5% emulsion of blood cells in each test tube. It had been found that a 0.05% solution of sapotoxin in a 0.85% solution of NaCl was best suited for this purpose. It was prepared in each experiment fresh from a standard 1% solution, to which 0.1% of salicylic acid had been added.

After both systems had been kept together for 2 hours in the incubator at 37°C., in both of them the test tube was determined in which complete hemolysis had occurred. It was assumed that the amount of serum which could produce complete hemolysis was equivalent to the amount of sapotoxin which had produced the same effect. For practical purposes that amount of sapotoxin was calculated in milligrams which was equivalent to the hemolytic action of 100 cc. of serum. This figure was called "Sapotoxin Units". Example: In the first system 0.025 cc. of serum produced complete hemolysis of 1 cc. of 5% blood. In the second system 0.290 cc. of the sapotoxin solution had the same effect. 0.290 cc. of a 0.05% solution of sapotoxin are equal to 0.145 mg. sapotoxin. The sapotoxin units are $0.145 \times 100/0.025 = 580$ sapotoxin units.

TABLE I.*

Sapotoxin units of different hemolytic amboceptors. Blood was injected intra-abdominally in intervals of three days. The serum was taken nine days after the last injection.

Blood from	Injected into	Amount of blood injected		Sapotoxin Units
		%	cc.	
Rat	Dog	50	3-3-3-3	95
Rat	Dog	50	4-8-12	315
Cow	Dog	50	10-10-10	800
Cow	Dog	50	10-10-10-10	1070

* E. Sieburg. *In Handbuch der biologischen Arbeitsmethoden*, E. Abderhalden Abt. I, Teil 10, pp. 545-584. 1923.

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Denaturation of Hemoglobin.

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In a recent series of papers by Anson and Mirsky,[1] the claim is made that hemoglobin, in N/20 HCl, is completely denatured in

[1] Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, 1930, xiii, 121, 133, 469, 477.

3 minutes at 0°C. These authors claim, further, that this denaturation may be reversed to the extent of 75% by simply neutralizing the acid added with an equivalent amount of NaOH containing a small amount of NaCN. These experiments on the reversal of hemoglobin denaturation are, in essential details, substantially the same as those published almost 3 years earlier by Wu and Lin.[2] Wu and Lin, however, considered that they were dealing not with true denaturation but with a pseudo-denaturation which was peculiar to hemoglobin and dependent upon its prosthetic group. In order to test these divergent views, we repeated the experiments of Anson and Mirsky at temperatures which would really denature the protein. Instead of insolubility at the iso-electric point, we made use of precipitin reactions against a specific antiserum to follow the course of denaturation. Since true denaturation of a protein results in a gradual diminution of its ability to react with its antiserum, we may expect that any reversal of this denaturation would manifest itself by a return of its precipitinogenic properties.

A 3% solution of ox carbon monoxide hemoglobin, prepared by the method previously described by Boor and Hektoen[3] was used in these experiments. One volume of this solution was treated with 3 volumes of N/15 HCl and, after standing at room temperature for 10 minutes, the brown mixture was placed in a boiling water bath. At stated intervals, 4 cc. of this mixture was removed and neutralized by the addition of 1 cc. N/5 NaOH and 1/20 cc. N. NaCN. After standing for one hour at room temperature, 3 cc. of this solution was treated with an equal volume of saturated ammonium sulphate solution while the remainder was used for precipitin reactions against an ox carbon monoxide hemoglobin antiserum. Similar portions of solution, taken at the same time, were also permitted to stand at room temperature for one hour and then used, without neutralization, for precipitin and ammonium sulphate precipitation reactions. The results obtained in a typical series of experiments are summarized in the table.

These preliminary experiments indicate very definitely that heating of the acid solution results in a gradual loss of ability to react with the specific antiserum, in agreement with results observed in the denaturation of other proteins. Parallel experiments heated the same length of time, one neutralized while the other was not, show practically no differences in precipitin titer in spite of the fact that the unneutralized solutions had stood for an additional hour at room

[2] Wu, H., and Lin, K., *Chinese J. Physiol.*, 1927, i, 219.

[3] Boor, A. K., and Hektoen, L., *J. Infect. Dis.*, 1930, xlvi, 1.

TABLE I.

Denaturation of Ox Carbon Monoxide Hemoglobin.

3% solution of ox carbon monoxide hemoglobin treated with 3 volumes of N/15 HCl. Solution placed in boiling water bath after 10 minutes at room temperature.

