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Calcium Content of Tissues from Rats Receiving Different Sulfonamides.*

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During studies[1] on the enzymatic changes in tissues from rats fed sulfasuxidine, a marked increase in the calcium content of the liver tissue was observed. Indirect evidence that concentrates from liver were effective in counteracting the change was also obtained. In this paper we wish to report a more detailed investigation of these changes and of the efficacy of liver concentrates in reversing the accumulation of excess calcium. Attempts have also been made to elucidate the mechanism of the action by studying the effect of other compounds which have pharmacological effects in common with those of some of the sulfonamides. Martin[2] found several sulfonamides, thiourea and *p*-aminobenzoic acid to produce a hyperplasia of the thyroid and hypothyroidism and to cause a "pseudopotentiation" of the action of insulin. However, sulfadiazine, the sulfonamide showing the greatest anti-thyroid activity,[3] failed to produce changes in the calcium content of any of the tissues except the kidney, and this change may be attributed to other causes. *p*-Aminobenzoic acid and thiourea caused no change in the amount of calcium in any of the tissues analyzed.

Experimental. Animals. Twenty-one day-old male rats of the Sprague-Dawley strain weighing 35-45 g were placed on the following purified ration: casein (Labco, vitamin-free), 18; sucrose, 73; salts IV,[4] 4; corn oil, 5. The drugs were incorporated individually in place of part of the sucrose at the following percentages: succinylsulfathiazole, 0.75; sulfathiazole, 0.75; sulfadiazine, 0.50; *p*-aminobenzoic acid, 1.0; thiourea, 0.50. Each rat received the following B vitamins daily in 0.5 cc of 20% EtOH in a supplement dish: thiamin hydrochloride, 20 γ; pyridoxin hydrochloride, 25 γ; nicotinic acid, 25 γ; riboflavin, 30 γ; calcium pantothenate, 200 γ; choline hydrochloride, 10 mg; biotin (as SMA conc. No. 1000), 1 γ. Each rat received on alternate days 2 drops of corn oil containing the fat-soluble vitamins, in concentrations to allow the following *daily* intake: A, 30 I.U.; D, 30 I.U.; E (α-tocopherol), 0.4 mg; K (2-methyl-1,4-naphthoquinone), 20 γ. When solubilized liver (Wilson fraction "L") was fed as a therapeutic measure it was placed in the B-vitamin supplement at a level of 0.2 g per day.

The animals were maintained on these regimens for 2-12 weeks. Rats from each group were sacrificed at frequent intervals for calcium and histological studies, and also for correlation of calcium content with changes in enzymatic activity previously reported.[1] The rats were decapitated and drained of blood preparatory to removal of the tissues for the various studies.

Calcium Determination. Samples of liver, kidney, and leg muscle were blotted with moistened filter paper, weighed, dried at

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2 Martin, G. J., *Arch. Biochem.*, 1943, **3**, 61.

3 Astwood, E. B., Sullivan, J., Bissell, A., and Tyslowitz, R., *Endocrinol.*, 1943, **32**, 210.

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

TABLE I.
Calcium Content of Livers of Rats Fed Sulfonamides.

Weeks on exp.	Sulfasuxidine basal*	Sulfasuxidine plus Sol. liver*†	Sulfathiazole basal*	Sulfathiazole plus Sol. liver*†	Sulfadiazine basal*	Control, no drug*
2-4	39.5		7.5			
	43.0		12.5			
4-6	85.0		10.5		10.5	20.5
	876		85.5		11.0	23.0
	1480		284			
	1660		1287			
6-8	15.0		9.5		18.0	10.0
	88.5		9.5		25.0	19.0
	191		23.5		38.0	19.5
	577		785			22.0
	944		1605			24.0
	1065					
	2380					
8-10	159	12.0	791	7.0	11.0	7.5
	620	63.5		17.0	23.0	13.5
	1230		1410			18.0
	1270					
	1280					
10-12	360	14.5	24.0	7.5	10.0	9.5
	850	14.5		24.0	11.5	12.5
	2640	23.5			14.5	
					16.0	

* In milligrams percent of dry tissue.
† 0.2 g per day of solubilized liver was fed for 10-37 days after rats had been on basal sulfonamide ration at least 6 weeks and were continued on the sulfonamide during therapy.

