TABLE IV.
Effect of Various Supplements to the Purified Diet on Susceptibility of Mice to Pneumococcal Infection.

Supplement to basal diet	No. of mice	No. surviving 6-days	Avg survival time, days	% survival
Biotin, inositol*	5	5	6.0	100
Biotin, inositol, folic acid†	20	18	5.7	90
Alcoholic extr. of liver‡	10	9	5.6	90
75% corn starch	11	8	5.2	73
3% salts	6	6	6.0	100
12% casein	10	8	5.3	80
12% proteose-peptone	10	9	5.7	90
2% 70 A.I. liver§	10	10	6.0	100
5% 70 A.I. liver	10	6	4.5	60
10% brewer's yeast	20	15	5.3	75
5% asparagine	10	10	6.0	100

Each supplement was incorporated into the basal diet by replacement of an equal weight of sucrose.

* 40 mg inositol, 400 μg biotin per kg diet.

† 2 mg biotin, 80 mg inositol, and 15 g folic acid concentrate (equivalent to 6 mg of folic acid $\pi = 40{,}000$) per kg diet.

‡ Fraction soluble in 70% alcohol equivalent to 0.8 g liver per g diet.

§ Fraction insoluble in 70% alcohol.

Dose = 10^{-6} ml culture per mouse.

the mouse, except insofar as higher levels were maintained by a more or less continuous supply from exogenous sources. Such an hypothesis would explain the rapid change in susceptibility of the mouse which occurs when the dietary regimen is changed.

The conclusions to be drawn from the present experiments may or may not be applicable to other types and strains of pneumococci. However, Robinson and Siegel[2] found mice on all purified diets to be less susceptible to pneumococcal infection than those on their stock diet. The data reported here show similar comparative effects from their stock diet and the purified diets. It seems likely, therefore, that the same factors influencing susceptibility may be operative in both laboratories.

Summary. The resistance of mice to the intraperitoneal injection of Type I pneumococcus was found to be markedly dependent on the type of diet on which they were maintained. Those on a purified diet survived the equivalent of 100,000 lethal doses for those on a commercial laboratory diet. This difference could not be ascribed to any known dietary essential.

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Further Purification of the Follicle-Stimulating Hormone and Its Effect in Normal and Hypophysectomized Rats.*

W. H. McSHAN AND ROLAND K. MEYER.

From the Department of Zoology, University of Wisconsin, Madison.

Follicle-stimulating hormone preparations have been obtained previously by the digestion of an aqueous extract of sheep pituitary powder with trypsin.[1,2] The extracts ob-

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1 McShan, W. H., and Meyer, Roland K., *J. Biol. Chem.*, 1938, **126**, 361.

2 McShan, W. H., and Meyer, Roland K., *J. Biol. Chem.*, 1940, **135**, 473.

tained by this method were biologically tested mainly by use of normal immature female rats, although the effect of a few of these preparations in hypophysectomized rats was reported.[1] The preparations obtained by the method reported in 1940[2] when used in the human being caused local reactions at the site of injection, which made it necessary to do further work in order to remove this local reacting factor. This report is concerned with the method of removal of this factor, and with the assay of several preparations in normal and hypophysectomized rats.

Experimental. Removal of local reacting factor. The procedure followed is the same as that reported previously[2] until the fraction of the digest which is soluble at pH 4 is obtained. Instead of precipitating the active material at this stage with ethyl alcohol, it was dialyzed in the cold against distilled water which was changed 5 times. This caused the formation of a light inactive precipitate which was separated by centrifuging. The hormone was precipitated from the solution by addition of 4 volumes of 95% alcohol. Precipitation was aided by addition of 4 drops of 2N HCl while the mixture was being stirred.

