The results of these studies characterize vitamin B_6 as a substance of very low toxicity corresponding with the low toxicity found for other members of the vitamin B complex, thiamin,[9] riboflavin,[10] and nicotinic acid.[11] Excessively large doses (3.0 g per kg) of vitamin B_6 produced convulsions and death.

The difference in subcutaneous and oral toxicity, as shown by the L.D. 50, is small and suggests a rapid and complete absorption from the intestinal tract.

Prolonged feeding of sublethal doses failed to produce toxic symptoms, thus indicating that excessive doses of vitamin B_6 are either rapidly excreted or destroyed. Studies on this problem are now in progress.

We wish to acknowledge the technical assistance of Mr. Joseph Greslin.

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Urinary Excretion of Vitamin B_6 in the Rat.

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(Introduced by H. Molitor.)

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Following the isolation,[1] identification,[2] and synthesis[3] of vitamin B_6, as reported from these laboratories, the urinary excretion of the vitamin has been studied in the rat.

Recently, Kuhn and Low[4] reported on the use of the Folin-Denis reagent for the colorimetric determination of the vitamin in aqueous solutions. Stiller, Keresztesy and Stevens[2] observed that the vitamin gave a positive Gibbs reaction.[5] This reaction has been modified and

9 Molitor, H., and Sampson, W. L., *E. Merck's Jahresber.*, 1936, **50**, 51.

10 Kuhn, R., *Klin. Wchnschr.*, 1938, **17**, 222.

11 Unna, K., *J. Pharm. and Exp. Therap.*, 1939, **65**, 95.

1 Keresztesy, J. C., and Stevens, J. R., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 64.

2 Stiller, E. T., Keresztesy, J. C., and Stevens, J. R., *J. Am. Chem. Soc.*, 1939, **61**, 1237; Harris, S. A., and Folkers, K., *J. Am. Chem. Soc.*, 1939, **61**, 1242.

3 Harris, S. A., and Folkers, K., *J. Am. Chem. Soc.*, 1939, **61**, 1245.

4 Kuhn, R., and Low, I., *Ber.*, 1939, **72**, 1453.

5 Gibbs, H. D., *J. Biol. Chem.*, 1927, **72**, 649.

applied to the estimation of the vitamin in urine. A more detailed study of this method will be presented in the near future.

Two groups of rats were used for this investigation. One group was kept on a complete diet, the other on a diet deficient in vitamin B_6. All rats were maintained on these initial diets throughout the test period. Urine collected over the 5- to 24-hour period following the oral administration of 5 cc of water per 100 g body weight was used for control purposes. The rats were then dosed with the vitamin, accompanied by 5 cc of water per 100 g body weight, by various routes.

Urine was collected over specified periods of time. Care was exercised to prevent feces from remaining in contact with urine, since this was found to introduce interfering substances. Cages were washed into the collecting flasks to prevent urine losses, and the approximate dilution was noted. The urine samples were made strongly alkaline to thymol blue (pH above 9.6) with 30% NaOH, the volume was measured, and the sample was allowed to stand over night. This treatment suffices to destroy interfering reducing substances.

The following day 1 cc samples of the urine were neutralized to pH 7 to 7.5 and the volume was adjusted to 25, 50 or 100 cc as necessary. The adjustment of pH is of paramount importance. This is achieved by using brom thymol blue which gives a distinctive green color over the required pH range. The indicator was used externally (spot plate).

The calibration curve for the instrument was established with aqueous solutions of the vitamin at concentrations of 2 to 10 γ per cc. Urine samples were diluted to fall within this range. No interfering substances were observed when it was possible to dilute the urine samples from 1 to 50 or above. When low vitamin concentrations required a 1 to 25 dilution, occasionally interfering substances were present. These were usually negligible, although they became significant at lower dilutions. This phase of the problem is being investigated further.

Under these conditions 100% recoveries were obtained when the vitamin was added to urine.

Method. To 5 cc of the adjusted and diluted urine, 5 cc of the veronal buffer* and 20 cc of the butanol solution of the chlorimide reagent† were added.

* This was prepared by dissolving 18 g of sodium diethylbarbiturate (Merck) in 700 cc of distilled water and titrating to pH 7.6 with dilute hydrochloric acid, using the glass electrode. The solution was filtered from the precipitated barbituric acid and the pH was checked from time to time.

The tubes were briefly, but vigorously shaken, and after 5 minutes they were shaken again. After an additional 10 minutes the two layers were separated by centrifuging. The supernatant butanol layer was pipetted into 10 cc of fresh veronal solution. After shaking out extraneous colored substances‡ the two layers were separated by centrifugation, and the washing process was repeated. This treatment required less than 10 minutes. Fifteen cc of the washed butanol layer was then pipetted into a colorimeter tube which contained 5 cc of absolute ethyl alcohol, the contents were thoroughly mixed and the colors were read 40 minutes after the addition of the reagent. The colorimeter was adjusted to 100% transmission for pure butanol using a No. 660 filter. Solutions of the vitamin indophenol showed an absorption peak at 6600 Å. The color was found to be stable between 40 and 60 minutes after the addition of the reagent to the test solution.

Experimental. A series of experiments were designed to measure the total output following different modes of administration of a constant dose of 10 mg of the vitamin. Normal rats, weighing 175 to 225 g each, were placed in cages in groups of 3 or 4. Each rat was given 10 cc of water and 10 mg of vitamin B_6 and this dose was maintained daily throughout the test period of 2 weeks to insure maximum saturation of the animal. Twenty-four-hour samples of urine were analyzed, although excretion was complete in considerably less time.

