THE ORIGIN OF FAT FROM PROTEIN IN THE SO-CALLED FATTY METAMORPHOSIS OF PHOSPHORUS POISONING.

By ALONZO ENGLEBERT TAYLOR, M. D., Assistant Director of the Laboratory, and Woodward Associate in Clinical Medicine.

(From the William Pepper Laboratory of Clinical Medicine, University of Pennsylvania, Phœbe A. Hearst Foundation.)

The idea of employing hibernating animals for the study of fatty degeneration seems to have originated with Leo (1). Later Polimanti (2) took up the plan along similar but less uncontrolled lines, and reached the same conclusions as Leo,—that in phosphorus poisoning in frogs fat is formed from the protein. In the work of both were obvious errors and also opportunities for further error. Polimanti, as Pflueger (3) promptly pointed out, erred in not having determined the initial weight of his live frogs, and in assuming that the weight at death and the dried residue of the poisoned and control frogs could be properly compared; he erred further in employing heavier frogs for the poisoning, while lighter frogs were used as controls; and most important of all, Pflueger demonstrated that hibernating frogs contain sufficient glycogen to account for the fat apparently formed. In such experiments it must obviously be demonstrated that the fat in the tissues of a poisoned animal could not be derived from any other source than protein, before it can be held as demonstrated that in fatty degenerations fats are produced from protein.

I have repeated the experiments of producing phosphorus poisoning in frogs, and, I believe, have arranged the conditions so rigidly that the results must be indubitable, in so far at least as this particular procedure is concerned. I have utilized the Rana palustris.

The frogs were gathered about the first of December, 1898, and held in moist chambers exposed to the light for nearly four months at a temperature of 18-20° C. This was done in order to exhaust as much as

possible the store-houses of fat and glycogen. Twenty-eight males were then selected, and divided into groups of fourteen each; the total weight of each group was almost identical with that of the other, and the frogs were so paired as to make the series balance well. The frogs were wiped dry with filter paper, the urine expressed, and they were then carefully weighed. The frogs of one group were then poisoned by phosphorus. I wished to prolong the action of the phosphorus, and the frogs were given two small doses rather than one large dose. The phosphorus was administered in an emulsion of acacia, and each frog was given 0.001 grm. at each administration, the second dose being given on the fourth day. Whenever a frog died his control fellow was at once killed. Such as did not die were killed upon the twelfth day, and their controls at the same time. The animals were weighed when found dead or when killed. An interesting point was the œdematous condition of many of the poisoned frogs, especially those found dead over night. The original weights were as follows:

CONTROLS.	TO BE POISONED.
C1=21.990	P1=22.450
C2=20.600	P2=21.000
C3=21.330	P3=21.000
C4=22.850	P4=21.690
C5=20.090	P5=20.620
C6=21.200	P6=21.450
C7=38.000	P7=43.700
C8=28.030	P8=26.770
C9=19.870	P9=21.100
C10=19.850+	P10=18.280
C11=18.150	P11=16.260
C12=14.950	P12=15.480
C13=14.320	P13=14.150
C14=12.840	P14=10.400
294.430 grm.	294.350 grm.

One control frog, C10, weight 19.850, was lost. The total weight of the dead animals was:

CONTROLS.	POISONED.
C1=19.300	P1=22.100 killed.
C2=19.850	P2=22.720 dead.
C3=20.270	P3=25.480 dead.
C4=22.500	P4=21.200 killed.
C5=20.250	P5=24.100 dead.
C6=21.000	P6=21.450 killed.
C7=36.150	P7=42.500 killed.
C8=25.650	P8=27.900 dead.
C9=16.650	P9=20.900 killed.
C10=lost	P10=18.900 killed.
C11=17.300	P11=13.900 dead.
C12=12.900	P12=14.450 killed.
C13=14.400	P13=13.500 killed.
C14=12.500	P14=13.000 dead.
258.720	302.100 grm.
19.000 lost, approx.	
277.720 grm.	

