5. The blood sugar level does not necessarily indicate the quantity of glycogen stored in the organism. In Dog 20, 1 cc. of adrenalin, intravenously, had no effect upon the blood sugar, although the blood contained 82 mg. sugar per 100 cc. One hour later this animal was in hypoglycemic shock with a blood sugar of 52 mg. Dog No. 15 showed no effect of adrenalin upon the blood sugar, although the blood sugar was 90 mg., and in dog No. 57, 4½ hours before convulsions and death of the animal, the adrenalin had no effect on the blood sugar level at 86 mg. This is important from a clinical standpoint for it would mean that the sugar content of the blood is not an index of the amount of glycogen stored in the tissues.

These observations seem to confirm our previously reported results, that ligation of the hepatic artery causes an abnormally high degree of carbohydrate oxidation with a total depletion of glycogen stores of the body.

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A color reaction associated with vitamin D.

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"The chemical relationship between activated cholesterol and the naturally antirachitic substances, such as cod liver oil, yolk of egg, and bone marrow, is one of prime importance in a consideration of the etiology of rickets."[1]

It is definitely established that substances which contain either cholesterol or phytosterol can be made antirachitic by exposure to ultra-violet light. Cholesterol and phytosterol themselves, ordinarily without any curative effect on rickets, can be made antirachitic by irradiation. The criterion for the presence of the antirachitic factor (here called vitamin D) is the "line test" of

[1] Hess, A. F., Weinstock, M., and Sherman, E., *J. Biol. Chem.*, 1926, lxvii, 420.

McCollum and his coworkers.[2] Rachitic animals showing a wide metaphysis free from calcification are fed the substance to be tested in addition to the rickets-producing diet. Deposition of calcium salts, giving a positive line test, shows the presence of vitamin D in the food. This biological test is both expensive and time consuming. A chemical test for vitamin D would save time and effort, and in addition might lead to an understanding of the chemical nature of the vitamin. Hess and Weinstock[3] studied the changes in the ultra-violet light transmission of cholesterol before and after irradiation. Although they obtained a difference, its significance is at present not established. Besides, the method is not generally applicable. The color reactions of substances containing the fat soluble vitamins have been studied by Drummond, Rosenheim and their associates. Of the reagents studied, Rosenheim and Drummond[4] found $AsCl_3$ the most sensitive. They claim that their color test parallels the biological test for the presence of vitamin A.

Harden and Robison[5] state that the purple color given by liver oils when treated with H_2SO_4 can be closely simulated by adding furfural or a substituted furfural to cholesterol or butter. This suggested that the color producing substance in the oil might be related to furfural in behavior. When aniline and HCl are added to a solution containing furfural an intensely red color is obtained. Accordingly, this test was applied to cod liver oil. The aniline reagent was made by adding 1 part conc. HCl to approximately 15 parts aniline. Three cc. of the aniline reagent were added to an equal volume of cod liver oil in a wide test tube. The contents were mixed, heated to boiling with constant shaking, and boiled for about half a minute.

The yellow emulsion turned green, and in a few seconds changed to red. Within a minute or two, the emulsion separated into two layers, the lower one being colored an intense red. On standing the red color deepened. Sometimes the green color reappeared, but further boiling restored the green color. To determine whether the chromogenic substance was destroyed by mild oxidation, 100 cc. of cod liver oil was kept at 100° for an hour

[2] McCollum, E. V., Simmonds, N., Shipley, P. G., and Park, E. A., *J. Biol. Chem.*, 1922, li, 41.

[3] Hess, A. F., and Weinstock, M., *J. Biol. Chem.*, 1925, lxiv, 193.

[4] Rosenheim, O., and Drummond, J. C., *Biochem. J.*, 1925, xix, 753.

[5] Harden, A., and Robison, R., *Biochem. J.*, 1923, xvii, 115.

while air was bubbled through. At the end of that time, the tests with $AsCl_3$ and P_2O_5 were negative, as the English investigators reported; but the test with the aniline reagent was more positive than before. The red color developed rapidly even in the cold. This was taken as an indication that some substance other than vitamin A gave the red color in the oxidized oil.

