A NOTE ON THE CHEMICAL COMPOSITION AND BIOLOGICAL ACTIVITY OF LIQUOR FOLLICULI.

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Fellner has published several[1] interesting articles showing that the subcutaneous or intraperitoneal injection of the lipoids of the placenta, corpus luteum and of testes[2] into immature female rabbits stimulates the growth of the uterus in each case.

When, however, these lipoids were injected into immature male rabbits, the size of the growing testicle was restrained.[3] He also observed that the time of the beginning of spermatogenesis was delayed. If these injections were continued, the testicular connective tissue widened to the detriment of the lumen, in which detritis masses were conspicuous. The general condition of the testes is said to be similar to that noted as a result of the Roentgen ray.

Herrmann[4] practically duplicated the histological findings of Fellner so far as observing the stimulation of the growth of the female genitals upon injection of lipoidal preparations from placenta and corpus luteum. Herrmann, however, elaborated the methods of purification of the injected lipoid. He prepared a phosphatide-free fat, from which he isolated the unsaponifiable fraction. This proved to be active. The same procedure on the two lipoids led to similar results indicating the possible identity of the active constituents of placenta and corpus luteum. Herrmann states (page 26) that when the lipoids of ovarian residue were examined in parallel with the above-mentioned extracts dissolved in olive oil that they proved to be physiologically active[5]. Herrmann gives the boiling point of his substance as 193° to 230° at 0.06 mm., and states that it gives cholesterol color tests. Most of Herrmann's physiological tests were made in immature and castrated animals. In general he gave five injections (10 days time) amounting to 0.24 g. and observed the maturity of the genitals in 8-week old rabbits.

Fränkel[6] and Fonda have followed up Herrmann's work but the fractionation does not yield a fraction of constant boiling point, all further work being done on a fraction that boiled at 194° at 0.06 mm., but closely associated with substances of far different composition. To the intermediate fraction boiling at 194° the formula $C_{32}H_{52}O_2$ is assigned. The activity appears to be lost on distillation.

Goetsch[7] reported that the feeding of anterior pituitary lobe produced sexual maturity in young rats even more rapidly than corpus luteum. It is to be noted that Goetsch reports growth stimulation with corpus luteum, however.

Frank[8] reported results with placenta and corpus luteum lipoids in agreement with Herrmann, stating that the latter is richer in active lipoidal material.

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[1] *Centrabl. f. Allgemeine Path. u. Path. Anat.*, Aug., 1912. *Arch. Gyn.*, 100, 641 (1914), and *Arch. ges. Physiol. (Pflügers)*, 189, 199 (1921).

[2] Thymus lipoids also gave positive results, in contrast to brain lipoids.

[3] Herrmann and Stein, *Wien. klin. Wochenschr.*, 6, 25 (1916).

[4] *Monatsch f. Geburts. u. Gyn.*, XLI, 1 (1915).

[5] Fellner reports the reverse.

[6] *Biochem. Zeit.*, 41, 379 (1923).

[7] *Surg. Gyn. and Obstet.*, 25, 229 (1917).

[8] *Surgery, Gyn. and Obstet.*, 329, (1917).

Frank states that the chemical substance which produces these changes is thermostable, very resistant to alkalies and acids.

Recently Allen and Doisy[1] *et al.*, using chemical methods which lead to the unsaponifiable fraction of the lipoids found in the follicular fluid of sows, have demonstrated the presence of active material in this fluid. And when using corpus luteum they have been unable to demonstrate, by the method of Stockard and Papanicolau,[2] the production of estrus in spayed rats. Frank[3] states that corpus luteum gives positive anabolic results, identical with those obtained by the use of follicular fluid.

The evidence in these papers points out the importance of a fat fraction which has received very little attention. The growth-stimulating substance which has apparently a specific selective action on certain tissue, such as the uterus, is always found in the syrupy filtrates which systematically result after all the cholesterol (or phytosterol in plant fats) has been removed from the unsaponifiable. These syrups have up to this time failed to yield any substances of interest (except cetyl alcohol etc.) and are usually discarded.

It is of interest to point out in this connection that evidence[4] is accumulating that the antirachitic substance in cod liver oil is similarly a sterol or cholesterol derivative found in this fraction. It is in this fraction that we find substances having the property of emitting ultraviolet rays upon spontaneous oxidation. The general growth promoting vitamine A, and the more specific growth stimulating oil-soluble substances of the glands, which we designate as hormones are probably stored in these particular oil solutions by the animal body and have very similar properties. That these hormones can be varied by dietary revisions has been pointed out by Evans and Bishop.[5]

The œstrus cycle is in fact very sensitive to dietary deficiencies and it would appear to be a well-founded hypothesis that the content of these active substances in commercial products depends on the diet of the animals from which they were prepared; that they are in fact, like the carotins, not elaborated by the animal.