Twelve rats given the vitamin intravenously (femoral vein) showed an average output of 56% (±8, maximum deviation, 12%). When administered intraperitoneally, 36 rats excreted 50% (±8, maximum deviation 14%) of the vitamin, and 48 rats put out 59% (±11, maximum deviation 24%) after oral administration of the vitamin.

The recoveries were essentially the same on the first and last days of the test. The amounts of the vitamin recovered were the same regardless of the mode of administration, thus suggesting complete

† 2,6-Dichloroquinone chlorimide (Eastman No. 2483). 100 mg were dissolved in 1600 cc of acid-free butanol and stored cold in a brown, glass-stoppered bottle. Portions withdrawn daily were allowed to warm to room temperature before use. Under these conditions the reagent is stable for at least 2 weeks. Although excellent results were obtained with the Bausch and Lomb spectrophotometer, it was thought desirable to standardize the present method for the Evelyn colorimeter. Control tests in the absence of vitamin gave 14 to 16% absorption. Solutions of the reagent which gave control readings of 20% or more were discarded.

‡ These are negligible in dilute rat urine, but are significant in other samples. These will be considered in a forthcoming communication.

absorption of the vitamin. The total average recovery of the vitamin or metabolites retaining the beta-hydroxypyridine free para position structure was 57%. The fate of the remainder has not been established, although the darkening of these urines in contrast to the stable color of the controls is significant.

A second series of 22 rats, of equal weight, were given 10 cc of water containing 1.0 mg of the vitamin orally. The average output of 67% (±7, maximum deviation 15%) is approximately equal to that obtained at the higher level.

The average output of the vitamin 5 hours after the oral administration of 36 mg of crystalline B_6 in 10 cc of water to each of 8 rats was of the same order of magnitude (71%) as that found in the 24-hour samples mentioned above. Thus it appears that the vitamin is rapidly excreted.

A group of 24 rats weighing 40 to 50 g each, was maintained on a modified Sherman-Spohn diet. When these animals showed the characteristic lesions of acrodynia, they were placed in groups of 4 for a series of 3 experiments. Eight rats were given 3 cc of water orally and 2 hours later they were given 9 mg of the vitamin in 3 cc of water orally. The urine, collected over a 5-hour period, showed a 54% recovery. This experiment repeated with the same animals the following day gave a 71% recovery. Thus, at high dose levels deficient and normal rats excrete essentially the same percentage of administered vitamin.

This experiment was repeated with a second group of 8 B_6-deficient rats, reducing the dose to 0.5 mg of the vitamin. The recovery was 65% on the first day, and 58% on the second day.

To establish differences in the output of normal and deficient rats, the remaining 8 rats were used in the same manner, except that the dose was reduced to 0.1 mg. A control group of normal rats weighing 40 to 50 g were similarly treated. Since it was not possible to dilute the urine samples sufficiently to eliminate the influence of interfering substances, quantitative data are not presented here. However, the normal rats excreted more of the vitamin than the deficient rats.

At this dose level (2 mg per kg body weight) a differentiation of normal and deficient urinary output should be possible in larger animals. Studies in the dog and human subjects are being continued along these lines.

Thanks are due to Dr. Klaus Unna for the B_6-deficient animals. The assistance of Mr. Albert Schnerring is greatly appreciated.

Summary. A colorimetric method for the determination of vita-

min B_6 has been used to study the urinary excretion of the vitamin in the rat. At high dose levels (10 mg per kg and above) 50 to 70% of the vitamin is excreted by both normal and deficient rats. The vitamin is rapidly and completely absorbed and is rapidly excreted. At low levels (2 mg per kg) the data are qualitative, but normal rats appear to excrete a higher percentage of the ingested vitamin than B_6-deficient rats.

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Antidermatitic Effect of Vitamin B_6 Analogues.

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The following report presents data on the vitamin activity of 10 pyridine compounds closely related to vitamin B_6 These substances were synthesized in the Research Laboratories of Merck & Co., Inc., in connection with studies on crystalline vitamin B_6.

The vitamin B_6 activity of these compounds was determined by the single dose curative assay on rats, first proposed by Moll.[1] In this procedure 21-day-old rats were placed on a synthetic diet consisting of cornstarch 68%, casein 18%, Crisco 8%, salt mixture No. 1 (U.S.P. XI) 4%, cod liver oil 2%, and supplemented with 40 micrograms each of thiamin chloride and riboflavin per rat per day. After 30 days on this diet, the animals reached stationary weights and the first symptoms of dermatitis appeared. Within 7 to 10 weeks, dermatitis was fully developed in 35% of the animals. By this procedure it has been shown[2] that a single dose of 100 micrograms of vitamin B_6 cures 100% of the deficient animals within 14 days, and that a dose of 50 micrograms produces complete cures in 75% of the animals. Lower doses fail to produce complete cures, but signs of partial healing were obtained regularly with 25 micrograms and in some instances with a single dose of 15 micrograms.

In the present study 5 to 6 depleted animals were used for each dose level. Their weight and symptoms were recorded over a period of 14 days. The results obtained with the different pyridine derivatives, as

1 Moll, Th., and Schnittspahn, M., *E. Merck's Jahresberichte*, 1938, **52**, 10.

2 Reedman, E. J., Sampson, W. L., and Unna, K., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 112.