Skelly-solve B. The nonsaponifiable material thus obtained was dissolved in alcohol and most of the sterols were removed from this solution by fractional crystallization.

Attempted Isolation of the Tocopherols by Means of Their Lead Salts.—An alcoholic solution of normal lead acetate was added to the alcoholic solution of the nonsaponifiable material. Precipitation did not occur, even after concentration or cooling in the ice chest. The alcohol was completely removed on the steam bath and the residue treated with Skelly-solve B. The material insoluble in this solvent was suspended in alcohol and decomposed by hydrogen sulfide. A very small amount of a semi-solid was obtained.

When basic lead acetate was substituted for normal lead acetate the same results were obtained.

Attempted Isolation of the Tocopherols by Means of Their Barium Salts.—Essentially the same procedure was followed as outlined above with the exception that methanol was used as the solvent and a methanol solution of barium hydroxide as the precipitant. The results were negative.

Attempted Isolation of the Tocopherols by Extraction of a Skelly-solve B Solution of the Nonsaponifiables by Means of Aqueous and Alcoholic Sodium Hydroxide.—The nonsaponifiable material was dissolved in Skelly-solve B. It was extracted with 5, 10 and 20 per cent solutions of aqueous sodium hydroxide. When these solutions were neutralized with phosphoric acid and extracted with ether, a very small amount of a semisolid material was obtained. The nonsaponifiable portion was then extracted with an approximately 25 per cent solution of sodium hydroxide in 25 to 33 per cent alcohol. The alkaline extractive was colored and, upon decomposition with phosphoric acid and extraction with ether, yielded a small amount of an oil. From this oil an exceedingly small amount of an allophanate, m. p. 145° C., was obtained. This allophanate failed to give the color reactions characteristic of sterols and by virtue of its melting point was thought to be one of the tocopherols.

Crystalline Xanthophyll.—The petroleum ether solution that had been extracted with an alcoholic solution of alkali was allowed to stand in the ice chest for some time. Rosettes of garnet red leaflets separated from this solution. They were collected upon a fritted glass filter, washed with Skelly-solve B and dried. They melted at 175° C. and exhibited an absorption max. at 475, 450 in alcohol. A portion of the material when dissolved in Skelly-solve B would partition itself into 85 per cent methanol. A solution of the pigment in chloroform gave a bluish violet color when carefully stratified with strong sulfuric acid.

SUMMARY

Crystalline xanthophyll was obtained from the nonsaponifiable portion of wheat germ oil while attempting to isolate the tocopherols by new methods.

Colormetric Determination of Thiamin Chloride in Certain Pharmaceutical Preparations

*By M. E. Auerbach**

Recently, Prebluda and McCollum (1) have described in detail a chemical reagent for thiamin chloride. Using the same reagent Melnick and Field (2) have developed procedures which make possible a quantitative determination in a variety of substances. The principle elaborated by these investigators is that thiamin will couple with diazotized *p*-amino acetophenone to produce a purplish red compound. This compound is insoluble in water, but is readily soluble in various organic solvents, thus providing a colored solution suitable for comparison with standards. Melnick and Field prefer to use xylol as the color solvent. We use toluol, which is equally serviceable. For the analysis of thiamin dissolved in a simple, colorless menstruum, it is unnecessary to extract the colored compound from its mother liquor. In such cases, we simply add enough isopropanol to dissolve the color, and proceed directly to the quantitative comparison with a reference solution.

A certain drawback appears in the work quoted, in that at least 12 hours are stated to be necessary for the development of the maximum color intensity. During the past two years, we have employed a modification of the Prebluda-McCollum test which permits a complete analysis of thiamin in certain pharmaceutical products within 15 to 30 minutes. The same method, with minor alterations, has served for the determination of the stability and incompatibilities of thiamin chloride under various conditions and in various media.

EXPERIMENTAL

Conditions for the Analysis.—A quantity of test sample is taken which will yield as nearly as possible 100 micrograms of thiamin. A standard solution is made by dissolving exactly 50 mg. of pure dry thiamin chloride (or an equivalent calculated from actual analysis) in 500 cc. of 50% (volume) ethanol containing one drop of 4*N* HCl. This standard is stable at room temperature for several months, but

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to be on the safe side, we prepare a fresh standard every four weeks.

