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Liebermann-Burchard Reaction with Carotene.

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We have postulated some common chemical configuration, actually present or easily produced as a result of chemical interaction, in sterols, in carotene, in the fat-soluble vitamins A, D, and E, in terpenes, and in 5-membered monoheterocyclic compounds.[1] We have recently demonstrated that a mixture of formaldehyde and concentrated sulphuric acid constituted a very sensitive reagent for the detection of carotene in chloroform solution.[2] Whitby,[3] employing the same reagent, reported for cholesterol a color reaction which, however, is not identical with the one obtained for carotene. In this communication we report our findings with reference to the Liebermann-Burchard reaction with carotene. The reaction is the familiar one used in the qualitative detection and in the quantitative estimation of cholesterol. We have already shown that this reagent reacts with heterocyclic compounds like thiophene, furfurane and derivatives.[4]

Carotene gives a positive response with the Liebermann-Burchard reagent, using 10 cc. of chloroform solution, 2 cc. of acetic anhydride and 0.2 cc. concentrated sulphuric acid. With low concentrations of carotene a green color is obtained, which matches in color the one developed when cholesterol is treated with the same reagent. In higher concentrations the green color is tinged faintly blue, but can still be matched with the color obtained with cholesterol. Copper-colored crystals of carotene exposed to sunlight and air turn yellow brown. Carotene, thus oxidized, does not give the characteristic color when dissolved in chloroform and treated with acetic anhydride and sulphuric acid. A brown color instead of the green develops. Old solutions of carotene in chloroform also react in the

1 Levine, V. E., and Richman, E., *J. Biol. Chem.*, 1933, **101**, 373; *Biochem. J.*, 1933, **27**, No. 6, 2051; Levine, V. E., and Seaman, C. L., *Biochem. J.*, 1933, **27**, No. 6, 2047; Levine, V. E., and Shaughnessy, E. J., *Biochem. J.*, 1933, No. 6, **27**, 2048.

2 Levine, V. E., and Bien, G. E., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 581.

3 Whitby, G. S., *Biochem. J.*, 1923, **17**, 5.

4 Levine, V. E., and Richman, E., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 582.

negative. Baumann and Steenbock[5] have recently shown that carotene in organic solvents deteriorates on standing. We therefore used freshly prepared chloroform solutions of carotene for our tests.

The color obtained with carotene reaches its maximum intensity immediately and fades within 2 minutes, leaving a dirty yellow brown color. The green color obtained with cholesterol develops but slowly, reaching its maximum intensity within 15 minutes. The limit of the sensitivity for carotene is 0.01 mg. in 10 cc. of chloroform. The limit of sensitivity for cholesterol is 0.01 mg. in 10 cc. of chloroform as judged with the naked eye and 0.04 mg. in 10 cc. of chloroform as judged in the colorimeter. No attempt was made to determine the sensitivity of carotene with reference to a colorimetric reading owing to the rapid fading of the color.

The Liebermann-Burchard reaction evidently is not given by sterols alone, but also by carotene and certain 5-membered monoheterocyclic compounds. In addition, caryophillin, urson (ursolic acid), rosin, rosin acids, and rosin oil have been reported to give positive tests.[6]

Pure cholesterol in chloroform solution yields higher figures with the Liebermann-Burchard quantitative procedure when carotene is added.

TABLE I.
Effect of Carotene on Cholesterol Determinations.

Reading after	Mg. Cholesterol in terms of 100 cc. of blood	% error
Series I. 0.72 mg. cholesterol + 0.2 mg. carotene in 10 cc. of chloroform, corresponding in the Myers and Wardell[7] method for cholesterol to 180 mg. of the sterol and 50 mg. carotene in 100 cc. of blood.		
8 min.	218	21.10
10 "	208	15.55
12 "	205	13.88
15 "	198	10.00
Series II. 0.72 mg. cholesterol + 0.1 mg. carotene in 10 cc. of chloroform, corresponding to 180 mg. cholesterol and 25 mg. carotene in 100 cc. of blood.		
8 min.	202	11.22
10 "	201	11.66
12 "	194	7.78
15 "	191	6.11
Series III. 0.72 mg. cholesterol + 0.05 mg. carotene in 10 cc. of chloroform, corresponding to 180 mg. cholesterol and 12.5 mg. carotene in 100 cc. of blood.		
8 min.	202	12.22
10 "	200	11.11
12 "	194	7.38
15 "	186	3.33

[5] Baumann, C. A., and Steenbock, H., *J. Biol. Chem.*, 1933, **101**, 561.

