15658

Changes Produced by Starvation in the Vitamin Content of Rat Tissues.

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(Introduced by Ralph R. Mellon.)
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It has been observed in this laboratory that the topical application of β,β' dichlorethyl sulfide (mustard gas) in rats causes marked changes in the vitamin content of some tissues. Since severe starvation is a prominent feature of mustard gas poisoning, it was deemed necessary to determine the effect of starvation *per se*. This report describes the effects of mustard gas and severe starvation upon the liver, kidney, and bone marrow contents of 6 of the B vitamins.

Methods. Animals. Twenty-five-day-old, male, albino rats of the Sprague-Dawley strain were housed in individual wire-mesh cages and were fed the following ration: casein (Labco, "vitamin-free") 18; sucrose, 76; salts IV,[1] 4; and corn oil, 2. To each kilogram of ration were added: thiamine hydrochloride, 3.0 mg; riboflavin, 3.0 mg; pyridoxine hydrochloride, 3.0 mg; calcium pantothenate, 10.0 mg; *i*-inositol, 0.3 g; choline chloride, 1.0 g; and 2-methyl-1,4-naphthoquinone, 10.0 mg. At the beginning of the experiment, the rats were given one drop of halibut liver oil (Abbott-plain) containing 1.3 mg of added *dl*-α-tocopherol acetate. All of the rats received this ration *ad libitum* for 4 days, at which time the experimental procedures were initiated.

Tissues. At the conclusion of the experiments the rats were decapitated, and the tissues removed for analysis. The total weights of the livers were determined, and weighed portions of both the liver and kidney were dried for 48 hours at 45°C *in vacuo*. The samples were reweighed to determine the dry weights, then stored in the cold until prepared for analysis. The dried livers and kidneys were ground and a weighed portion (approximately 50 mg) homogenized in 10 ml of water with a glass homogenizer.[2] Each sample was incubated at 37°C under toluene with clarase and papain (each enzyme added at a level equivalent to 5% of the weight of the tissue) for 24 hours at the natural pH, and then for 24 hours in .05 N sodium acetate buffer at pH 4.5.

The 6 long bones (femora, tibiae, and humeri) were split with a scalpel and the marrow, which was removed with a hypodermic needle filed on one side to form a scoop, was suspended in water and stored in the cold under toluene until prepared for assay. The bone marrow was homogenized and the nitrogen content determined on an aliquot by a micro digestion and nesslerization procedure.[3] One gram of each of the enzymes per gram of nitrogen was added and the tissue digested as above, except that .02 N sodium acetate buffer was used.

In all the tissues, the digests were adjusted to pH 6-7, steamed and rehomogenized. These preparations were used for the assays of vitamin B_C and pantothenic acid; for biotin determinations an aliquot was autoclaved at 120°C for 2 hours in 6 N sulfuric acid and neutralized; for nicotinic acid an aliquot was autoclaved at 120°C for 20 minutes in 1 N sulfuric acid and neutralized; and for riboflavin and pyridoxine an aliquot was steamed at 100°C for one-half hour in 0.1 N hydrochloric acid, adjusted to pH 4.5 with sodium acetate, and filtered.

Vitamin Assays. The analyses of these tissues for their content of vitamin B_C (employing *L. casei* with the vitamin B_C reference standard of Parke, Davis & Co.), biotin, pantothenic acid, nicotinic acid, and riboflavin were performed by modifications of the

[1] Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, **138**, 459.

[2] Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1936, **114**, 495.

[3] Johnson, M. J., *J. Biol. Chem.*, 1941, **137**, 575.

TABLE I.
Constituents of Media for Microbiological Assays.