The precipitate was recovered by centrifuging and the active material was recovered by extracting the precipitate with cold distilled water. It was necessary to make at least 5 extractions using 75 ml of water each time for each 200 g of pituitary powder. Thorough extraction was accomplished by shaking the mixture with glass beads. During the third extraction the insoluble residue became gummy so that it was necessary to break the insoluble particles with a glass rod. Each extraction was continued for 2 to 4 hours followed by centrifuging and decantation of the extract into 1.5 liters of cold acetone. After each extraction the extract was added to the acetone to which the others had been mixed. A few drops of 2N HCl were added followed by stirring to cause precipitation after which the mixture was allowed to stand overnight in the cold.

The precipitate was collected by centrifuging. It was dried either by washing with acetone, or by lyophilizing after solution in 0.02 M phosphate buffer of pH 7.4. The product obtained by either of these methods of drying is soluble in water, or in 0.9% saline and can be sterilized by Seitz filtration.

Assay—Normal rats. The total amount of the hormone preparation to be given to a single rat was dissolved in 4.5 ml of 0.9% sodium chloride. Solutions of this kind were injected subcutaneously into 21 day old female rats in nine injections of 0.5 ml each beginning on the afternoon of the first day and followed by morning and afternoon injections for the next 4 days. Autopsy was done during the morning of the sixth day. The ovaries were removed, examined grossly with the aid of transmitted light for the presence of follicles and corpora lutea, and weighed. These ovaries were prepared for microscopic study and later they were examined for the presence of lutein tissue.

Hypophysectomized rats. Immature female rats 28 days old were obtained from Sprague-Dawley, Inc. These animals were hypophysectomized on the 29th day, and the injections were started the following day when they were 30 days old. The total amount of hormone to be given each rat, which was usually 2.5 g,† was dissolved in 10 ml of 0.9% sodium chloride. They were injected subcutaneously with 0.5 ml on the morning and afternoon of each day for 10 days, and were killed on the morning of the eleventh day at which time the ovaries were removed, examined, weighed and prepared for histological study. Completeness of hypophysectomy was determined at the time of autopsy by use of a binocular microscope.

Results and Discussion. Assay of preparations that contained the local reacting factor in normal and hypophysectomized rats. The 10 preparations listed in Table I were prepared according to the tryptic digestion method reported in 1940,[2] and thus contained the local reacting factor. Five hun-

† Indicates in all cases grams equivalent of original pituitary powder used in making the preparation.

TABLE I.
Assay of FSH Preparations Which Caused Local Reactions in the Human Female.*

Prep. No.	Mg per g. eq.	Total‡ dose, g	No. of rats	Wt of ovaries, avg mg	Qualitative response: Ovaries	Qualitative response: Vagina
			Normal rats injected 4.5 days.			
5000†	11.57	0.5	6	85	follicles only	O§
508	15.10	0.5	6	56	" "	No
514	—	0.5	3	27	" "	No
		1.0	3	99	" "	O
515	13.50	0.5	3	32	" "	No
516	12.40	0.5	6	50	" "	O
517	8.33	0.5	6	62	" "	O
519	—	0.5	6	105	" "	O
520	—	0.5	3	38	" "	No
		1.0	3	100	" "	O
522	11 80	0.5	7	41	" "	No
523	13.24	0.5	3	110	corpora	O
Avg	12.13					
			Hypophysectomized rats injected 10 days			
5000†	11.57	2.5	4	183	follicles only	O
508	15.10	2.5	5	232	" and few bl. pts.	O
514	—	2.5	5	65	" only	O
516	12.40	2.5	5	263	" "	O

* Made by method reported in 1940.2
† FSH5000 was made by combining parts of preparations 508, 510, 513, 515, 516, and 517.
‡ Indicates g equivalent of pituitary powder.
§ O indicates that vagina was open; No that vagina was not open.

dred grams of pituitary powder was used in making each of these preparations. The total dose used for assay in normal rats in most cases was 0.5 g. The ovarian weights obtained by injecting this amount of hormone weighed from 27 to 110 mg and the average was 61 mg. These ovaries, with the exception of those produced by preparation 523, contained follicles only as indicated by microscopic examination at the time of autopsy. The uteri were highly distended in all cases, and certain of the preparations caused the vaginas to open. The fact that preparation 523 produced ovaries that contained corpora lutea suggests that the extract was not properly digested. The failure of the extract to clear during digestion as it usually does, further supports this idea, and emphasizes the need for making a systematic study to determine the optimum conditions for digestion.