We have been studying this fraction of corpus luteum and some other glands and after the publication of the Allen and Doisy paper we were interested in finding out what the prospects might be of utilizing the follicular fluid of the sow as a source of supply of this material, but the yield is extremely low; only 1.2% total fat was found as compared with 0.7% of the unsaponifiable fraction, cholesterol free in corpus luteum.

Incidentally as we could find no report in the literature we have conducted a brief analysis of the liquor folliculi, a résumé of which is found at the end of the article.

EXPERIMENTAL.

Corpus Luteum.—Previous work, in which 5,673 g. of desiccated glandular material was exhaustively extracted with cold acetone gave, after the separation

[1] *J Biol. Chem.*, 61, 711 (1924). *Am. J. Anatomy*, 34, 133 (1924); *ibid.*, 34, 445 (1924).
[2] *Am. J. Anat.*, 22, 225 (1917).
[3] *J. A. M. A.*, 81, 1133 (1923).
[4] T. F. Zucker, Pappenheimer, Barnett, *Proc. Soc. Exptl. Biol. Med.*, 19, 167 (1921); Steenbock, Jones and Hart, *J. Biol. Chem.*, 55, XXVI (1922); also 46, XXXII (1921).
[5] *J. A. M. A.*, 81, 889 (1923).

of phosphatides, a fat fraction yielding 720 grams. This fraction, after removal of free fatty acids by shaking the ethereal solution with alkali, was saponified and there resulted 110 grams of unsaponifiable material containing 72 grams cholesterol (both as ester and free). The faction under discussion therefore amounts to about 38 grams or 0.7% of the desiccated gland.

In comparing this yield with follicular fluid we found that 7.3% solids of this fluid yielded 0.088% of fat so that this substance in dry form will yield 1.2% of fat, *i. e.*, both saponifiable, free fatty acids and cholestrol. This quantity of fat upon further analysis will be found to contain much less than 0.7%, found in the corpus luteum fraction referred to above.

	Fat.	Unsaponifiable.	Unsaponifiable, minus cholesterol, etc.
100 Gm. Corpus Luteum	= 12.33%	= 1.93	0.7
100 Gm. dry Follicular Fluid	= 1.20%	...	...

Follicular Fluid.—The material examined consisted of the liquor folliculi from hog ovaries, obtained through the kindness of Dr. D. Klein of the Wilson Laboratories. A sample of follicular liquid, consisting of 230 cc. was immediately placed with 460 cc. of ethyl alcohol. A heavy white precipitate of protein formed. This was centrifuged off and washed thrice with 500 cc. of 75 per cent. alcohol, twice with 500 cc. of absolute alcohol at 40° C., and twice with 500 cc. of dry ether. It was then dried to constant weight *in vacuo*. It was a white impalpable powder weighing 14.9870 grams = 13.93 grams dried at 110° or 6.06%.

A sample was dried to constant weight at 110° C.
Sample, 0.5188 gram, loss at 110° C., 0.0367 gram moisture, 7.07%.

The dry protein[1] was analyzed for nitrogen by the micro-Kjeldahl method and for phosphorus by the Neumann method.

Samples 0.0349, 0.0337; *N*/50 ammonia, 17.90; 17.43 cc.
Found N, 14.37; 14.49
Samples 0.1660, 0.1776; *N*/20 sodium hydroxide, 2.80; 3.18 cc.
Found P, 0.09; 0.10.

The alcoholic ethereal filtrates from the protein were evaporated under reduced pressure to a volume of about 500 cc. A flocculent precipitate separated on standing in the ice box. This was centrifuged off, washed with alcohol and ether and dried *in vacuo*. This was a brownish solid weighing 0.6554 Gm. = 0.28% further or 6.34% total protein. This material appeared to be impure protein and was not further analyzed.

The filtrates and washings from the above precipitate were concentrated again under reduced pressure and made up to a volume of 500 cc.

10 cc. gave 0.0446 gram solids. Total solids (not protein) 2.2300 grams = 0.97%
0.0446 gram solids gave ash 0.0290 gram. Total ash, 1.450 grams = 0.63%
Organic material 0.7800 gram = 0.34%
Samples 5.00 cc., 5.00 cc. *N*/50 ammonia 1.51 cc.; 1.67 cc.
Total non-protein N, 0.0445 gram = 0.019%.

[1] The nitrogen distribution will be reported later.