It was hoped that this would prove to be a color test for vitamin D. To test further this hypothesis, a number of substances were irradiated and tested with aniline reagent. The results obtained with the specimens examined are given in the table.

TABLE I.

Substance.	Treatment	Color	Conclusion
Cod liver oil	none	lower layer intensely red	markedly positive
Cod liver oil	mildly ox'zed at 100°	lower layer intensely red	markedly positive
Unsaponifiable fraction of c. l. o.	none	deep red solution	markedly positive
ditto	freed from bulk of cholesterol by CH_3OH	deep red solution	markedly positive
Cottonseed oil	none	lower layer light yellow	negative
Cottonseed oil	mildly ox'zed at 100°	lower layer brown	negative
Cottonseed oil	irrad. 50 min. at 1 ft.	lower layer red	positive
Cottonseed oil	irrad. 65 min. at 1 ft.	lower layer decidedly red	strongly positive
Raw linseed oil	none	lower layer slightly orange	very faintly positive
Raw linseed oil	heated in open dish at low temp. for 15 min.	lower layer orange	faintly positive
Raw linseed oil	heated more strongly for 15 min.	lower layer brown	negative
Raw linseed oil	irrad. 70 min. at 1 ft.	lower layer intensely red	strongly positive
Boiled linseed oil	none	lower layer reddish*	positive?
Olive oil	none	lower layer orange	faintly positive
Olive oil	irrad. 90 min. at 1 ft.	lower layer red	positive
Salt butter	none	lower layer orange	faintly positive
Cocoanut oil	none	lower layer light brown	negative
Cocoanut oil	irrad. 50 min. at 1 ft.	lower layer decidedly red	positive
Cholesterol	none	yellow solution, light brown meniscus	negative
Cholesterol	irrad. 15 min. at 1 ft.	darker soln.,reddish meniscus	faintly positive
Cholesterol	irrad. 1 hr. at 1 ft.	red solution	positive
Colorless mineral oil	none	lower layer light yellow	negative
Colorless mineral oil	irrad. 45 min. at 1 ft.	lower layer light brown	negative
Colorless mineral oil	irrad. 2½ hr. at 1 ft.	lower layer brown with an orange tinge	very faintly positive

* Test obscured by dark color of the boiled oil.

Of the particular specimens examined, only cod liver oil and its unsaponified fraction gave a decidedly red color without any preliminary treatment. The unsaponified fraction developed a strong red color immediately even in the cold. Although some of the oils before irradiation gave a reddish yellow, or reddish brown color, they all gave a decidedly and unmistakably red color after irradiation. The only exception was the mineral oil. The red color does not fade; it rather grows stronger on standing and lasts at least several days. Longer heating with the reagent appeared to produce a more marked reaction. In the test with cholesterol itself, the greater the quantity of irradiated cholesterol dissolved in a given volume of reagent, the deeper was the red color obtained. With non-irradiated cholesterol, the color was yellow even at saturation. The irradiated oils reacted negatively with $AsCl_3$.

Apparently these preliminary tests indicate a rough parallelism between the color reaction and the presence of the antirachitic factor. It is emphasized that the substances examined for this reaction have not yet been subjected to the biological test, and it therefore cannot be stated with certainty that substances which contain vitamin D give this reaction, and substances lacking vitamin D do not. However, since usually cod liver oil and its unsaponifiable fraction alone (of the substances studied here) are antirachitic, and since the other substances, excepting the mineral oil, become antirachitic on irradiation, it seems possible that further study may show a close connection, if not an identity, between the antirachitic factor and the chromogenic substance. This relationship is being studied in this laboratory from a number of angles. If such is found to be the case, this color reaction may be utilized for a quantitative estimation, as it is a permanent color.

After the above described work was completed, the author became cognizant of a paper by Bezssonov[6] in which he describes color tests associated with vitamins A and D, obtained with a reagent which he calls phosphomolybdotungstic acid. A comparative study of the two tests is being undertaken.

[6] Bezssonov, N., *Comptes Rendus*, 1924, clxxix, 572.