Isolation of the Saponin.—Air-dried and powdered seeds (200 Gm.) were refluxed four times with 45% alcohol. The combined extracts were concentrated to a small bulk, filtered and treated with an aqueous solution of lead acetate. The precipitate obtained was suspended in 75% alcohol and the lead was removed by a current of hydrogen sulfide. The filtrate was concentrated and a little alcohol added. The precipitate formed, consisting of starchy matter, was filtered off and rejected. Further amounts of alcohol were added and the first lot of crude saponin (0.81 Gm.) precipitated was filtered off. Ether was added to the alcoholic filtrate and the second crop of the crude saponin (0.75 Gm.) was obtained. On drying over concentrated sulfuric acid in vacuum the saponin was obtained as a yellow powder (yield 0.78%). The crude saponin was purified by dissolving it in the smallest quantity of water and precipitating it with alcohol. The flocculent precipitate formed was collected and dried over concentrated sulfuric acid and was obtained as a white amorphous powder. Addition of ether to the alcoholic filtrate gave a precipitate which on drying formed a yellow amorphous powder. The white and the yellow powders gave all the tests for saponin. The white powder was further purified by dissolving it in smallest quantity of water and precipitation by alcohol. The saponin was thus obtained as a white amorphous powder which decomposed at 148° C.

The filtrate obtained after removing the lead acetate precipitate was treated with lead subacetate but nothing of interest could be obtained either from the lead subacetate precipitate or the filtrate.

The Saponin.—The white amorphous saponin, acid in nature, was very soluble in water and insoluble in petroleum ether, ether, chloroform and alcohol. On hydrolysis the saponin gave sugars. Alcoholic cholesterol and baryta water gave a white precipitate with the saponin.

Pharmacological Action.[1]—The saponin possessed strong hæmolytic action and was found to kill fish. *Macrones vittatus* were experimented with and the opercular movement of the fish, kept under water, was recorded on a kymograph before and after the addition of the saponin.

SUMMARY

The toxic principle of the seeds of *Barringtonia acutangula* is an amorphous acid saponin. Other substances such as starch, fatty oil and sugars are present. The poisonous action of the seeds on fish could be accounted for by the presence of the saponin.

[1] The authors desire to express their thanks to Dr. N. N. Das for carrying out the pharmacological experiments.

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Interrelated Vitamin Requirements

The Influence of Thiamin, Riboflavin, Pantothenic Acid and Vitamin B_6 on Liver Glycogen Reserves*

By G. C. Supplee, R. C. Bender and Z. M. Hanford

The degree and rate of glycogen formation and hydrolysis in the liver are influenced by many factors, including dietary components, energy demands of the organism, state of metabolism and the degree of normality of glandular secretions. As yet, only fragmentary and incidental data concerning the influence of vitamin entities are available, notwithstanding much evidence of an inferential character indicating that important reactions involving these factors are largely localized in the liver. Although glycogenesis and glycogenolysis are recognized as liver functions, the chemical reactions involved are only generally known to be of an oxidative or reducing character, and it has not been determined to what degree individual vitamin entities may be directly or indirectly concerned. The investigations reported herein were designed to determine the interrelationship and effect of each of four entities of the vitamin B complex on glycogen formation

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and retention in the liver. The controls and techniques adopted for the study, as will be described hereinafter, are believed to be an improvement over methods frequently employed.

EXPERIMENTAL

The investigation involved the use of about 600 white rats and extended over a period of about sixteen months. The procedure involved the depletion of a large number of animals of each of the vitamins to be tested. Following depletion, a 24-hr. starvation period was imposed prior to administration of the test substance. Forced feeding with a blunt hypodermic needle used as a stomach tube, of 1 Gm. of the basal ration and vitamin supplements other than the particular one under investigation was the common practice for the controls and test animals immediately following the 24-hr. fast period. (The basal ration with a fluidity suitable for handling in this manner was prepared by mixing 50 Gm. of the dry product with sufficient water to make a 60-ml. volume.) The vitamins tested were thiamin, riboflavin, pantothenic acid and vitamin B_6. These were administered in 100-γ quantities (with exceptions noted hereinafter) in 0.5 ml. of physiological salt solution by injection directly into the heart. This method of administration obviates loss through excretion, variable absorption and other uncontrolled features inherent in oral feeding or injection at other sites. Less than 2% of casualties have resulted at the hands of experienced operators during the injection of several hundred animals involved in this and other studies.