The remainder of the 500 cc. (480 cc.) was taken down to 10 cc.; 75 cc. of absolute alcohol and 75 cc. of ether were added. A gummy precipitate formed which was centrifuged off. This solid was very thoroughly washed with alcohol and ether. The filtrates and washings were then taken down to about 3 cc. at 40° C., treated with 5 cc. of absolute alcohol and 50 cc. of dry ether. More of the gummy precipitate formed. This was washed with alcohol and ether, joined with the first precipitate, dissolved in water in which it was practically all soluble, filtered and evaporated to dryness. The material consisted of cubical crystals, presumably sodium chloride and varnish-like extractive material.

Proximate analysis of extractive fraction.—It was dissolved in water and made up to a volume of 100 cc. (1 cc. = 2.208 cc. follicular fluid).

Total nitrogen.—Five cc. by micro Kjeldahl required 5.94 cc. $N/50$ acid, equivalent to 0.001663 gram N. This amounts to 0.015% of the original follicular fluid.

Ammonia and urea.—One cc. (=2.208 cc. follicular fluid) was examined by the urease method of Marshall[1] and required 0.84 cc. of $N/50$ acid, equivalent to 0.000235 Gm. urea N equivalent to 0.0106% urea N in follicular fluid. The *urea* content is 0.023% since urea contains 46.6% nitrogen.

The ammonia blank made on 2 cc. (=4.416 cc. of follicular fluid) required only 0.1 cc. N 150 acid and was not calculated.

Creatinine (total).—One cc. (= 2.208 cc. of follicular fluid) + 1.8 cc. 25 % H_2SO_4 + 6 cc. water was heated on the steam-bath and made up to 10 cc.[2] Of this 9 cc. (= 1.99 cc. follicular fluid) was neutralized and read against a standard creatinine solution containing 0.1 mg. Colorimetrical readings showed 0.000105 Gm. creatinine, or 0.005% of the follicular fluid = 0.0019% creatinine nitrogen (× 37.2%).

Phosphotungstic acid precipitable bases.—The urea N (0.0106%) + creatinine (0.0019%) = 0.0125% N of a total of 0.015%.

The remainder of the solution, 85 cc. (= 187.7 cc. follicular fluid) was made acid with 5% sulphuric and precipitated with phosphotungstic acid. The small precipitate was removed by centrifugation, decomposed with baryta in the usual manner, and the filtrate concentrated and then made up to 50 cc.

Ten cc. (37.5 cc. follicular fluid) required by micro-Kjeldahl 2.6 cc. $N/50$ acid equivalent to 0.000728 Gm. N = 0.0019% follicular fluid.

The phosphotungstic precipitate yielded but little creatinine, therefore most of this is derived from the original presence of creatine.

Two cc. (= 7.5 cc. of follicular fluid) + 1.8 cc. 25% H_2SO_4 + 5 cc. water was hydrolysed on the steam-bath and made up to exactly 10 cc. Of this 9 cc. (= 6.75 cc. of original follicular fluid) when compared to a standard of 0.05 mg. creatinine showed the presence of 0.028 mg. equivalent to 0.000414% creatinine, or 0.00015% creatinine N. Obviously the creatinine in the precipitate is so slight that it indicates the original presence of creatine.

We may therefore conclude that the basic constituents precipitable with phosphotungstic acid (not creatine) amount to approximately 0.0019% N.

The filtrates and washings from the above alcohol plus ether insoluble material were taken down to a syrup and taken up again in 5 cc. of ether, in which it was completely soluble, and the phosphatides precipitated with 20 cc. of pure acetone. A white flocculent precipitate of the acetone insoluble lipoids was obtained. The mixture was cooled and the phosphatides centrifuged off. This precipitate was taken up in 2 cc. of ether and reprecipitated with 8 cc. of acetone. The mixture was

[1] *J. A. M. A.*, 62, 1558 (1914).

[2] Janney and Blatherwick, *J. Biol. Chem.*, 21, 579 (1915); Falk, Baumann, and McGuire, *ibid.*, 37, 528 (1919).

again cooled and the precipitate centrifuged off. It was washed with 5 cc. of acetone and dried *in vacuo*. This weighed 0.0613 Gm. (= 0.027% phosphatide). It was analyzed for phosphorus and nitrogen.

Sample, 0.0219 Gm.; 4.14 cc. $N/20$ sodium hydroxide: P, 1.05% of Phosphatides.
Sample, 0.0371 Gm.; 1.59 cc. $N/50$ ammonia: N, 1.20% of Phosphatides.
Nitrogen: phosphorus = 2.53:1.

There was not sufficient material for emulsification with water so the high nitrogen of this fraction was probably due to contamination of a carnithin[1] like nature.