Constituent	Amt./liter double-strength medium	
	Lactobacilli	*S. carlsbergensis*
Casein hydrolysate* (SMACO, 10% soln.)	100.00 ml	80.00 ml
Alkali treated peptone†	10.00 g	—
Yeast extract supplement†	2.00 "	—
Norit treated peptone‡	0.40 "	—
dl-Alanine‡	0.40 "	—
dl-Tryptophane	0.40 "	—
l-Asparagine	0.20 "	—
l-Cystine	0.20 "	—
Glucose	40.00 "	40.00 g
Sodium acetate (anhyd.)	24.00 "	—
Potassium citrate · H_2O§	1.00 "	10.00 "
Citric acid · H_2O	—	2.00 "
K_2HPO_4	1.00 "	0.60 "
KH_2PO_4	1.00 "	0.60 "
$MgSO_4 \cdot 7H_2O$	0.40 "	0.20 "
$FeSO_4 \cdot 7H_2O$	20.00 mg	10.00 mg
$MnSO_4 \cdot 4H_2O$	20.00 "	10.00 "
NaCl	20.00 "	10.00 "
Adenine sulfate	20.00 "	—
Guanine hydrochloride	20.00 "	—
Uracil	20.00 "	—
Xanthine	20.00 "	—
i-inositol	—	20.00 "
Nicotinic acid	2.00 "	—
Pyridoxine hydrochloride	2.00 "	—
Calcium pantothenate	1.00 "	2.00 "
Riboflavin	1.00 "	—
Thiamine hydrochloride	1.00 "	1.00 "
p-Aminobenzoic acid	0.10 "	—
Biotin	0.01 "	0.01 "
Adjust to pH	6.5-6.6	5.2-5.5

* Omitted in the riboflavin assay.[8]
† Added only in the riboflavin assay.[8]
‡ Added only in the vitamin B_c assay.[4]
§ Prevents precipitation of the mineral salts in the *Lactobacilli* assays.

existing microbiological methods.[4-8] The various media proposed for assays with the *Lactobacilli* have been incorporated into a single medium which, with the modifications indicated, serves for all of the *Lactobacilli* assays. The constituents of the medium are given in Table I, requiring only the omission of the vitamin under consideration for its assay. The assays employing the *Lactobacilli* were carried out in 5 ml quantities and inoculated by the convenient procedure suggested by Black and Arnold.[9] The *L. casei* were incubated at 37°C and the *L. arabinosus* at 30°C for 3 days and titrated electrometrically with 0.05 N NaOH. Pyridoxine was determined by the *S. carlsbergensis* method[10] employing the medium shown in Table I. The assays were run in 5 ml quantities contained in 50 ml Erlenmeyer flasks. The flasks were incubated for 18 hours at

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5 Wright, L. D., and Skeggs, H. R., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 95.

6 Skeggs, H. R., and Wright, L. D., *J. Biol. Chem.*, 1944, **156**, 21.

7 Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, **1941**, **139**, 675.

8 Snell, E. E., and Strong, F. M., *Ind. and Eng. Chem., Anal. Ed.*, 1939, **11**, 346.

9 Black, T. L., and Arnold, A., *Ind. and Eng. Chem., Anal. Ed.*, 1940, **12**, 344.

10 Atkin, L., Schultz, A. S., Williams, W. L., and Frey, C. N., *Ind. and Eng. Chem., Anal. Ed.*, 1943, **15**, 141.

30°C and growth was determined turbidimetrically in the Evelyn colorimeter.

Vitamin B_C assays on bone marrow samples extracted with ether at pH 4.5[11] indicated that fatty materials did not interfere with the assay. Recovery experiments on bone marrow for vitamin B_C and on liver for vitamin B_C and pantothenic acid also showed that the assays were functioning satisfactorily. The vitamin B_c contents of some samples of liver and bone marrow were also determined by the *Streptococcus fecalis R* method.[12] These values were 10-20% lower than those obtained by the *L. casei* method. Assays on the enzymes used for digestion of the tissues showed that there were insufficient quantities of the vitamins present to necessitate any correction in the values obtained.

The vitamin content of the livers and kidneys was expressed in amount per gram of dry tissue; while that of the bone marrow was expressed on the basis of amount per gram of nitrogen. We chose to express our vitamin concentrations per gram of nitrogen in the case of bone marrow since the constituents of this tissue are frequently expressed on the basis of nitrogen or phosphorus and because this procedure is experimentally convenient for bone marrow.

Experimental. Each of the experiments presented in this paper (Series I and Series II) consisted of 3 groups of animals: a *mustard-treated* group (fed *ad libitum*) which received topically 8 mg of mustard gas per kg of body weight and 2 *control* groups, one of which was fed *ad libitum* while the food allowance of the other was restricted to varying degrees as described below. Mustard gas was not administered to the control groups. The daily food consumption of each animal was recorded. The average weight of the animals at the beginning of the experimental period was 50 g in Series I and 46 g in Series II.