In all probability much of the loss in weight in the control frogs was due to the fact that the dead frogs could be wiped more completely dry than the living frogs. Poisoned frogs Nos. 2, 3, 5, 8, and 14 gained notably in weight; they were œdematous and the gain seemed obviously water. A comparison of these tables of weights illustrates how inaccurate it would be to use as the basis of calculation the dead weight of the frogs at the end of the experiment. The only correct basis of calculation is the original live weight before the experiment.

The bodies of the frogs were first partly dried in ovens at 70° C., and then completely dried in a vacuum over sulphuric acid. They were then finely ground in a small closed hand-mill, again dried in a vacuum over sulphuric acid, and weighed. The dried residues were:

Control frogs: 49.855 grm.	Poisoned Frogs: 44.620 grm.
or 18.15% of the original weight.	or 15.15% of the original weight.

In order to make the comparison complete, allowance must be made for the lost control frog: assuming his dried residue the same as his fellows the dried residue of the control frogs was 18.15 per cent of 294.430, or 53.441 grm., as against 44.620 grm. for the poisoned frogs, representing a loss of dried residue during the process of poisoning of 8.821 grm., or 16.5 per cent.

Three portions each of the control and poisoned frogs were then submitted to the Kjeldahl process for the estimation of nitrogen. The results were as follows:

CONTROLS:	POISONED:
1—0.604 grm. subs.=0.0708 N.	1—0.735 grm. subs.=0.0865 N.
2—0.570 grm. subs.=0.0666 N.	2—0.550 grm. subs.=0.0645 N.
3—0.575 grm. subs.=0.0656 N.	3—0.650 grm. subs.=0.0766 N.
On an average, 11.55% of the dried residue, or 5.762 grm. total nitrogen. Including the lost control frog, however, the dried residue was 53.441 grm., of which the total nitrogen (11.55 × 53.441) would amount to 6.403 grm., or 2.173% of the original weight. This with the factor 6.25 would correspond to a total protein of 40.019 grm.	An average of 11.70% of the dried residue, or 5.221 grm. total nitrogen, or 1.776% of the original weight. This with the factor 6.25 would correspond to a protein of 32.631 grm.

Thus during the process of the poisoning by phosphorus 1.182 grm. of nitrogen were lost as compared to the control frogs; this corresponds to a loss in protein, using the factor 6.25, of 7.388 grm., or 18.37 per cent. These 7.388 grm. of proteid contain about 3.500 grm. of carbon, which could conceivably correspond to about 4.600 grm. of fat.

The material of each series was then subjected to the same analyses, as follows:

The entire remainder of the dried residue, after the small portions for the nitrogen determinations had been taken, was placed in a beaker, 500 cc. of water added, hydrochloric acid until the mixture contained one-half of one per cent HCl, and 2 grm. of pepsin which was free of fat and glycogen. The beaker was then placed in an oven at 38° C. and allowed to remain there with the further addition of HCl in the third day, until digestion was completed, which was at the end of the fourth day, when the frog powder had gone into solution except for a few minute particles. The acidity was then partially neutralized by the addition of NaHO, and the fluid transferred into modified Sohxlet tubes designed for the extraction of fats from liquids, and there extracted with ether for about seven hours per day during five days. To make sure of complete extractions the fluid was then thoroughly shaken with ether in glass-stoppered cylinders. The combined ethereal extractions were then allowed to evaporate, and the fat collected in pure ether, filtered, the ether allowed to evaporate, the fat then placed in an oven at 95° for several days, cooled and weighed as crude fat. The crude fats were then saponified with alcoholic KHO, the mixture rendered acid with H_2SO_4, and the fatty acids extracted with ether. The ether was then allowed to evaporate, the fatty acids then dissolved in alcohol, and titrated with N/10 alcoholic potassium hydrate, with phenolphthalein as an indicator. From the titration the fats were calculated upon the basis of a mixture of stearin, palmatin, and olein, a calculation certainly not accurately applicable to frog's fat. Of the fat in the control frogs, 4.534 grm., 86.1 per cent was fat, being 3.904 grm.; the remainder, 0.630 grm., being lecithin, cholesterin, pigments, etc. Of the fat in the poisoned frogs, 3.508 grm., 84.6 per cent was pure, being 2.968 grm.; the remainder, 0.540 grm. being lecithin, cholesterin, pigments, etc. Thus of pure fat the control frogs had 3.904 grm., the poisoned frogs, 2.965 grm., a loss of 0.936 grm. The chief value of this study of the fats lies in the fact that it demonstrated that the ethereal extracts from the two series contained approximately the same amount of fat.

