tered subcutaneously. In a second series of experiments the meat meal was replaced by 120 cc of 7% alcohol administered by stomach tube. In all cases the gastric juice was collected over a 3-hour period and titrated for free acid (Topfer's reagent) and total acid (phenolphthalein).

Results. The results, in the form of averages for the different experiments are presented in Table I. It can be seen that 1 mg of atropine sulfate completely abolished the gastric secretory response to the meat meal, but only partially reduced the response to 7% alcohol. Similarly, the alcohol stimulus proved to be more resistant than the meat meal to the inhibitory action of olive oil.

The available evidence suggests that a close resemblance exists between the actions of alcohol and histamine on the gastric glands. The action of both is resistant to the inhibitory action of atropine and fat, and Kreuger and MacIntosh[8] have reported that both stimulate the production of a juice of high acidity and low pepsin concentration.

Conclusions. In dogs with Pavlov pouches the gastric secretory response to dilute alcohol is resistant to the inhibitory effects of atropine or fat.

11087

Photoelectric Study of Liebermann-Burchard Reaction and Its Significance in Determination of Cholesterol.

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The common colorimetric method for the determination of cholesterol is based upon the Liebermann-Burchard[1] reaction, in which acetic anhydride and concentrated H_2SO_4 are added to a dilute solution of cholesterol in chloroform. The color produced is at first blue, then becomes green and finally, on long standing, a yellow-brown. This reaction, in spite of its apparent simplicity, has proved difficult to control, for the intensity as well as the shade of the color is markedly influenced by small differences in the concentrations of the reagents, the presence of traces of water or other impurities, time, and temperature. The many previous efforts to control these factors

1 Liebermann, C., *Ber. Deut. Chem. Ges.*, 1885, **18**, 1803.

by modification of the technique of Autenrieth and Funk[2] and of Bloor[3] have been for the most part empirical. A study of the reaction, however, with the aid of the photoelectric colorimeter and varying filters gives a clue to the nature of the reaction and the principles that must be followed for exact, duplicable, colorimetric analysis of cholesterol.

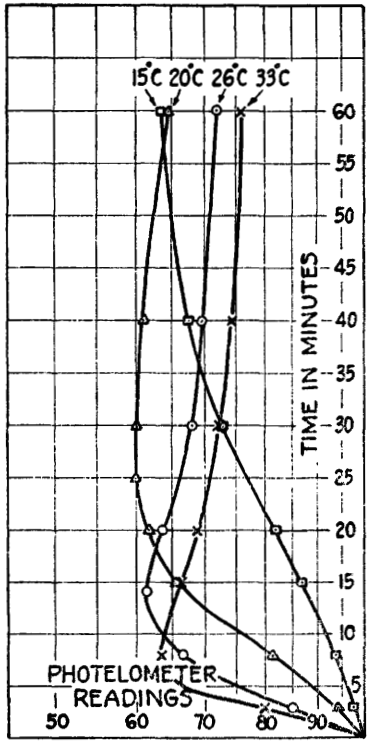
A mixture of 15 cc of acetic anhydride, 1 cc of concentrated H_2SO_4 and 24 cc of chloroform, C.P. was used as the reagent. This mixture was prepared immediately before using, since it decomposes on standing for more than an hour. Five cc were added with thorough mixing to 5 cc of a chloroform solution containing 0.48 mg of cholesterol in a glass-stoppered cylinder. The vessel was immediately placed in a water bath maintained at the proper temperature. This procedure, which was also followed in the routine analysis of cholesterol, eliminated errors due to inaccuracy of measurements of small quantities of acetic anhydride and H_2SO_4 and to irregular initial temperatures produced by direct addition of H_2SO_4, and allowed accurate measurement of the effect of time and temperature. Readings were made with a photoelectric colorimeter with an orange filter with a maximal transmission at 620 mμ, or with a blue filter with a maximal transmission at 430 mμ.* The curves obtained were plotted on semi-logarithmic paper, since the concentration of the light-absorbing substance is proportional to the negative logarithm of the reading.

At 26°C, with an orange filter, the absorption progresses to a maximum in 13 minutes, then slowly recedes and approaches zero (Fig. 1). With a blue filter, absorption increases continuously with time, shows no maximum in an hour, but at that time is approaching a constant value (Fig. 2). At higher temperatures with the orange filter, a maximal absorption is reached earlier, but the value is less. At temperatures lower than 26°C, a maximal absorption is more delayed, but when reached has a higher value and is maintained for a longer time before recession occurs. At 15°C, a maximal absorption is not obtained in an hour. Further readings at 15°C indicate that the reagent decomposes before a true maximum is reached. With a blue filter, curves at various temperatures are similar to each other, but the absorption at 60 minutes is proportional to the temperature (Fig. 2). At 10 minutes, the effect of temperature change

[2] Autenrieth, W., and Funk, A., *Münch. Med. Woch.*, 1913, **60**, 1243.

[3] Bloor, W. R., *J. Biol. Chem.*, 1915, **23**, 317.

* The Cenco-Sheard-Sanford Photelometer, manufactured by the Central Scientific Company, was used in these experiments.