Series I. In the *mustard-treated* group, analyses were performed upon the tissues of 8 animals which died 3-7 days after the application of mustard. The average daily food consumption of these animals was 3.4 g and the average daily loss in weight was 3.1 g.

The food intake of each rat in the *control-restricted* group (8 rats) was limited to that consumed during the previous 24 hours by its paired member in the mustard group. Each animal was sacrificed 24 hours after the death of its paired-fed mate. The average daily loss of weight in this group was 1.3 g with considerable variation among animals in the total loss of weight over the 3-7-day period. The average daily food consumption of the *control-ad libitum* group (8 rats) was 8.5 g and the average daily weight gain was 3.2 g. The animals were sacrificed on the fifth and sixth days of the experiment.

In general, the concentrations of vitamins in tissues from the mustard-treated and food-restricted groups were similar and differed from those of the groups fed *ad libitum.* These changes were particularly evident in the liver, where marked increases were noted. The changes observed in this series were similar to those tabulated for Series II (Table III). Of significance for the purposes of this report is the fact that the deviations from the normal in the partially-starved group could be correlated with the degree of weight loss of the animals. This was particularly true for the concentrations of vitamin B_C which increased in the liver and decreased in bone marrow. As mentioned previously, the weight changes in the members of this group were variable and could be accounted for by the variations in the food intakes of their partners in the mustardized group. It was also apparent that the utilization of food by the rats poisoned with mustard was impaired to varying degrees in individual members of this group. Thus, the weight losses in these animals were uniform, in spite of the differences in food intake. These observations led to the conclusion that the vitamin changes in the mustard-treated animals were secondary to inanition and that the extent of the changes in the paired-fed rats was conditioned by the weight loss of the animals. Accordingly, in Series II, the food allowance of one control group was

[11] Bauernfeind, J. C., Sotier, A. L., and Boruff, C. S., *Ind. and Eng. Chem., Anal. Ed.*, 1942, **14**, 666.

[12] Luckey, T. D., Briggs, G. M., and Elvehjem, C. A., *J. Biol. Chem.*, 1944, **152**, 157.

TABLE II.
Changes in Liver Size Resulting from Mustard Treatment or Severe Starvation for 4 Days (Series II).*

Group	Starting wt (g)	Final wt (g)	Wt of liver (g)	% liver†
Control—*ad lib.* (10 rats)	46 (40-51)	54 (45-59)	3.10 (2.35-4.58)	5.74 (5.06-6.18)
Control—restricted (10 rats)	46 (39-50)	35 (27-39)	1.22 (0.69-1.67)	3.48 (2.56-4.00)
Mustard-treated (10 rats)	44 (40-46)	34 (31-36)	1.30 (1.03-1.60)	3.82 (2.86-4.56)

* Averages are given with the range of values in parentheses.
† Calculated from the final body weight.

TABLE III.
Effects of Mustard Treatment and of Severe Starvation upon the Concentrations of Vitamins of Rat Tissues (Series II).*

Group	Vitamin B_c	Pantothenic Acid	Biotin	Riboflavin	Pyridoxine	Nicotinic Acid
			Liver.			
Control—*ad lib.*	7.02 (5.50-8.30)	225 (151-304)	2.31 (1.50-3.82)	88.3 (62.4-116)	16.2 (10.4-24.8)	377 (334-426)
" —Restricted	19.7 (10.9-30.3)	428 (378-515)	5.01 (3.70-6.62)	134. (110-164)	42.6 (35.8-53.4)	554 (513-640)
Mustard-treated	24.3 (14.7-33.6)	433 (326-540)	4.71 (3.70-5.98)	131. (106-147)	39.5 (34.2-46.5)	546 (505-589)
			Kidney.			
Control—*ad lib.*	11.1 (7.78-13.6)	158 (146-168)	3.17 (2.28-3.76)	112. (104-124)	15.7 (11.4-22.0)	300 (280-320)
" —Restricted	14.4 (7.20-23.9)	260 (218-317)	4.94 (3.98-5.90)	111. (87.0-146)	25.4 (18.7-32.0)	310 (283-338)
Mustard-treated	16.2 (9.30-27.8)	293 (243-356)	5.04 (4.36-6.62)	104. (91.0-117)	23.3 (18.8-27.9)	306 (290-335)
			Bone Marrow.			
Control—*ad lib.*	26.1 (22.4-30.4)	360 (322-451)	1.56 (1.05-2.18)	262. (216-296)	36.2 (16.7-67.6)	1033 (815-1325)
" —Restricted	16.7 (14.3-20.2)	332 (262-430)	1.31 (0.73-1.86)	359. (200-547)	63.2 (35.6-93.0)	1079 (953-1250)
Mustard-treated	8.8 (4.9-14.6)	381 (285-500)	1.32 (0.54-1.82)	263. (171-398)	42.5 (32.4-50.7)	1220 (940-1570)