Exp. No.	Time of Heating in min.	Solution neutralized with	Precipitin Reactions with anti-ox COHb serum	Half Saturation with $(NH_4)_2SO_4$	
				Ppt.	Filtrate
1	5	NaOH+NaCN	+++[1]	Slight	Red
2	5	——	+++	Heavy	Colorless
3	15	NaOH+NaCN	++	Small	Light Red
4	15	——	++	Heavy	Colorless
5	30	NaOH+NaCN	++	"	Pale Red
6	30	——	++	"	Colorless
7	60	NaOH+NaCN	+[2]	"	Faint Red
8	60	——	+	"	Colorless
9	135	NaOH+NaCN	+	"	"
10	135	——	+	"	"
	Room Temp.				
11	10	NaOH+NaCN	+++	Very slight	Deep Red
12	10	——	+++	Heavy	Colorless
13	60	——	+++	"	"
14	Control with ox COHb		+++++[3]	No ppt.	Deep Red

[1] Highest dilutions of test solutions which gave precipitates with antiserum in 1 hour at room temperature. + = dilution of 1 to 10, ++ = dilution of 1 to 100, +++ = dilution of 1 to 1,000, etc.

[2] Probably represents a non-specific reaction since most solutions also gave precipitates with normal rabbit serum at this dilution.

[3] Control gives precipitate with antiserum in dilution of 1:13,300,000.

temperature before the precipitin reactions were carried out. The precipitation reactions with ammonium sulphate, which Anson and Mirsky use as an index of the extent of reversal of denaturation, show that the amount of hemoglobin, as indicated by the color in the supernatant liquid, decreases with time of heating until, in about one hour, the supernatant liquid is colorless. These results differ markedly from those obtained in similar experiments at room temperature. In contrast with the neutralized solutions, all of the protein in the acid solutions is completely precipitated by half saturation with ammonium sulphate. That this result is peculiar to hemoglobin is indicated by the fact that hemoglobin, treated with HCl as above at room temperature and then immediately half saturated with ammonium sulphate is also completely precipitated, whereas crystallized ovalbumin gives practically no precipitate under similar conditions.

We are thus led to the conclusion that Anson and Mirsky, in their experiments, dealt not with true denaturation but with an even more obscure reaction which is peculiar to hemoglobin. We find no evidence for reversal of denaturation, by their method, when the pro-

tein is subjected to conditions which actually denature it. Although Anson and Mirsky consider hemoglobin a typical coagulable protein, they do not differentiate between coagulation and denaturation. We cannot agree with their point of view that the effects of acid on hemoglobin are the same as on other coagulable proteins. Besides a conceivable denaturation of the entire hemoglobin molecule, acids exert other effects such as the splitting of hemoglobin into hematin and globin and the denaturation of the globin which are characteristic of hemoglobin and are entirely absent when dealing with the simple proteins. We feel that insolubility at the iso-electric point cannot be used as a criterion for denaturation of hemoglobin because of the complex transformations which this particular protein undergoes upon treatment with acids. Such a physical property, though valuable in the case of simple proteins like ovalbumin, is of little significance in the case of hemoglobin because of a lack of definite information on solubility and other relationships between the various components of the reaction mixtures.

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Some Observations on the Growth of Rats on "Fat-Free" and Fat-Containing Diets.

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Recently Burr and Burr[1] and McAmis, Anderson, and Mendel[2] have shown that the growth of rats on a diet from which all true fat has been excluded is distinctly inferior to that of rats on a diet which contains some fat, even though the fat used is devoid of vitamin A. Furthermore the health of the animals raised on the fat-free diet also suffers. Burr and Burr[1] believe that the total deprivation of food fat leads to the development of a deficiency disease, the symptoms of which are impaired growth, scaliness of the feet and tail, excessive production of dandruff, with ultimate hematuria and albuminuria, loss in weight, and premature death. In a later paper[3] Burr and Burr state that fat is an essential constituent of the diet

[1] Burr, G. O., and Burr, M. M., *J. Biol. Chem.*, 1929, lxxxii, 345.

[2] McAmis, A. J., Anderson, W. E., and Mendel, L. B., *J. Biol. Chem.*, 1929, lxxxii, 247.

[3] Burr, G. O., and Burr, M. M., *J. Biol. Chem.*, 1930, lxxxvi, 571.