The weighed fats were not deeply pigmented. The fat from the control frogs weighed 4.0846 grm. The fat from the poisoned frogs weighed 3.356 grm. Since these quantities were obtained from the powders minus the quantities used for the nitrogen analysis, they must be corrected. From the residue of the control frogs, 49.885 grm., 1.749 grm. were removed from the three nitrogen analyses; the 4.0846 grm. fat in the control frogs were therefore 48.136/49.885 of the total, which would

be 4.232 grm. From the dried residue of the poisoned frogs, 44.620 grm., 1.935 grm. were removed for the nitrogen analyses; the 3.356 grm. fat were therefore 42.685/44.620 of the total, which would be 3.508 grm. The 4.232 grm. of fat in the control frogs is, however, still too small by reason of the absence from the analysis of the one lost frog. As calculated for the dried residue, this was raised from 49.885 to 53.441 grm.; the 4.232 grm. of fat therefore represented 49.885/53.441 of the real total, which would be 4.534 grm. Since 294.430 grm. of control frogs contained 4.534 grm. of fat, the percentage was 1.54 per cent; and since 294.350 grm. of poisoned frogs contained 3.508 grm. of fat, the percentage was 1.19 per cent. Thus the control frogs contained 4.534 grm. of fat, and the poisoned animals 3.508 grm.—a loss of fat, therefore, during the course of the phosphorus poisoning, of 1.026 grm.—or 22.64 per cent.

Following the extraction of the fats the digested fluid was neutralized, wherein a rather heavy precipitate was produced. The precipitate was separated by filtration, and in the filtrate and precipitate the Brücke-Külz method for glycogen was conducted separately. The use of this method after the digestion of the substance has been recommended by Austin (4).

The precipitate was heated in a 2 per cent KHO until homogeneous, then cooled and HCl and Brücke's reagent added until no further precipitate appeared. The precipitate was then filtered off, redissolved in 2 per cent KHO, reprecipitated with HCl and Brücke's reagent, and this process was repeated four times; the filtrates were then united.

The original filtrate was then mixed with twice its volume of 96 vol. per cent alcohol and allowed to stand 24 hours. By this time the glycogen was entirely precipitated, and it was hoped that the albumoses would remain entirely in solution. This they did not do; a portion was precipitated. The precipitate was washed twice in 62 vol. per cent alcohol containing a little NaCl, and then dissolved in warm water. In order to attempt to avoid the milky cloudiness which would surely be produced by the Brücke reagent in a liquid containing albumoses, the solution was again mixed with double its volume of 96 vol. per cent alcohol; as before, albumoses were precipitated and could not be separated from the glycogen in this way. Upon the following day, the precipitate was dissolved in warm water, the required amount of KHO added to bring it up to 2 per cent KHO, and then submitted to the precipitation with HCl and Brücke's reagent; a small precipitate and a milky cloudiness appeared, and the solution was allowed to stand 24 hours, but the cloudiness still persisted. Thereupon the entire Brücke-Külz procedure was repeated, but with the same result. This time the precipitate was sepa-