* The liver and kidney values are expressed as micrograms of vitamin per gram of dry weight; the bone marrow values are expressed as micrograms of vitamin per gram of nitrogen. Values given represent the averages of determinations on 10 samples, with the exception of vitamin B_c in bone marrow, in which case 20 samples were analyzed. Ranges are given in parentheses.

limited to amounts which kept the weight losses on a parity with those of the mustardized rats.

Series II. In the mustard-treated group, analyses were made of the tissues of animals which died on the fifth day after mustard gas application. The animals in the *restricted* group were each given 2 g of control ration daily for 3 days and no food on the fourth day. The following day the animals in this group, as well as those in the control group fed *ad libitum* were sacrificed. This degree of food restriction resulted in weight losses equal to those of the mustardized rats (Table II). This experiment was repeated exactly in order to obtain sufficient bone marrow to complete the vitamin analyses. The vitamin contents of the liver, kidney, and bone marrow from 10 animals of each group were determined, with the exception of the bone marrow vitamin B_C in which case 20 tissues from each group were analyzed.

Weight changes in the 3 groups, along with the total liver weight and the percentage of liver in the rats (based on final body weight) are given in Table II for purposes of calculations to be discussed. The vitamin contents of the tissues are given in Table III.

Neither mustard treatment nor starvation produced any change in the percentage moisture content of the livers or kidneys.

Discussion. The foregoing data (Table III) demonstrate that severe starvation produces a marked rise in the content per gram of tissue of several of the B vitamins in the liver and that these changes also occur in the kidney, though to a lesser extent. The only notable change in the bone marrow is the fall in the vitamin B_C content. Although the riboflavin and pyridoxine values in the bone marrow show a trend in the opposite direction, it must be pointed out that the values in the starved and normal groups overlap to a great extent. Increases in the succinoxidase[13] and nucleoprotein[14] contents of liver during starvation have been reported.

It is also apparent (Table III) that the concentrations of the vitamins in the starved group are very similar to those in the mustard-treated group. It appears, therefore, that the changes in the vitamin content induced by mustard treatment are secondary to the concomitant inanition. The effect of mustard upon the vitamin B_C content of the bone marrow, however, appears to be specific. In this case the decrease in the vitamin due to the mustard treatment cannot be accounted for solely by inanition. This observation is of interest in view of the well-known deleterious effect of mustard upon the leukopoietic activity of the bone marrow. The prominent role of vitamin B_C in leukopoiesis suggests that the action of mustard upon the bone marrow is exerted through its effect upon the metabolism of vitamin B_C.

Table II shows that both mustard treatment and starvation cause a considerable decrease in liver size, both on an absolute basis and expressed as percentage of body weight. Under such circumstances it becomes necessary to reassess the significance of values based upon a unit weight of dry tissue. Two alternate methods of calculation present themselves: (a) determination of the total content of the constituent in the liver; or (b) calculation of the liver content per 100 g of body weight (percentage basis). Various investigators have elected to calculate the amounts of the following constituents in terms of one of these methods: vitamin,[15] enzyme,[13,16,17] nucleoprotein and phospholipid.[18]

Comparisons based upon the total amount of the constituent in the liver would appear to be valid only when the weights of the various groups of animals are the same. On the other hand, the expression of values in terms of the liver constituent per 100 g of body weight compensates for slight differences in body weight. Since the ratio of liver weight to body weight in normal rats varies during growth, such a calculation when applied to growing rats should be employed only when the ages of the different groups of animals under comparison are similar.