Animals properly depleted of each substance were divided into two major groups with an equal number of each sex. One group served as negative controls which received the 1 Gm. of food only; the other group received both the food and the injected vitamin. From 8 to 12 animals were immediately sacrificed and the liver removed for analysis. The remaining animals in each group were returned to their respective cages and supplied with water only. At 4-hr. intervals throughout a 24-hr. period, not less than 4 and sometimes as many as 8 animals from both the control and injected groups were sacrificed and liver glycogen determined. Glycogen determinations were carried out according to the method of Good, Kramer and Somogyi (1).

The depletion of the animals preparatory to determining the influence of individual vitamin factors was as follows: White rats, 23 to 25 days old, were placed in individual screened bottom cages and supplied the following basal ration: vitamin-free casein,[1] 20 parts; sucrose, 69 parts; hydrogenated vegetable oil,[2] 3 parts; salt mixture No. 40,[3] 4 parts; powdered agar-agar, 2 parts; and cod liver oil (medicinal grade), 2 parts. At the end of one week, the requisite primary supplements were supplied as a dosage per rat per day.

Animals to be depleted of thiamin were supplied with 10 γ of riboflavin and 100 mg. of rice polish concentrate[4] autoclaved at pH 8.5 for five hours at 120° C. This autoclaved product contains no vitamin B_1 or riboflavin but does contain from 11 to 13 γ of pantothenic acid, and 7 to 8 γ of vitamin B_6 per 100 mg. following the heat treatment. The criteria for determining uniform depletion were constant or declining weight and manifestation of "medium" paralytic symptoms.

Animals to be depleted of riboflavin were supplied with 12.5 γ of thiamin and 100 mg. of alkaline (pH 8.5) autoclaved rice polish concentrate. The criterion of depletion was constant weight or slow growth not exceeding about 2 Gm. per week; this state of depletion was reached after six to eight weeks.

Animals to be depleted of vitamin B_6 were supplied with 12.5 γ of thiamin, 10 γ of riboflavin and 100 mg. of rice polish factor II.[5] This product contains no vitamin B_1, riboflavin or vitamin B_6 (2), but does contain approximately 20 γ of pantothenic acid per 100 mg. The criteria of depletion were cessation of growth and development of the acrodynia type dermatitis, usually manifested in five to seven weeks.

The animals to be depleted of pantothenic acid were supplied 12.5 γ of thiamin, 10 γ of riboflavin, 10 γ of vitamin B_6 and 100 mg. of the rice polish factor II autoclaved at pH 11 for five hours at 120° C. This alkaline heat-treated product contains 0.3 γ or less of pantothenic acid per 100 mg., but no riboflavin, thiamin or vitamin B_6. Evidence of depletion was slow growth or constant weight and occasional sudden and unpredictable deaths among the group; these manifestations usually occurred within six to eight weeks and pantothenic acid depletion could frequently be confirmed by evidence of adrenal and/or kidney damage upon autopsy (3, 4).

Certain variations in these procedures were carried out with other groups of animals in which it was desired to determine the effect of the absence of all of the factors or variations in the amount of a particular factor on glycogen formation and retention; specifically, a comparison of the influence of 25-γ and 100-γ injections of riboflavin, the influence of previous supplements of pantothenic acid, and the influence of previous saturation of the tissues with thiamin. The only departures from the basic plan of procedure and techniques required for these particular studies were: in the riboflavin comparison, the injection of 25 γ in lieu of 100 γ; for the pantothenic acid variation, a daily supplement of 50 γ of pantothenic acid in addition to the other primary supplements was provided; and for the study involving tissue saturation with thiamin, the animals were first depleted to the paralytic state

[1] Labco brand. [2] Crisco. [3] Steenbock, H., and Nelson, E. M., *J. Biol. Chem.*, 56 (1923), 355.

[4] Labco brand. [5] Labco brand.

and then supplied with 100 γ of thiamin per day for five days in addition to the other primary supplements. All groups of animals prepared according to these particular requirements were subjected to the usual 24-hr. starvation period prior to the forced feeding of 1 Gm. of the basal ration and supplements and injection of the test substance.