Reagents:

(1) 0.03% *p*-amino acetophenone (Eastman Kodak Co.) in 0.2*N* HCl. This solution is stable indefinitely.
(2) 0.1% sodium nitrite. Prepare fresh daily.
(3) Normal NaOH and 2.5*N* NaOH.
(4) Ethanol, 50% (volume).
(5) Toluol.
(6) Isopropanol.
(7) 4*N* HCl.

The solution referred to as "diazo solution A" is prepared as follows: In a small flask mix 10 cc. of the amino acetophenone solution and 2 cc. of the sodium nitrite solution. Let stand in ice water for at least two minutes. Just before the solution is to be used, add 3 cc. of normal NaOH and mix. "Diazo solution B" is similarly prepared, 2.5*N* NaOH being used instead of normal NaOH.

COURSE OF THE ANALYSIS

A. Ampule Solutions.—Dilute the vitamin solution with distilled water, so that each cc. is estimated to contain about 100γ. In a clean test-tube with a 10-cc. calibration mark, take 1 cc. of this dilution, add 2 cc. of 50% ethanol and place the tube in a water-bath kept at 60° C. After a minute, add one cc. of diazo solution A. Mix well and let stand at 60° C. for another 2–3 minutes. Cool, add one drop of 4*N* HCl, then add isopropanol to the calibration mark, mix, transfer to a colorimeter tube and compare with the reference solution. The reference solution is prepared by taking one cc. of distilled water in a tube, then adding one cc. of the standard thiamin solution, one cc. 50% ethanol and one cc. diazo solution A. in the manner already described.

B. Thiamin Chloride Elixir.—The elixir in question contains about 17 mg. thiamin per 100 cc. If 6 cc. of elixir are mixed with 4 cc. of water, the mixture will contain close to 100γ per cc. Take one cc. of this dilution in a clean test-tube, add 3 cc. 50% ethanol, warm to 60° C. in a water-bath, then add 2 cc. of diazo solution B. Keep at 60° C. for another 15 minutes. Cool, acidify with a few drops of 4*N* HCl and transfer to a small separatory funnel. Dilute with water to 25–30 cc., then add exactly 8 cc. toluol and shake. The water insoluble, colored vitamin derivative is readily taken up by toluol, especially from acid solutions. Add 3 cc. of 2.5*N* NaOH, shake, allow to separate, discard the aqueous layer, wash the toluol layer once with distilled water, then transfer to a centrifuge tube. Centrifuge for a few minutes (until perfectly clear and bright) and compare with the reference solution. The latter is prepared by diluting 6 cc. of vitamin-free elixir with 4 cc. water. To one cc. of the dilution add one cc. of standard thiamin solution, then 2 cc. 50% ethanol, and continue as given above. The vitamin-free elixir can be prepared by shaking out the original elixir with a suitable adsorbent, such as Decalso (2), (3).

C. Tablets Containing Thiamin Chloride.—Thoroughly pulverize ten tablets. Weigh out an amount which will contain about one mg. thiamin, and transfer to a test-tube with a 10-cc. calibration mark. Fill to the mark with 50% ethanol, and let the tube stand in a 60° C. water-bath for ten minutes, with occasional gentle mixing. Cool, and if necessary add alcohol to bring the level back to the mark. Mix well and centrifuge until clear. To one cc. of the clear centrifugate add 1 cc. 50% ethanol and 1 cc. water, warm to 60° C. and add 1 cc. diazo solution B. Keep at 60° C. for another 2–3 minutes, cool, acidify with 4*N* HCl, then extract with toluol as described in section B. Compare the colored toluol solution with a reference solution prepared from a mixture of 1 cc. water, 1 cc. 50% ethanol, 1 cc. standard solution and 1 cc. diazo solution B.

GENERAL DISCUSSION

When 100 micrograms are taken for a standard reference solution, and the quantity in the test sample is in the range 90–110 micrograms, the error of the method is not greater than ±3%. The error increases in a fairly definite way as the discrepancy between the test quantity and standard quantity increases.

We have found it possible to determine thiamin chloride in such varied solvents as milk, wine, orange juice, mineral oil, propylene glycol, alcohol, glycerol and sugar syrup and in aqueous or/and alcoholic solutions also containing phenol, chlorbutanol, benzoates, citrates, bromides, iodides, lactates, salicylates, phosphates, glycerophosphates, ferric ammonium citrate, vitamins A and D_2, riboflavin, ascorbic acid, barbiturates, amidopyrine, pepsin, liver extract. In some cases a simple preliminary treatment was necessary. For example, solutions containing liver extract are best handled by diluting with three or four parts of methanol, centrifuging off the alcohol-insoluble constituents, and taking a convenient aliquot of the centrifugate for the determination. The presence of ascorbic acid in the test sample involves minor additional treatment which we hope to describe in the near future.