The filtrates and washings from the above phosphatide, containing the fats, cholesterol, etc., were concentrated down to a syrup, taken up in 2 cc. of dry ether and treated with 10 cc. of pure acetone. The solution turned slightly cloudy on cooling. It was filtered and made up to a volume of 20 cc. It contained 0.195 Gm. equivalent to 0.088% of the follicular fluids.[2]

Sample, 2 cc.; Solids 0.0195. Total solids, 0.1950 Gm.
Sample, 2 cc.; 0.91 cc. $N/20$ sodium hydroxide, P, 0.000050 Gm.
P = 0.26% of fat fraction.
Sample, 2 cc.; 3.03 cc. $N/50$ ammonia. N, 0.00085. N = 4.35% of fat fraction.

Material thus prepared was used for physiological testing.

We have been testing the effects of glandular feeding and of various corpus luteum fractions on the œstrus cycle in guinea-pigs, using the methods of Stockard and Papanicolau. According to Allen,[3] subcutaneous injections of oil solutions of the fat fraction of follicular fluid into spayed mice and rats, in which the anabolic phase of the œstrus cycle is 2 to 3 days, produced œstrus in 3 injections (containing roughly 1 to 2 milligrams of the fat fraction). Continued injections caused an extended period of œstrous growth (freedom from leucocytic infiltration). This test is therefore based on the stimulation of growth in the vaginal and uterine mucosa by the injection and the subsequent induction of metoestrum by its withdrawal.

The guinea-pigs used in these experiments showed an average dioestrous cycle of 15.3 days (22 periods). The injections of the fat fraction of follicular fluid were made subcutaneously, using an oil base. Three injections were made in each experiment. When total quantities not in excess of 0.05 Gm. (fat fraction) were injected, no change in the cycle was observed. In larger quantities, however, (0.06 gram) the œstrous cycle was prolonged. With this quantity the period was extended to 18 days, while the average of those receiving the smaller doses average 15.3 days, no lengthening whatever being observed. All these injections were made at three-day intervals, beginning at the 8th day from the last heat period.

The proximate analysis of follicular fluid may be stated as follows:

	Per cent.
Total Solids	7.310
Protein	6.340

[1] H. MacLean, *Biochem. J.*, 9, 353 (1915).

[2] Doisy *et al.*, report, *J. Biol. Chem.*, 61, 721 (1924), their active fraction in purified extracts to vary from 0.02 to 0.19%.

[3] Allen *et al.*, *Am. J. Anat.*, 34, 149 (1924).

Solids not protein	0.970
Ash	0.630
Lipoids (total)	0.115
Phosphatides (crude)	0.27
Fat, cholesterol, etc	0.88
Total extractive N	0.019
Lipoid N	0.004
Extractive N	0.015
Urea N	0.0106
Creatinine	0.0019
Basic N	0.0019

It is therefore shown that of the total solids in follicular fluid, the chief mass is protein. The concentration of the salts are apparently somewhat lower than in the blood and a considerable quantity of urea is present, but a trace only of phosphotungstic precipitable substances. The fat fraction amounted to 0.088%.

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A PALATABLE COD LIVER OIL CONCENTRATE POSSESSING THE THERAPEUTIC PROPERTIES OF COD LIVER OIL.*

BY HARRY E. DUBIN.

The therapeutic value of cod liver oil has been common knowledge for a great many years, but until recently its use was on a purely empiric basis.[1] With the indisputable proof at hand that cod liver oil is really a specific in the treatment of rickets, interest has been aroused in the possibility of isolating the active principle responsible for its therapeutic effect.

Regarding the mechanism of the action of cod liver oil, investigation has revealed the fact that the metabolism of calcium and phosphorus is in some way favorably influenced. It has also been found that cod liver oil has a stimulating effect on the intestinal mucous membrane and possibly on the formation of blood platelets.

Whatever the mode of action, the efficacy of cod liver oil is unquestioned. While it is a specific remedy for rickets, it has in addition a general restorative and vitalizing value. The vitamines inherent in cod liver oil are necessary for growth and health and for the normal development of bones and teeth. They promote growth and development in the adolescent period and are of great help in combating infections, by increasing body resistance.

The healthy adult does not need cod liver oil, but there are numerous cases where it could be used to advantage. While the adult does not suffer from active

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* Detailed experimental data obtained by Dr. Dubin and Dr. Funk appears in *J. Metabolic Research*, 4, 467 (1923).

[1] The results of the clinical investigation made by Dr. Louis Fischer, Director of the Infantorium and Nursery of the Heckscher Foundation, New York City, are published in *J. Metabolic Research*, 4, 480 (1923).