The results from the entire study are shown in the accompanying area graphs in Figs. 1 to 4. A higher liver glycogen level at specified periods up to 24 hrs. resulted from all vitamin injections with the possible exception of the group which received a simultaneous injection of all four factors (Fig. 1, group I). The period of impoverishment during which the animals in this group could be maintained without any of the supplementing factors was only three weeks. Since it is well established that experimental animals cannot be depleted of any one of these vitamins in a 3-week period if the other factors are supplied, it is reasonable to assume that a substantial residuum of body stores existed in the impoverished animals to a degree which did not permit the variations shown by the groups more fully depleted of a single factor. All such groups showed characteristic glycogen storage patterns with variations attributable to the particular vitamin factor tested. These variations are essentially of the same character irrespective of whether the glycogen is compared on the basis of the total amount in the entire liver, or as the concentration per Gm. of water-free liver tissue. While the data are presented as average values, the relationship between animals in comparable control and test groups was in no case found to be in opposition to the average of the relationships shown in the graphs

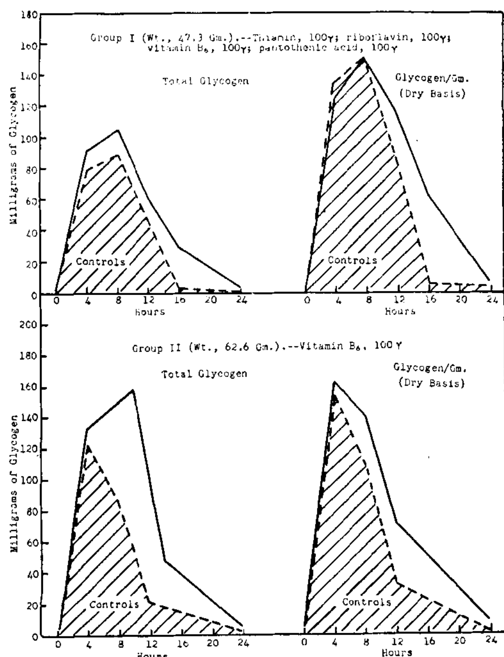

Fig. 1.—Liver Glycogen as Affected by Vitamin Supplements.

An examination of the data from the animals receiving the 100-γ and 25-γ injections of riboflavin indicates (Fig. 2) that the influence of this factor upon glycogen storage in the liver is relatively less than the influence of the other factors. Complete tissue depletion of riboflavin has never been reported and it is probable that such an accomplishment is impossible in the living animal from either a practical or theoretical point of view. Furthermore, unpublished data have shown that riboflavin is mobilized in the liver during digestion and assimilation even though body stores are greatly impoverished as a result of the absence of this factor in the dietary.

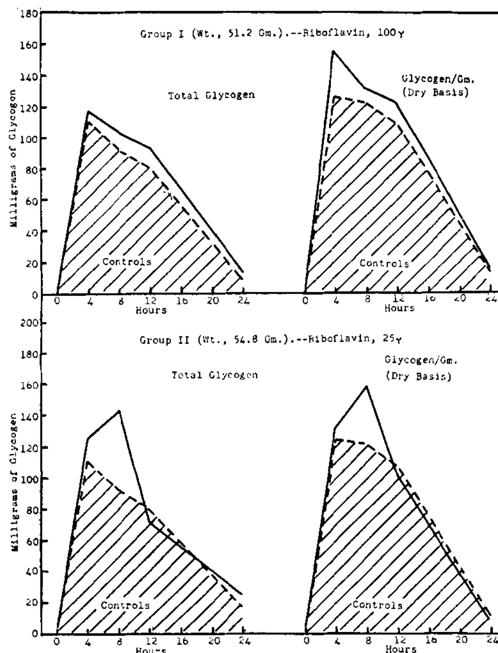

Fig. 2.—Liver Glycogen as Affected by Vitamin Supplements.

The data from the thiamin series (Fig. 3) show that the injection of 100 γ of this factor causes a marked increase in glycogen formation in depleted animals. The groups receiving 100 γ orally for five days following depletion in order to obtain a uniform and relatively high degree of tissue saturation prior to determining the effect of the 100-γ injection, did not show the same quantitative effect of the injection exhibited by the depleted group. This comparative evidence is interpreted as indicating a greater and more direct effect of thiamin deficiency on liver glycogen formation than results from riboflavin impoverishment.