[6] Honeywell, E. M., and Bills, C. E., *J. Biol. Chem.*, 1933, **103**, 515; Sando, C. E., *J. Biol. Chem.*, 1923, **56**, 464; Dodge, F. D., *J. Am. Chem. Soc.*, 1918, **40**, 1931; Wiley, H. W., and Bigelow, W. D., *U. S. Dept. Agr. Bur. of Chem. Bull.*, **65**, 1902.

[7] Myers, V. C., and Wardell, E. L., *J. Biol. Chem.*, 1918, **36**, 147.

The error due to carotene diminished with time. This fact is important since in the cholesterol determination of blood the colorimetric reading is made 15 minutes after the addition of acetic anhydride and sulphuric acid to the chloroform extract.

In the method for blood cholesterol the blood is taken up with plaster of Paris. This is dried, pulverized, and extracted with chloroform. The plaster of Paris retains the carotene. The pure pigment, however, is not adsorbed by plaster of Paris. Carotene was added to a chloroform solution of cholesterol, incorporated with plaster of Paris, and the organic solvent allowed to evaporate. The dried mixture was extracted from 1 to 2 hours with chloroform in a Soxhlet extractor. The chloroform solution assumed the color characteristic of carotene in this solvent. As the heating and extraction proceeded the chloroform turned lighter and finally assumed a faint yellow tint. The change in color is probably due to oxidation. The faintly colored chloroform solution gave but a slight green with the Liebermann-Burchard procedure.

Carotene added to blood in the proportion of 100 mg. per 100 cc. and treated with plaster of Paris as in the Myers and Wardell procedure for cholesterol did not increase the original cholesterol value of the blood. This quantity of carotene is extremely high and is hardly to be expected under any physiological or pathological condition. White and Gordon[8] reported in 18 normal human beings a mean value of 0.063 mg. of carotene per 100 cc. of serum, the highest value being 0.108 mg. In 14 diabetics they found a mean value of 0.213 mg. in 100 cc. of serum, the highest figure being 0.379 mg. Curtis and Kleinschmidt[9] reported a case of carotenemia in which the carotene content at the height of the condition was 1.65 per mg. 100 cc. of serum.

Carotene seems to be easily adsorbed. Palmer[10] demonstrated that carotene cannot be determined in whole blood due to the interference of the erythrocytes and in serum only when serum proteins are removed. A mixture of serum and plaster of Paris must be treated with alcohol to precipitate the proteins and to release the carotene preliminary to extraction with petroleum ether.

Crampton and Simons[11] in 1905 applied the Liebermann-Storch reaction for rosin oil for the detection of palm oil used in coloring other fats and oils. Their method consisted in developing a blue

8 White, F. D., and Gordon, E. M., *J. Lab and Clin. Med.*, 1931, **32**, 17, 53.

9 Curtis, A. G., and Kleinschmidt, E., *Ann. Int. Med.*, **1932, 6**, 751.

10 Palmer, L. S., Carotinoids and Related Pigments. The Chemical Catalogue Co., N. Y., 1922, p. 207.

11 Crampton, C. A., and Simons, F. D., *J. Am. Chem. Soc.*, 1905, **27**, 270.

color with a faint green tint in a mixture of equal parts of melted fat or oil and of acetic anhydride to which a drop of concentrated sulphuric acid was subsequently added. Chloroform was not used as a solvent for the fat or oil. Bryan and Gardner[12] maintained that the test was not specific, since other oils responded, that the color faded rapidly and varied greatly with the quantity of concentrated sulphuric acid used. Gill[13] reported that the test for palm oil as made by Crampton and Simons was due to the presence of carotene. He obtained positive spectographic reactions for carotene in carbon disulphide extracts of the non-saponifiable residue secured from palm oil and from the fat of orange peel, yellow corn, yellow squash, flax seed, mustard seed and black sesame seed. These extracts also gave positive tests with the Crampton and Simons procedure.