Preparations 5000, 508, 514 and 516 were tested in hypophysectomized rats by administering a total dose of 2.5 g over a period of 10 days. The ovaries obtained ranged in weight from 65 to 263 mg; the average weight was 186 mg. The uteri were distended with fluid and the vaginas were open in all cases. Macroscopic examination of these ovaries showed that they contained only follicles with the exception of those from one rat injected with FSH508 which contained a few hemorrhagic follicles.

It was found on microscopic examination that certain of these ovaries contained several patches of hypertrophied theca cells. One ovary contained some lutein cells, and there were a total of 8 small or partly formed corpora in four ovaries. Thus these preparations as indicated by assays in hypophysectomized rats contained very little luteinizing hormone but evidently were not completely devoid of luteinizing activity.

Assay of preparations that were free of the local reacting factor in normal and hypophysectomized rats. The results of the assays in normal rats of 15 different follicle stimulating preparations that were essentially free of the local reacting factor, are given in Table II. These preparations were tested by administration of 0.5 g and 1 g total doses. The ovaries as indicated by macroscopic examination at the time of autopsy contained only follicles with the exception of 3 pairs produced by 3 different preparations each given in a total dose of one gram. The

TABLE II.

Results of the Assay in Normal and Hypophysectomized Rats of Follicle-stimulating Preparations.*

Prep. No.	Total dose, g. eq.	No. of rats	Wt of ovaries	Qualitative response: Ovaries	Qualitative response: Vagina
230	1.0	3	87	follicles only	O†
234	0.5	6	38	" "	No
234	1.0	6	90	" "	O
235	0.5	6	50	" "	O
235	1.0	3	90	" "	O
236	0.5	3	68	" "	O
236	1.0	3	90	" " (few CL in 1 pair ov.)	O
237	0.5	6	35	follicles only	No
320	0.5	3	42	" "	No
321	0.5	3	35	" "	No
321	1.0	3	104	" "	No
322	0.5	6	46	" "	No
322	1.0	3	55	" "	No
323	0.5	3	34	" "	No
323	1.0	9	60	" " (1 pair ov. weighed 114 mg, contained few CL)	No
327	0.5	6	61	follicles only	No
327	1.0	3	102	" " (few CL in 1 pair ov.)	O
328	0.5	9	32	follicles only	O
328	1.0	15	75	" "	O
330A	0.5	3	41	" "	
330B	0.25	6	34	" "	No
330B	0.5	9	68	" "	O
331	0.5	3	36	" "	No
331	1.0	3	69	" "	No
333	0.5	6	25	" "	No
333	1.0	7	57	" "	No
			Hypophysectomized Rats. (2.5 g given over 10 days.)		
333	2.5	4	75	follicles only	O

* These preparations were made after the method was changed to remove the local reacting factor, and several of them were administered to human clinical cases.[3]

† O indicates that vagina was open; No that vagina was not open.

uteri were usually distended with fluid and in many cases the vaginas were not open. It is of interest to note that 0.25 g of FSH330B produced ovaries that had an average weight of 34 mg which was almost as great as that obtained with 0.5 g of the other preparations. This difference is believed to be due to the fact that the aqueous extract used in making FSH330B was not supercentrifuged before it was digested with trypsin. This suggests that more active preparations may be obtained without supercentrifuging but further work is necessary to test this possibility. Several of these preparations were effective in stimulating the ovaries of the human female, and in addition they caused little trouble from the standpoint of producing local reactions at the injection sites.[3]