105°C for 24 hours, and again weighed to determine the dry matter content. The dried tissue was ground and a 0.1-0.2 g sample ashed in a platinum crucible at 550-600°C. This procedure retains all of the calcium;[5] a slight residue of carbon does not interfere.[6] The ashed samples were treated essentially according to the method of Kuttner and Cohen.[7] They were dissolved in 0.2 ml of 2 N HCl, transferred with several small portions of water (total, 2 ml) to a special 15 ml centrifuge tube having a finely-drawn tip (an aliquot was taken for samples having a high calcium content), and precipitated with 0.5 ml of 1% Na_3PO_4 and 0.4 ml of 25% NaOH. The precipitate was washed twice with alkaline 50% ethanol and the phosphate determined in the Evelyn colorimeter by the method of Fiske and Subbarow.[8]

Histology. Within 15 minutes after the death of the animal, small portions of the liver were placed in Bouin's Fixative. After 24-48 hours they were dehydrated, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Results. Values for the calcium content, in milligrams percent of dry tissue, of the individual livers of the rats on the various sulfonamide-containing diets are presented in Table I. With but one exception, all animals receiving the basal sulfasuxidine ration without liver exhibited a moderate to marked rise in calcium content within the experimental period of 2 to 12 weeks. Nevertheless, rats on this diet for 8 to 12 weeks, when fed 0.2 g per day of solubilized liver for 10 to 37 days, although continued on the sulfasuxidine ration, showed a return to normal values, except for one animal that was moderately above normal.

[5] St. John, J. L., and Midgley, M. C., *J. Assn. Official Agr. Chem.*, 1941, **24**, 932.

[6] Linder, G. C., *Biochem. J.*, 1940, **34**, 1574.

[7] Kuttner, T., and Cohen, H. R., *J. Biol. Chem.*, 1927, **75**, 517.

[8] Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, **66**, 375.

TABLE II.
Dry Matter Content of Livers of Rats on Various Regimens.

Group	No. of samples	% dry wt*	Standard deviation†
Sulfasuxidine‡	8	27.85	1.21
"	9	26.81	0.95
Sulfathiazole	7	28.20	0.57
Basal control (food *ad libitum*)	7	30.06	0.80
Control§ (restricted food intake)	8	30.75	0.56
Biotin deficient§	8	31.04	1.18

* The liver was rinsed, blotted, to remove excess fluid, and dried at 105°C for 24 hours.
† Calculated by the formula for a small number of samples:

$$\sigma = \sqrt{\frac{\Sigma d^2}{N-1}}$$

‡ Animals from a previous experiment.[1]
§ Animals from other experiments[10,11] included for purposes of comparison.

Only one-half of the rats fed sulfathiazole had abnormal calcium levels, but these were as marked as those of the sulfasuxidine group. The calcium values of the livers of all animals in this group fed solubilized liver were normal. None of the livers of rats fed sulfadiazine, nor any of those fed thiourea or *p*-aminobenzoic acid showed any variations outside of the range of those of the normal control animals.

Histological examination showed no evidence of deposition of *inorganic* calcium salts. Several livers of the sulfasuxidine and sulfathiazole groups showed changes similar to those which have been reported.[9] There was moderate to severe swelling of a few to numerous liver cells both in central and portal areas. The cytoplasm of these cells was clear, suggesting hydropic changes or the presence of lipoid substances. Occasional foci of multinucleated liver cells and a few small areas of necrosis were noted in several of these sections. Some proliferation of Kupffer cells also occurred in some areas of a few sections. Occasional sections showed mild typical fatty change with small vacuoles in the cytoplasm of the liver cells of central zones. None of the sections of livers from the sulfadiazine group showed significant changes.