rated by filtration, and four times redissolved in KHO, reprecipitated by HCl and Brücke's reagent and filtered. All the filtrates were united and finally added to the filtrates obtained from the manipulation of the original residue, and thus all the glycogen in each series of frogs was finally brought into one solution. This solution was of a pale milky color, and all efforts to clarify it were fruitless. The same phenomena occurred in both series of analyses, and were in all probability due to albumoses which arose during the digestion of the frogs. It was obvious that the glycogen would not be pure preparations. Double analyses of aliquot parts of each solution were then made according to the Külz (5) method—precipitation by 2 volumes of 96 vol. per cent alcohol, careful washing upon the weighed filter paper, first with 66 per cent alcohol, then 95 per cent, three times with absolute alcohol, three times with ether, and finally again with absolute alcohol, and drying to a constant weight at 90° C., which required about four days. The glycogen in the control frogs was 2.029 grm. and 2.070 grm. respectively for two analyses, the average being 2.049 grm. The glycogen in the poisoned frogs was 1.784 grm. and 1.802 grm. respectively for two analyses, the average being 1.793 grm. These must both be corrected, as were the fats, for the quantity of dried residue removed for the nitrogen analyses. The 2.049 grm. in the control frogs was 48.136/49.885 of the total, therefore 2.123 grm. The 1.793 grm. in the poisoned frogs was 42.685/44.620 of the total, therefore 1.874 grm. The 2.123 grm. in the control frogs must be further corrected for the lost frog; it was 49.885/53.441 of the real total, which was therefore 2.274 grm.

It was obviously necessary to determine the purity of the glycogen. This was done by inverting it and making a quantitative analysis of the sugar. For inversion I employed a 2.5 per cent HCl solution, using a boiling water bath for four hours. The sugar was determined by the cupric oxide method as elaborated by Pflueger (6), a method I have repeatedly employed with entire satisfaction. According to the formula for glycogen of Hueppert (7), 11 parts of glycogen should furnish 12 parts of dextrose. Of the glycogen from the control frogs I inverted 0.109 grm., which should have produced 0.119 grm. dextrose; my analysis furnished 0.102 grm.; the glycogen was therefore 85.8 per cent pure; 85.8 per cent of the glycogen in the control frogs, 2.274 grm., equals 1.951 grm., the final figure for glycogen in the control frogs, 0.66 per cent of the original weight of the frogs. Of the glycogen from the poisoned frogs, 0.0942 grm. were inverted, and should have produced 0.102 grm. of dextrose; the analysis furnished 0.0924 grm., the glycogen from the poisoned frogs was therefore 91.5 per cent pure; 91.5 per cent of the

glycogen in the poisoned frogs, 1.847 grm., would amount to 1.690 grm., which is the final figure for the glycogen in the poisoned frogs, or 0.57 per cent of the original weight of the frogs. Thus during the course of the phosphorus poisoning the frogs lost 0.261 grm. of glycogen, or 13.3 per cent. As a matter of fact, since all the glycogen results are too low—the Külz method giving always too low results—the loss in glycogen was probably greater.

SUMMARY AND DISCUSSION.

588.780 grm. of frogs, all of the same sex, of the same comparative approximate weights, taken from the ground about the same time, kept awake and without food for nearly the same time, were divided into equal groups; the one group was poisoned with phosphorus, the other group held as a control. The frogs in the poisoned group *lost* in dried residue 8.821 grm. or 16.5 per cent of the dried residue of the control group; 1.182 grm. of nitrogen, corresponding to 7.388 of proteid, or 18.45 per cent of the nitrogen and protein in the control frogs; 1.026 grm. of fat, or 22.64 per cent of the fat in the control animals; and 0.261 grm. glycogen, or 13.3 + per cent of the glycogen in the control frogs.