FSH333 was assayed by use of hypophysectomized rats. The average weight of the ovaries was 75 mg and they consisted of follicles only as indicated by observation at the time of autopsy (Table II). These ovaries were sectioned and examined microscopically. One pair of ovaries contained no lutein cells; another pair showed one small group of hypertrophied theca interna or granulosa cells; the third pair contained 3 small groups of lutein cells; and the fourth pair had 5 very small corpora. Thus it is

[3] Davis, M. Edward, and Hellbaum, Arthur A., *J. Clin. Endocrinology*, 1944, **4**, 400.

clear that this preparation contained very little luteinizing activity as indicated by the administration of a total dose of 2.5 g over a period of 10 days. This demonstrates that follicle-stimulating preparations of a high degree of physiological purity can be obtained by the tryptic digestion method as described in this report.

The criteria used in assaying follicle-stimulating preparations obtained by the tryptic digestion method are relatively large increases in the weights of the ovaries of normal and hypophysectomized rats without the production of any lutein tissue or discrete corpora lutea. If these rigid specifications are to be the basis for determining the absolute purity of follicle-stimulating preparations, then not all preparations obtained by tryptic digestion are physiologically pure. These preparations are suitable for many practical purposes as demonstrated by their use in human beings,[3] and in laboratory and domestic animals.[4,5,6] The large doses and long injection periods used in testing the preparations are believed to be suitable for the maximum expression of any small amount of luteinizer that might be present in the preparations. When intact immature rats were used for assay most of our preparations have produced ovaries that contained only follicles as indicated by macroscopic examination. We believe that results of this kind, when obtained consistently, indicate that this method in which intact rats are used is as sensitive for detecting small amounts of luteinizer in the presence of follicle-stimulating hormone as are hypophysectomized rats. This is because the intact rat has a normal endocrine balance and the secretion of luteinizer by the animal's own pituitary gland would be added to any traces present in the follicle-stimulating preparation injected. If, under these conditions, a preparation produces ovaries that contain only follicles, it is believed the preparation has a high degree of physiological purity.

The product obtained by the tryptic digestion process is undoubtedly different chemically from preparations prepared by other methods such as salt fractionation reported by Greep *et al.*[7,8] It is also probable that the product obtained by the method developed by these workers is different in its physiological action from that obtained by tryptic digestion as little increase in ovarian weight resulted and no secretion of estrogen was obtained, even when large doses were given to hypophysectomized rats.[9] Another difference is that these workers used fresh hog pituitary glands in their work on chemical fractionation while whole sheep pituitary powder was used in the tryptic digestion method. Further study is needed to determine whether these variables are responsible for the different physiological effects produced by the two kinds of preparations.

Summary. A method is described for the preparation of follicle-stimulating preparations by tryptic digestion and the removal of a fraction which caused reactions in the human being at the site of injection.

Preparations made by the tryptic digestion method before and after it was changed to remove the local reacting factor were assayed both in normal and hypophysectomized rats. By macroscopic observation the ovaries from both kinds of rats appeared to contain only follicles but microscopic examination showed that a few contained lutein tissue indicating that not all the preparations were entirely devoid of luteinizing activity.

[4] Casida, L. E., Meyer, R. K., McShan, W. H., and Wisnicky, W., *Am. J. Vet. Res.*, 1943, **4**, 76.

[5] Warwick, E. J., Murphree, R. L., Casida, L. E., and Meyer, R. K., *Anat. Rec.*, 1943, **87**, 279.

[6] Casida, L. E., Warwick, E. J., and Meyer, R. K., *J. Animal Science*, 1944, **3**, 22.

[7] Greep, R. O., Van Dyke, H. B., and Chow, B. F., *Endocrinology*, 1942, **30**, 635.

[8] Chow, B. F., Van Dyke, H. B., Greep, R. O., Rothen, A., and Shedlovsky, T., *Endocrinology*, 1942, **30**, 650.

[9] Greep, R. O., Van Dyke, H. B., and Chow, B. F., *J. Biol. Chem.*, 1940, **133**, 289.