Livers containing large quantities of calcium were, on the average, approximately 50% larger than those in normal rats on the basis of total body weight. Enlargement of the livers of rats receiving sulfonamides has been reported by others.[9]

The livers of rats receiving the basal sulfasuxidine or sulfathiazole ration without liver also showed (by statistical analysis) a significant decrease in dry matter content. These data are presented in Table II, and values obtained from animals on other experiments[10,11] are included for comparison. Livers of animals supplemented with solubilized liver had a normal moisture content. Variations in moisture content of the livers from the sulfadiazine group do not permit proper evaluation of the results. These varied from 27-30% dry matter content.

In Table III are presented the calcium values for kidney and muscle tissues from the various groups of animals. Sulfadiazine produced (except for one value not included in the table) a definite increase in the calcium levels of the kidney. Sulfasuxidine and sulfathiazole caused a slight decrease in the calcium content of muscle. The calcium of tissues of rats fed *p*-aminobenzoic acid or thiourea was normal.

Discussion. Numerous reports in the literature indicate that sulfonamides exert toxic effects on animals and that they also decrease the synthesis of nutritional factors by bacteria in the gastro-intestinal tract (*cf.* [1]). Our data show that sulfasuxidine and sulfathiazole produce a marked accumulation of calcium in the liver and that this effect is reversed or prevented by feeding solubilized liver (Wilson

[9] Gross, P., Axelrod, A. E., and Bosse, M. D., *Am. J. Med. Sci.*, 1944, **208**, 642.

[10] Pilgrim, F. J., Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1942, **145**, 237.

[11] Pilgrim, F. J., and Elvehjem, C. A., *J. Biol. Chem.*, 1944, **156**, 257.

TABLE III.
Calcium Content of Kidney and Muscle Tissues of Rats Receiving Sulfonamides.

Group	Kidney		Muscle	
	No. of samples	Calcium content*	No. of samples	Calcium content*
Sulfasuxidine	6	34.3 (18.5-68.0)	4	16.8† (11.0-23.5)
Sulfathiazole	5	31.1 (19.5-46.0)	5	19.3 (14.0-29.5)
Sulfadiazine	9	165.0 (99.0-336.)	4	33.1 (21.0-48.0)
Basal control	8	39.1 (19.5-54.0)	6	32.2 (20.5-49.5)

* In milligrams percent of dry tissue.
† One value of 183 mg% is omitted because the muscle appeared to be bruised.

fraction "L"). Only those livers having an increased calcium content exhibited a decreased enzymatic activity; the results previously reported[1] showing that feeding of "norite eluate" concentrates allowed normal enzymatic activity, are, therefore, an indirect indication that concentrates high in the "norit eluate factor," as assayed by *S. fecalis* (*S. lactis*) or *L. casei,* are effective in preventing this disturbance in calcium metabolism.

The alteration of the calcium metabolism can be attributed to: (a) a nutritional deficiency caused by a decreased rate of synthesis of factor(s) by the intestinal bacteria; (b) a toxicity of the sulfonamide which is evident only in the nutritionally-deficient animals; (c) a direct toxicity of the drug requiring a more or less specific antagonist. It seems most likely that the effect is the result of a nutritional deficiency, because: (a) sulfasuxidine is the least readily absorbed of the drugs tested, yet it produces the change most consistently; (b) the change can be prevented or reversed by feeding a concentrate known to be rich in nutritional factors.

The histological studies show that there are no deposits of inorganic calcium salts such as those that follow necrosis or other degeneration. The accumulation of calcium is apparently the primary result of the deficiency or toxicity.

There may be a correlation between the increase in water content of the liver and the hydropic degeneration observed microscopically by others,[9,12] and noted in a few of our sections. However, histological observations do not show the change as consistently as the dry weight determinations reported here. It is interesting to note that deficiencies of the known B vitamins do not alter the moisture content of the tissues;[13,14] see also Table II.