We believe that, under the conditions of the present experiment, calculations based upon the amount of liver constituent per 100 g of body weight furnish the most reliable basis for evaluating the significance of our data. Such calculations made from data for the liver given in Tables II and III (using amount of vitamin per unit of dry weight since the percentage dry weight is the same in the 3 groups) demonstrate that both starvation and mustard treatment cause increases ranging from 15 to 132% in the vitamin B_C, pantothenic acid, biotin and pyridoxine contents. No changes in the riboflavin and nicotinic acid values are observed. It is of interest that in the kidney, where the vitamin values are expressed per unit of dry weight of tissue, riboflavin and nicotinic acid are also the only 2 vitamins whose contents are not affected by starvation or mustard.

It is evident that the effects of inanition upon the contents of various liver constituents must be considered in cases where severe starvation for a short period of time

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[14] Davidson, J. N., and Waymouth, C., *Biochem. J.*, 1944, **38**, 379.

[15] Sure, B., *J. Biol. Chem.*, 1945, **157**, 543.

[16] Lightbody, H. D., and Kleinman, A., *J. Biol. Chem.*, 1939, **129**, 71.

[17] Schultze, M. O., *J. Biol. Chem.*, 1939, **129**, 729.

[18] Kosterlitz, H. W., and Cramb, I. D., *J. Physiol.*, 1943, **102**, 18P.

results from a given set of experimental conditions. This factor is of particular significance when one is dealing with pharmacological agents which often produce such a state of severe starvation.

Summary. 1. The vitamin B_C, pantothenic acid, biotin, riboflavin, pyridoxine, and nicotinic acid contents of the livers, kidneys, and bone marrows have been determined in rats: (a) fed *ad libitum;* (b) treated with mustard gas; and (c) whose food intake was restricted. 2. When expressed on the basis of unit weight of tissue, increases of 50 to 180% were noted in the vitamin concentrations of livers from the food-restricted group. Less marked increases occurred in the kidney, while a decrease of 35% in the vitamin B_C content of the bone marrow based on nitrogen content was observed. 3. With the exception of the vitamin B_C content of the bone marrow, the changes observed in the partially-starved group were also found in the mustard group. 4. Calculations based upon the vitamin content of the liver per 100 g of body weight indicate that both starvation and treatment with mustard gas cause increases ranging from 15 to 130% in the vitamin B_C, pantothenic acid, biotin, and pyridoxine contents of the liver. No significant changes occur in the riboflavin or nicotinic acid contents.

We are indebted to the Parke, Davis and Company for the vitamin B_c reference standard; and to Merck and Company for the synthetic vitamins. This work was done under a contract from the Medical Division of the Chemical Warfare Service.

15659

Toxicity of Amino Acids as Influenced by Riboflavin Deficiency.

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The therapeutic application of protein hydrolysates has become common and as a result it grows more and more important to understand interrelationships between amino acids, vitamins, minerals, etc. In the course of experiments involving toxicity studies of histidine[1] and tyrosine,[2] it became probable that riboflavin deficiency was a key to increased amino acid toxicities. To establish this point, histidine and tyrosine toxicities were rechecked and cystine, glycine, glutamic acid and tryptophane were investigated.

Experimental. The various amino acids were incorporated into a synthetic diet[3] at varying levels from 5 to 20% depending upon the relative toxicity of the amino acid under consideration. The amino acid replaced an equivalent amount of carbohydrate in the basic diet. Glycine, *l*-tyrosine, *l*-histidine, *l*-cystine, *d*-glutamic acid and *dl*-tryptophan were used. The rats in all series showed crusted and bloody snouts, spectacled eyes, and diarrhea. The animals receiving glycine with or without superimposed riboflavin deficiency lost their hair; some became almost hairless. The rats were lethargic and appeared to lack muscular coordination. In those receiving glycine without a superimposed B_2 deficiency the hair condition corrected itself after a few weeks. Emaciation was common.

The symptomatology seen in these rats on toxic levels of an amino acid is also seen in riboflavin deficiency[4] but it should be recalled that these manifestations are nonspecific in nature.

Table I summarizes our results.

Accentuation of the toxicities of amino

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2 Martin, G. J., in press.

3 Martin, G. J., *Arch. Biochem.*, 1943, **1**, 397.

4 McCollum, E. V., Orent-Keiles, E., and Day, H., *Newer Knowledge of Nutrition*, 1939, Macmillan, New York.