Employing the Liebermann-Burchard method of testing, we examined several oils, 5 drops of which were dissolved in 5 cc. of chloroform. Our samples of linseed oil and sesame oil gave a dark brown or mahogany color. Cod liver oil yielded a green, which became greenish brown and finally changed to brown. Cotton seed oil, olive oil, castor oil, and wheat germ oil responded with the characteristic green color. Evanescence of color was taken to indicate the presence of carotene rather than cholesterol. Crampton and Simons reported positive color reactions with sesame oil and mustard oil, and Gill noted a positive reaction with linseed oil. The results we obtained with our samples of sesame oil and linseed oil do not agree with those of Gill and Crampton and Simons, but indicate, however, the presence of oxidized carotene, since we obtained a dark brown color instead of the characteristic green color. McDonald[14] and Baumann and Steenbock have recently reported that carotene in solution in various oils oxidizes more or less rapidly on standing as a result of the action of light and air.

Conclusions. Carotene can readily be detected in as low a quantity as 0.01 mg. dissolved in chloroform by means of acetic anhydride and concentrated sulphuric acid. The reaction is similar in method of performance and in final color production to the one employed in the Liebermann-Burchard test for cholesterol. The difference in the duration of the green color developed may serve, however, to differentiate one compound from the other. The green

[12] Bryan, T. J., and Gardner, B. C., *U. S. Dept. Agr., Bur. Chem. Bull.*, **137**. 1911, 87.

[13] Gill, A. H., *J. Ind. Eng. Chem.*, 1917, **9**, 136; 1918, **10**, 612.

[14] McDonald, F. C., *J. Biol. Chem.*, 1933, **103**, 455.

color obtained with carotene develops its maximum intensity at once and fades within 2 minutes, leaving a dirty yellow brown color. The green color obtained with cholesterol develops its maximum intensity at the end of 15 minutes.

Carotene does not interfere with the quantitative estimation of cholesterol in the blood. The extraction with chloroform of a mixture of blood and plaster of Paris removes the cholesterol, but does not release the carotene present.

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Immunization of Guinea Pigs with Formalized Cultures of European Strain of Typhus Rickettsia.

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We have reported[1] the successful cultivation of the European strain of Typhus *rickettsia* in flask cultures containing guinea pig tunica. These cultures have now passed successfully through 7 subcultures during a period of 4 months.

In view of successful immunization of guinea pigs with formalized rickettsia suspensions from infected lice[2] and Zinsser's[3] experience with formalized rickettsia suspensions of the Mexican type from infected X-rayed rats, it appeared desirable to test the possibility of using flask tissue cultures for this purpose. Our efforts to immunize guinea pigs with formalized infected brain tissue proved unsatisfactory. Dead virus produced at best only a slight degree of immunity, when large amounts (10 gm.) of formalized infected tissue were injected. Solid immunity could only be obtained with tissue formalized for 1-2 hours, that is tissue still containing live (attenuated?) virus. The difficulty seems to lie in the quantity of organisms, or in other words the amount of antigen, injected. The cultures appeared to offer a satisfactory solution to the problem. On the one hand, the organisms are present in fairly large quantities and, on the other, the cultures are more easily handled than lice.

1 Kligler, I. J., and Aschner, M., PROC. SOC. EXP. BIOL. AND MED., 1933, **31**, 349.

2 Kligler, I. J., Olitzki, L., and Aschner, M., PROC. SOC. EXP. BIOL. AND MED., 1932, **29**, 456.

3 Zinsser, H., and Castaneda, M. R., *J. Exp. Med.*, 1933, **57**, 381.