7%), whereas 12 of 48 women in the "no heart disease" category exceeded this value.

The overall diagnostic accuracy of QX/QT ratio when applied to evaluation of any RS-T depression after the "2-Step" test is 92.8%. The technic, however, is 97% accurate in males.

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