injecting 2% ammonium hydroxide with ammonia gas into a loop of jejunum. The ammonia content of the lymph was increased 4 times when the intra-enteric pressure was raised to 60 mm. of mercury. In 4 cats the lymphatic pedicle at the base of the mesentery was ligated without prolongation of the survival period attending the establishment of simple ileal obstruction.

It would appear that the regional lymph nodes normally serve as an effective barrier against overwhelming absorption of bacteria into the blood stream via the mesenteric lymphatic channels. A true bacteremia probably does not occur and most likely is not the cause of death in uncomplicated simple intestinal obstruction as we were unable to obtain positive cultures of the test organism from the thoracic duct. The lymph nodes yielding positive cultures acted as efficient barriers and precluded more centripetal invasion. A toxemia of bacterial origin, however, has not been wholly excluded.

Conclusion. Intestinal obstruction and increased intra-enteric pressure are conducive to increased lymphatic absorption of dyes and bacteria into the regional lymph nodes. No evidence, however, was adduced to indicate that this occurrence is of great significance in the lethal issue of bowel obstruction.

8236 C

An Improved Method for Determination of Blood Carotene.*

E. DÁNIEL AND G. J. SCHEFF. (Introduced by L. F. Rettger.)

From the Departments of Surgery and Hygiene, University of Pécs, Hungary.

Following closely upon the rapid accumulation of our knowledge regarding the relation of plant carotene to vitamin A, increasing interest has been manifested by investigators in the study of carotenoids. Thus far the investigations have been concerned chiefly with the carotene content of human and animal organism under different physiological and pathological conditions. The need of a suitable method for determining small amounts of carotene in the circulating blood has become quite apparent.

The methods hitherto employed[1] are more or less unsatisfactory

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1 Van den Bergh, H., Muller, P., and Broekmayer, J., *Biochem. Z.*, 1920, **108**, 279; Connor, Ch. L., *J. Biol. Chem.*, 1928, **77**, 616; Van Eekelen, M., *Acta brevia*

in that they are impractical or non-specific. In some, fairly large amounts of blood are required for each estimation, which makes serial determinations difficult or impossible. Others are not specific and may lead to false results when they are used in the presence of other similarly reacting substances. The methods which involve the use of the yellow color of the carotin dye itself as an agent in the determination do not take into consideration that polyene dyes other than the carotenes, for example those of the xanthophyll type, may be part of the food intake and enter the blood. As a matter of fact, such instances were demonstrated by Palmer[2] and other investigators long ago. It is but natural, therefore, that under such conditions the yellow color of the blood serum extracts will represent the resultant of different dye substances, whereas the chief aim of all these methods should be the determination of the real carotene content of the blood. The present method was devised to eliminate the above-mentioned errors.

The carotenoids are precipitated completely from the blood serum by deproteination with alcohol. The dye is removed from the protein precipitate by repeated extraction with ether. After saponification of the lipoids and xanthophyll esters in the ether extract, the polyene hydrocarbons and xanthophyll dyes are separated from each other by dispersing them in a mixture of methyl alcohol and ether-petroleum-ether ("Entmischung"). The carotenes are contained in the upper layer, and may be determined in it colorimetrically.

Three cubic centimeters of the test serum are placed in a 20-25 cc. glass-stoppered centrifuge tube. After precipitation with 3 cc. of alcohol, the tube is carefully shaken and centrifuged. The supernatant fluid is discarded. The precipitate is repeatedly extracted with 3-4 cc. of ether. It is sufficient as a rule to repeat the extraction 4 times. To the combined dye-extract 0.5 cc. of a methyl alcohol solution of KOH (30%) is added; in order to prevent autoxydation, the liquid should be kept under nitrogen in an almost completely filled flask for about 24 hours. The fluid is then diluted with distilled water until separation into 2 layers takes place. An elongated graduated glass-stoppered separatory funnel of 25-30 cc. capacity proved to be the most convenient for this purpose. After

Neerlandica, 1931, **1**, 3; White, F. D., and Gordon, E. M., *J. Lab. and Clin. Med.*, 1931, **17**, 53; Menken, D., *Med. Wschr.*, 1932, 1484; Levine, V. E., and Bien, G. E., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 804; Latest synopsis of Zechmeister, L., 1934, Carotinoide, and Bomskov, Chr., 1935, Methodik der Vitaminforschung.

[2] Palmer, L. S., *J. Biol. Chem.*, 1914, **17**, 191.

removal of the lower layer, the washing with water is repeated from 10 to 12 times, or until the wash water becomes neutral to litmus. As may be expected, the amount of ether is gradually decreased during this procedure, so that of the original ether used (9-12 cc.) only about half is left. To this washed ether extract one part of petroleum ether (50-60°) is added.

The separation of the polyene hydrocarbons from the oxygen-containing dyes now follows. By adding one volume of methyl alcohol (85%) to the above-mentioned dye-extract, the carotenes are recovered in the upper (ether-petroleum ether) layer, while the xanthophylls are held in the lower (methyl-alcohol) layer.[3] By removing the lower layer and washing the carotene-containing solution twice with fresh methanol, the xanthophylls are completely removed from it. It is important, however, to recover the small traces of carotene which may have been removed by the methyl alcohol. This can be done by re-washing the methyl alcohol extract with fresh petroleum ether. The upper layer is combined with the main ether-petroleum-ether solution, and the combined extract washed three times with 10 cc. of water to eliminate the last traces of methanol. This last treatment was found necessary, because the intensity of the color of the carotenoids is largely dependent upon the quality of the solvent.