With few exceptions, it is necessary to run a blank of the same composition as the test sample, since many substances affect the

intensity and shade of the color produced. Within our experience, however, no thiamin-free substance has yielded a positive reation. Mixtures of unknown composition are readily de-vitaminized by shaking with Decalso at p_H 4.0–4.5 (2), (3).

For good results, it is important to plan the quantities of reagents used so that the coupling reaction takes place in a mixture containing 20–25% ethyl (or methyl) alcohol.

The specificity of the Prebluda-McCollum color test depends largely on the fact that the colored thiamin derivative is insoluble in both acids and alkalis. We always extract the color from an acid medium, to avoid the annoyance of the emulsions which tend to form when shaking alkaline solutions. We then wash the colored toluol solution with sodium hydroxide solution, to remove foreign colored substances containing acidic groups such as carboxyl or phenol hydroxyl.

SUMMARY

A simple and rapid method for the quantitative estimation of thiamin chloride has been described. The method is based upon the Prebluda-McCollum reaction. It has successfully been applied to the analysis of mixtures of pharmaceutical interest, containing as little as twenty micrograms of thiamin chloride per Gm. of sample.

REFERENCES

(1) Prebluda, H. J., and McCollum, E. V., *J. Biol. Chem.*, 127 (1939), 495.

(2) Melnick, D., and Field, H., *Ibid.*, 127 (1939), 505, 515, 531.

(3) Hennessy, D. J., and Cerecedo, L. R., *J. Am. Chem. Soc.*, 61 (1939), 179.

ADDENDUM

Colorimetric Determination of Thiamin Chloride in the Presence of Ascorbic Acid.— In the foregoing paper, several examples were given of a colorimetric determination of thiamin chloride in pharmaceutical products. It was stated that the presence of vitamins A, B_2 and D causes no difficulty with the method, but that the presence of vitamin C necessitates a special pre-treatment. Both ascorbic acid and its oxidation products prevent the development of the colored thiamin derivative upon which the determination depends. It was discovered that the addition of calcium ion (magnesium and barium are partially effective) to the mixture of thiamin chloride and oxidized ascorbic acid adequately prevented interfering reactions. The mechanism by which this is accomplished is not yet clarified.

The example given below presents details of the modified method. The product chosen for description consists of a suspension of vitamins A, B_1, B_2, C and D in a fat.

Method: Transfer about one Gm. of the thoroughly mixed fatty mass to a tared 50-cc. Erlenmeyer flask. Add 5 cc. monochlorobenzene and then, from a burette, 10 cc. of $N/10$ HCl for each mg. of thiamin chloride estimated to be in the sample taken. Stopper and shake the flask thoroughly for five minutes, then centrifuge the contents until the aqueous layer is perfectly clear. The fatty material and vitamins A and D are retained by the monochlorobenzene. Thiamin chloride, riboflavin and ascorbic acid are all quantitatively extracted by the acid water.

Take one cc. of the aqueous extract in a clean test-tube, add 1 cc. 50% ethanol, then, drop by drop, strong fresh bromine water until the color is permanently a deeper yellow than the original solution. Add one drop of 4% sodium salicylate solution to take up excess bromine, then 2 cc. of 50% ethanol and finally 1 cc. of approximately $N/1$ $CaCl_2$. Warm in a water-bath at 60° C. and add 2 cc. of freshly prepared diazo solution.

(The diazo solution is prepared by mixing 10 cc. of 0.03% *p*-amino acetophenone in $N/5$ HCl with 2-cc. 0.1% $NaNO_2$. Place in an ice-bath for at least 3–4 minutes, then add 3 cc. of 10% NaOH just before using.)

Allow the tube to stand at 60° C. for 10–15 minutes, cool, acidify with a few drops of $4N$ HCl, add 8 cc. toluol, shake well and transfer to a small separatory funnel. Discard the aqueous layer, then wash the colored toluol with (1) about 15 cc. water; (2) about 15 cc. 4% NaOH; (3) about 15 cc. water. Transfer the colored toluol to a small centri-