Histological studies have shown[15,16] that administration of sulfadiazine results in crystallization of the drug or its acetyl derivative in the kidney with consequent irritation and calcification. The increase in calcium in the kidneys may be related to this phenomenon. There is no sound basis for postulating any other mechanism for this effect of sulfadiazine.

The slight decrease in muscle calcium (Table III) of rats fed sulfasuxidine or sulfathiazole may also be a result of the disturbed calcium metabolism since the ration can readily supply the relatively small amount of calcium (in terms of total utilization) accumulated in the liver.

Summary. Rats receiving sulfasuxidine in a purified ration exhibit a marked increase in the calcium content of the liver within 4 to 8 weeks. The increased calcium content is apparently a primary metabolic defect and not a secondary calcification following necro-

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sis. Rats fed sulfathiazole show the same syndrome but not as consistently. The calcium of the kidney and muscle of rats fed sulfasuxidine or sulfathiazole is not increased. The altered calcium metabolism of the liver can be reversed or prevented by feeding solubilized liver for 10 to 40 days. Rats receiving sulfadiazine have an increased calcium content of the kidneys but not of liver or muscle.

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The Urinary Excretion of Sulfadiazine Determined with Thiobarbituric Acid, a New Specific Reagent.*

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By means of the thiobarbituric acid reagent described in another connection,[1] 2-aminopyrimidine and its derivatives sulfadiazine and acetylsulfadiazine can be determined quantitatively, the latter being recovered from urine to within 1%. The specificity is such that no reaction was obtained with 15 other pyrimidines, with sulfamerazine, or with sulfanilamide, sulfapyridine, sulfacetamide, sulfathiazole, sulfasuxidine, sulfapyrazine, and sulfaguanidine, with the common constituents of blood and urine, or with 50 control urines.

Procedure. Unknown and standard solutions are prepared to contain 0.5 to 2 mg% of sulfadiazine in 0.2 N HCl. 1 ml of unknown, of standard, and of 0.2 N HCl (the reagent blank) plus 4 ml of thiobarbituric acid reagent[1] are heated on the boiling water bath in chromic acid-cleaned tubes for 45 minutes, evaporation being minimized. The solutions are then made up to 10 ml with water, and within several hours the unknown and standard are read through filter 520 against the reagent blank in the Evelyn photoelectric colorimeter. The Beer-Lambert law is obeyed. The molar extinction coefficients of sulfadiazine and acetylsulfadiazine are equal. Preliminary hydrolysis of the urine (40 minutes, 0.2 N HCl) does not affect the analysis.

Urines from 30 subjects taking sulfadiazine were examined. In 25, the thiobarbituric acid analysis (TBA) of *fresh* urine agreed with the Bratton and Marshall[2] free sulfadiazine (BM-F). But after aging the urine at 5° or 20°C for one week, TBA agreed with the Bratton and Marshall free plus acetylated, or total (BM-T). The same change was obtained in 30 minutes by treating the fresh urine with ascorbic acid or sodium hydrosulfite (final concentration 0.6%). Example: analysis of fresh urine, TBA 61 mg%, BM-F 60.5 mg%, BM-T 91; after treatment with ascorbic acid, TBA 91.5 mg%.

In 5 subjects, however, aging had no effect, TBA agreeing with BM-T from the start. Example: analysis of fresh urine, TBA 113.5 mg%, BM-F 68 mg%, BM-T 115 mg%.

The analysis of a mixture of fresh urines from *both* types of subject equalled the sum of their individual analyses. This indicates that the difference between the types is not due to the presence of an interfering substance in one of them.

Following the ingestion of acetylsulfadiazine, both types of subject reacted in the same manner. De-acetylation was negligible, aging was without effect, and TBA equalled BM-T in fresh urine. Example: analysis of fresh

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