The results from the pantothenic acid series shown in Fig. 4 present a still further variation in the liver glycogen pattern. There was not so great a variation in glycogen levels between the control and injected groups as prevailed in the thiamin series. This may be due to less complete exhaustion of body stores, or it may be due to a less significant

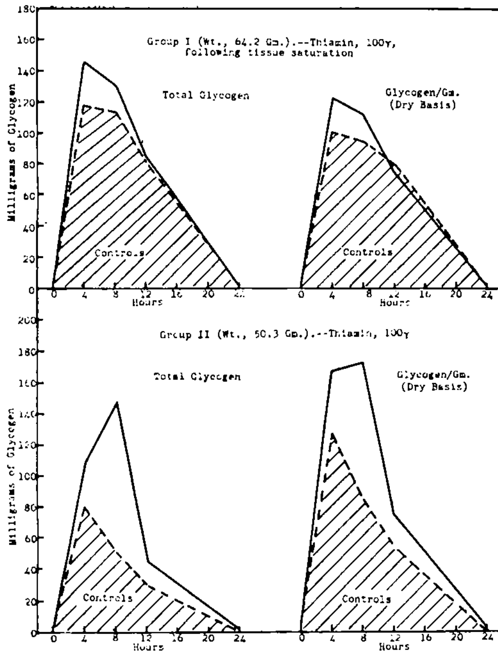

Fig. 3.—Liver Glycogen as Affected by Vitamin Supplements.

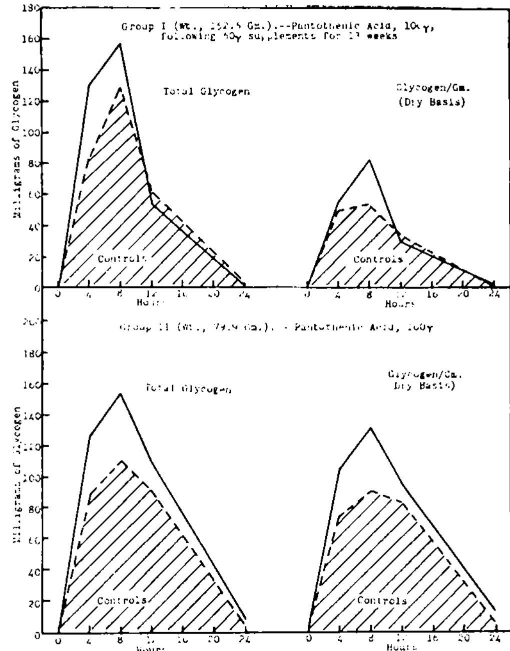

Fig. 4.—Liver Glycogen as Affected by Vitamin Supplements.

physiological role. It is significant, however, that the increase in liver glycogen caused by the injection of 100 γ is substantially of the same order of magnitude, irrespective of whether previous impoverishment was severe or only suboptimal.

The influence of vitamin B_6 impoverishment on the liver glycogen pattern (Fig. 1, group II) is characterized by a rapid decrease following the initial high level reached in a comparatively short time. Since an alteration of this pattern is brought about by injection of the vitamin, the results indicate that this factor may be involved in regulating the rate of glycogenolysis.

Since the present studies are believed to have been well controlled with respect to preparation of the animals, controlled intake of the supplementing and test substances, and uniform caloric intake during the 24-hr. test period, the data were further integrated in an attempt to evaluate the influence of each factor in terms of a comparable mathematical expression. The treatment of the data with this objective in view is briefly described as follows.

Various data showing the caloric requirements of white rats were reviewed for the purpose of selecting the value most applicable to the animals used and maintained under the conditions prevailing in these studies. The average basal metabolism rate of 744 calories per day per square meter of body surface as determined by Lewis and Luck (5) was selected as a suitable basis for the calculations. The caloric requirement per 4-hr. period for individual animals of given weight was readily determined by reference to the data reported by Diack (6). These calculated values varied from 1.11 calories for a 43-Gm. animal, the lowest in the thiamin series (Fig. 3, group II), to 2.85 calories for a 170-Gm. animal, the highest in the pantothenic acid series (Fig. 4, group I). Although the 4.11 calories provided by the 1-Gm.