I believe that it is obvious that in these experiments no fats were produced from protein. Mathematically, it is possible to conceive that fats could have been formed but entirely burned up. As previously stated, the carbon in the proteid lost during the poisoning was equivalent to 4.600 grm. of fat, and it is conceivable that these 4.600 grm. of fat were formed, but that they, together with the 1.026 grm. of fat actually lost during the experiments, were burned. In brief, the fat combustion might have been tremendously increased, and masked an actual fat formation. This however is unsupported by evidence, and is highly improbable. It is hard to conceive that in an organism whose katabolic functions were greatly augmented as the result of phosphorus poisoning, in which protein, fat, and glycogen were being burned in excess, the carbon of the protein would first have been converted into fat and then the fat burned as such. I believe the only conclusion which can be drawn from these experiments is that no fat was formed as the result of phosphorus poisoning. Thus the fatty

degenerations so-called which occurred in these frogs did not comprehend any formation of fat at all, but simply the deposition of fat.

These results are directly opposite to those of Polimanti. Polimanti apparently did not weigh his animals before the beginning of the experiment, and based his calculations upon the relation of the fat to the dried residue. Obviously his calculation was based upon the assumption that the dried residue of a frog was unaffected by phosphorus poisoning. Polimanti, in declining to base his calculations upon the weight of the animals when dead, states that as water is often increased, such a calculation would be misleading. But since the dried residue may and does vary, calculations based upon it are also misleading, and thus the only proper basis of calculation is the original weight of the frogs before the experimentation. Calculated upon the basis of the dried residue, in my material the percentage of fat in the control animals was 8.48 per cent, in the poisoned animals 7.86 per cent, so that, even upon the basis of Polimanti's incorrect calculation, in my experiments fat was lost in notable quantity.

Just before this study was completed, the publication of Athanasiu, (8) from Pflueger's laboratory, appeared. Operating with a large number of frogs, and under varying conditions, with careful methods and rigid controls, Athanasiu reached the conclusions: that phosphorus poisoning has no effect upon the total quantity of fat in frogs; that it has little effect upon the nitrogen; that it produces a diminution in the quantity of glycogen; and that the fatty degenerations are really fatty infiltrations. While my results agree with those of Athanasiu in the essential point that no fat was produced by phosphorus poisoning, they differ in that the poisoned frogs, in my experiments, lost fat and protein as well as glycogen, while his frogs lost only glycogen. Since our methods were almost the same, the differences must have resided either in the conditions surrounding the experiments, or in the animals. I do not believe that such differences exist between the Rana fusca and esculenta of Europe and the Rana palustris of America as to explain the differences in our results. These differences I believe may be explained by varying conditions. My animals were kept in a warm cellar, at a temperature

of from 18 to 20° C. The period of poisoning with Athanasiu's frogs varied from one to six days; all of my frogs lived over six days, most of them ten or twelve days. Since we know that the katabolic actions of most poisons are greater in prolonged intoxications, it is fair to assume that the time element was the factor in the production of my results.

While it would be unscientific and illogical to state that fat cannot be formed from protein, the fact stands that it has never been shown, either in physiology or pathology, that fats are formed from protein. On the contrary, nearly all of the careful work upon the question has yielded negative results. Not only has it never been shown that, in fatty degeneration so-called, fat is formed from the cellular protein, but it has never been demonstrated that fat is then formed at all, even from glucosides, etc., substances from which fats may be readily formed.

REFERENCES.

1. H. Leo.—*Ztschr. f. physiol. Chemie*, 1884-5, ix, 469.
2. Polimanti.—Pflueger's *Archiv*, 1898, lxx, 349.
3. Pflueger.—Pflueger's *Archiv*, 1898, lxxi.
4. Austin.—Virchow's *Archiv*, 1897, cl, 185.
5. Külz.—*Ztschr. f. Biol.*, 1886, xxii, 161; Beiträge zur Physiologie, Marburg, 1891, p. 69.
6. Pflueger.—Pflueger's *Archiv*, 1898, lxix, 399.
7. Hueppert.—*Ztschr. f. physiol. Chemie*, 1894, xviii.
8. Athanasiu.—Pflueger's *Archiv*, 1899, lxxiv.

For critical reviews of the entire subject see Taylor, Critical Summary of the Question of Fatty Degeneration, *American Journ. of the Med. Sciences*, 1899, cxvii, 569; and the article by Athanasiu cited above.