The next step in the process is the evaporation of the carotene solution to dryness, *in vacuo* at 50°C. The ochre-colored residue is dissolved in 0.75-1.00 cc. of ligroin (70-80°), and the solution so obtained dehydrated with ignited sodium sulphate. Solution usually occurs in a very short time. The ligroin solution is poured into the trough of a microcolorimeter having a marked capacity of 1.0 cc. The deficiency is made up to the mark with ligroin rinsing of the evaporating flask. The entire carotene content of the original 3 cc. of blood serum is contained in this 1 cc. of ligroin.

The final colorimetric readings are made on the ligroin extract. For comparative purposes the azobenzene standard recommended by Kuhn and Brockmann[4] was employed: 14.5 mg. of azobenzene "Schering Kahlbaum" are dissolved in 100 cc. of 96% alcohol. The standard is set at 10 mm. Diffuse daylight proved to be most satisfactory. Five quick readings were made and the average taken. Control experiments did not differ more than 5% from each other. Known amounts of crystalline carotene added to the ether extracts of serum were recovered quantitatively.

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[3] Willstätter, R., and Stoll, A., *Untersuchungen über Chlorophyll*, 1913.

[4] Kuhn, R., and Brockmann, H., *Z. f. physiol. Chem.*, 1932, **206**, 41.

Experiments are now in progress to determine the merits of the method on a large scale in clinical cases.

We believe the method described here has sufficient accuracy to be applicable to small quantities of blood serum (3 cc.), and to serial determinations. The use of whole blood appears unnecessary. The investigation of Karrer and his collaborators[5] showed that the red blood corpuscles do not contain carotene. Special tests were carried out to ascertain whether, in the preliminary precipitation with alcohol, all of the blood carotene is precipitated. Tests were made also to determine whether the recovery of the dye by the extraction of the precipitate with ether is complete. For this purpose, 2 kinds of controls were employed. First, the fluid decanted from the alcohol precipitate was extracted with ether, and the extraction procedure repeated with ether and ligroin after the addition of lye; second, the precipitate itself was treated with 5% NaOH and extracted with ether several times. Neither the ether nor the ligroin used for the extraction contained any carotene.

In other experiments the effectiveness of the separation of carotenes from xanthophylls was tested on larger quantities of serum. For this purpose about 5 liters of mixed human blood serum was prepared in a manner similar to that described above for small quantities. After separation of the 2 solvent layers, the isolated dyes were identified with the aid of Tswett's chromatogram method and the grid-spectroscope.[6] For better identification of these dyes, a chromatogram (Tswett) was prepared from the ligroin solution of each isolated dye by pouring the solution through a $Ca(OH)_2$ column.† The results indicate that even some of the more recent investigators (Wendt[7]) have erred, basing their carotene determinations on the yellow color extracted directly from the serum; a considerable amount of the extracted material may belong to the xanthophyll type of coloring matter. The colorimetric determinations showed that the xanthophyll coloring matter present in the mixed human blood serum examined was about 15% of the total lipochrome content. In the chromatogram of the epiphasic dye a yellowish-pink ring developed above the yellow ring which is characteristic for carotenes; in the chromatogram of the hypophysis the zone characteristic of xanthophylls was seen. Each dye layer was

[5] Karrer, P., Euler, H. v. Hellström, H., and Rydbom, M., *Arch. for Kemi, Mineralogie och Geologie,* **10**, No. 12.

[6] Winterstein, A., and Stein, G., *Z. f. physiol. Chem.*, 1933, **220**, 247.

† Our thanks are due to Miss Njhelyi for valuable assistance.

[7] Wendt, H., *Klin. Wschr.*, 1935, No. 1.

dissolved separately in CS_2, and the extinction maxima determined with the grid spectroscope, with the following results. The upper yellowish-pink ring of the polyene hydrocarbon portion did not show any characteristic absorption band (autoxydized layer). The yellow ring below this gave an extinction maximum between those which are characteristic for A and B carotenes, *i. e.*, 514 and 479mμ. On the other hand, spectroscopic examination showed the main portion of the other fraction to be xanthophyll, with absorption bands at 509 and 473mμ (in CS_2).

In the experiment just described it appears evident, therefore, that the blood serum contains a considerable quantity of xanthophyll dye, which has to be eliminated from the test material before the carotene determination is made.

It is to be emphasized, however, that the method described may lead to false results in instances where the blood contains lycopene. Lycopene has been, as is well known, identified in human fat tissue[8] to which it is carried by the blood stream. Being a much more intense dye than carotene, a very small amount of lycopene is sufficient to cause an error in the colorimetric determination of carotene. The 5 liters of mixed blood examined by the writers contained no lycopene. It is important to keep patients whose blood is to be tested for carotene on lycopene-free (tomato-free) diet for several days before the blood examination.

Summary. Human blood serum contains other lipochromes besides carotenes, which must be removed from the test material before determinations of provitamine A are made.

8237 P

Homologous Function in Supernumerary Limbs After Elimination of Sensory Control.

PAUL WEISS.

From the Department of Zoology, The University of Chicago.

When in a salamander a supernumerary limb is transplanted into the neighborhood of a normal limb so that it receives its innervation from some part of the limb plexus, every movement of the transplanted limb is an exact duplication of the simultaneous movement of the nearby normal limb. This phenomenon of "homo-

8 Zechmeister, L., and Tuzson, P., *Z. f. physiol. Chem.*, 1935, **231**, 259.