TABLE I.—RATIO OF EFFECTIVENESS OF THIAMIN, RIBOFLAVIN, VITAMIN B_6 AND PANTOTHENIC ACID IN INFLUENCING LIVER GLYCOGEN LEVELS[a]

Vitamin Factor	Amount, γ	Figure Reference	Ratio of Effectiveness 4 Hrs.	8 Hrs.	12 Hrs.	16 Hrs.
Thiamin, riboflavin, vitamin B_6 and pantothenic acid	100 of each	1, group I	1:1.20	1:1.23	1:1.22	1:1.15
Vitamin B_6	100	1, group II	1:1.11	1:1.94	1:2.21	....
Riboflavin	100	2, group I	1:1.07	1:1.49	1:1.22	1:0.70
Riboflavin	25	2, group II	1:1.18	1:1.13	1:0.81	....
Thiamin	100[b]	3, group I	1:1.10	1:0.98	1:0.99	....
Thiamin	100	3, group II	1:1.32	1:3.29	1:1.45	....
Pantothenic acid	100[c]	4, group I	1:1.45	1:1.28	1:0.90	....
Pantothenic acid	100	4, group II	1:1.43	1:1.49	1:1.01	....

[a] Unity is assigned to the results from the controls.
[b] Following 5-day tissue saturation by oral feeding of 100 γ per day.
[c] Following oral feeding of 50 γ per day for 13 weeks.

feeding of the basal ration does not enter into the following calculations, the value is recorded for reference purposes. The calories stored as liver glycogen at each of the 4-hr. periods at which determinations were made, were calculated from the glycogen values found. By formulating a suitable equation for calculating the theoretical calories stored as glycogen (based upon actual values found in the control animals) by animals of different weight in the control and vitamin injected groups, and comparing this calculated value with that found in the injected groups, a ratio of the effectiveness of the vitamin was readily determined, with unity assigned to the values found in the controls. The utility of this ratio calculated for the 4-, 8- and 12-hr. periods (Table I) is primarily one of convenience for evaluating the data presented in graphical form and also serves as a numerical index of the relative effectiveness of the particular factors in contributing to the liver glycogen levels found under the prevailing experimental conditions. Such a numerical expression likewise emphasizes the significance and complementing role of these vitamins and their interrelated functions which contribute to maintenance of normal metabolism and efficiency of food utilization.

SUMMARY

1. Impoverishment of body stores of thiamin, riboflavin, vitamin B_6 and pantothenic acid resulting from inadequate dietary intake causes lowered liver glycogen levels indicating inefficient food utilization, deficient reserve energy and an unbalanced metabolism.

2. Animals depleted of any one of these factors but supplied with the others showed a prompt and characteristic response in elevation of the liver glycogen level following administration of the missing factor.

3. Liver glycogen patterns obtained over a 24-hr. period following controlled forced feeding of unit amounts of food and vitamin administration were characteristically different for each of the vitamin entities studied.

4. The data do not disclose a specific glycogenic or glycogen hydrolyzing role for any of the factors. Each undoubtedly complements the others in maintaining a balanced state of metabolism.

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Inhibiting Effect of Various Compounds on Curative Action of Sulfanilamide and Sulfapyridine in Infected Mice*

By George W. Raiziss, M. Severac and J. C. Moetsch

Lockwood (1, 2), Stamp (3), Green (4) and Fleming (5) contributed to the study of substances inhibiting the bacteriostatic effect of sulfanilamide. Woods and Fildes (6, 7) found that *p*-aminobenzoic acid present in yeast inhibits the bacteriostatic effect of sulfanilamide. Selbie (8) showed that *p*-aminobenzoic acid administered orally inhibits the therapeutic effect of sulfanilamide in mice infected with *Streptococcus hæmolyticus*.

EXPERIMENTAL

We studied the inhibiting effect *in vivo* of *p*-aminobenzoic acid on sulfanilamide and sulfapyridine administered to infected mice. Closely related products of *p*-aminobenzoic acid and other chemical compounds were included in this investigation.[1] Some mice were infected intraperitoneally with pneumococcus type II and others with *Streptococcus haemolyticus*. Groups of 5 mice used for the studies on streptococcus were infected intraperitoneally with 200 minimum lethal doses of *Streptococcus haemolyticus*, strain C-203, of which the average minimum lethal dose was 0.5 cc. of 1:10,000,000 dilution of broth culture. Treatment with sulfanilamide or sulfapyridine was given by mouth in a dose of 10 mg. immediately after infection, and once daily for the following two days. Ten milligrams of *p*-aminobenzoic acid or other compound was administered by mouth one hour after each treatment.

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[1] While we were engaged in this research, McCarty (9) reported that *p*-aminobenzoic acid is capable of destroying the curative effect of sulfapyridine for type I pneumococcic infection in mice.