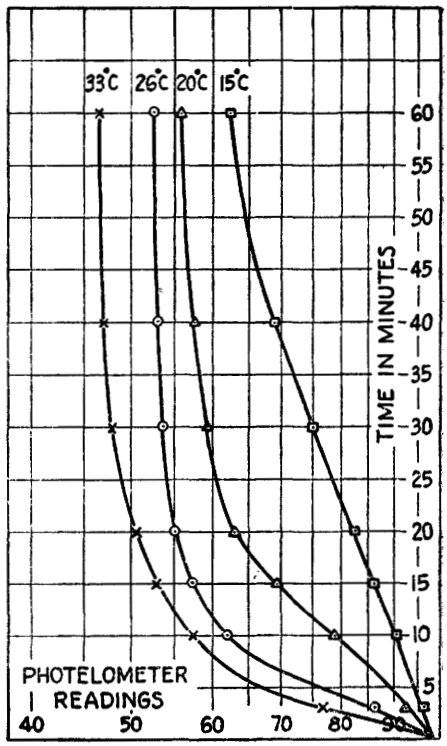


FIG. 1 (Left)
The relationship between light-absorption with an orange filter and the time of the development of the color, at various temperatures. A reading of 100 indicates no absorption.

FIG. 2 (Right)
The relationship between light-absorption and time at various temperatures, with a blue filter.

upon the reading is much greater for both filters in the lower temperature range than in the higher.

These results indicate that the Liebermann-Burchard reaction consists of 2 phases: A, the production of a blue substance which has absorption bands both in the region of the blue and of the orange; B, the decomposition of this blue substance, as soon as it is formed, into a yellow substance which has an absorption band only in the blue. Reaction B is affected by increased temperature more than is A. Therefore, at higher temperatures, the point is reached sooner where the decomposition of the blue substance is as fast as its formation, and the sooner this point is reached, the less there is of the blue substance.

Spectrophotometric examination of the colored solution with the

Cenco-Sheard Spectrophotelometer confirmed these findings.† There are 2 broad absorption bands, one between 600 and 660 mμ with a maximum at 630, and another between 380 and 440 mμ with a maximum at 420 mμ. As the solution changed from blue to green to yellow-brown, the absorption band in the orange-red became more shallow while that in the blue became deeper.

It is obvious that these facts must be considered in applying the Liebermann-Burchard reaction to the analysis of cholesterol. All the factors which determine the intensity and shade of the color, particularly time and temperature must be controlled. This is especially true of photoelectric methods, for here analyses of unknown and standard solutions are not made simultaneously. In the photoelectric method, either an orange or a blue filter may be used, but the former is preferable since the reading can be made at a maximal value after a short time. Besides, colored impurities in the reagents give little blank with an orange filter, but may show appreciable and variable blanks with a blue filter. Since the time of the reading can be fixed more readily than the temperature, it is best to make the readings with the orange filter at exactly 10 minutes at a temperature of 26°C or slightly above, for at this time, and this temperature range, there is the least effect of change of temperature upon the reading.

In the actual determination of cholesterol in blood, 0.2 cc of serum (or whole blood) are allowed to dry on a filter paper disc and then extracted with about 4 cc boiling chloroform for 60 minutes in a Leiboff[4] extraction tube. The cooled chloroform solution is then quantitatively transferred to a 10 cc glass stoppered cylinder, made up to 5 cc with chloroform, and treated with 5 cc of the freshly prepared reagent. The cylinder is placed in a water bath kept between

TABLE I.
Accuracy of Determination of Cholesterol.
The results are expressed as mg per 100 cc.

Sample	No. of Determinations	Concentration	Added Cholesterol	Total Cholesterol	
				Calculated	Found
Cholesterol solutions					
240 mg per 100 cc	8	240 ± 3			
160 mg per 100 cc	8	160 ± 2			
Serum	8	178 ± 5	80	258	252
Serum	8	184 ± 4	100	284	294
Whole blood	8	148 ± 5	160	308	310

† The author is indebted to Dr. M. N. States, of the Central Scientific Company, for his assistance in the spectrophotometric study.

[4] Leiboff, S. L., *J. Biol. Chem.*, 1924, **61**, 177.

27°C and 26°C for 9 minutes. The colored liquid is then transferred to a fused glass absorption cell and read in the photelometer with an orange filter at exactly 10 minutes. The value is read from a calibration curve obtained from the analyses of standard cholesterol solutions in chloroform made exactly in the same manner. The curve plotted on semi-logarithmic paper is a straight line.

The accuracy and duplicability of the method is attested to by the findings in Table I. Added cholesterol was recovered with the same degree of accuracy.

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Effect of Adrenocorticotropic Hormone in 4-Day-Old Rats.*

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Adrenocorticotropic hormone (A-C-T) has been assayed in this laboratory using the 21-23-day-old male rat.[1] This test requires relatively large amounts of A-C-T. For this reason the response of 4-day-old rats to A-C-T has been studied with the hope of finding them to be more sensitive.

On the day of birth, rats were grouped into litters of 8, containing both males and females. Beginning on the 4th day postpartum, the rats in each litter were injected intraperitoneally with 0.1 ml of A-C-T preparations (previously assayed in 21-23-day-old rats)

TABLE I.
Effect of A-C-T on 4-day-old Rats.

No. of rats	Dose units	Avg body wt		Avg wt of adrenals	Avg wt of thymus
		Init. grams	Final grams	mg	mg
16	0.05	9.5	12	3.7	10.5
16	0.10	9.6	14.2	4.0	9.5
16	0.15	9.5	11.5	4.3	5.1
16	0.25	9.0	11.2	4.4	5.3
16	0.50	—	10	6.1	2.5
40 controls		9.8	14.0	2.4	20.9

* Aided by grants from the Board of Research of the University of California and Rockefeller Foundation of New York City. Assistance was rendered by the Federal Works Progress Administration, Project O.P. 65-1-08-62 Unit A-5.

[1] Moon, H. D., PROC. SOC. EXP. BIOL. AND MED., 1937, **35**, 649.