demonstrate calcification until the inflammation subsided.

Conclusions. 1. In the dog, there is an initial local acidity in the hematoma resulting from a fracture, followed by a change to alkalinity beyond the value in the venous blood.

2. Biopsy of the fracture site after the fluid became alkaline, at the end of the experiment, showed calcification in 6 out of 8 cases.

3. Infection at the site of fracture produced a persistent local acidity.

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Granulocytopenia in Rats Given Thiourea and Thyroxin. The Therapeutic Effect of *L. casei* Factor.

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The effect of thiourea and its derivatives in depressing the functional activity of the thyroid gland in experimental animals and in man is well known and the literature pertaining to the discovery of this phenomenon has been repeatedly reviewed.[1,2] In the first report on the clinical use of thiourea and thiouracil in the treatment of thyrotoxicosis[1] there was noted the development of agranulocytosis in one patient. A number of additional cases of leucopenia, granulocytopenia or agranulocytosis following the use of these drugs have been described. The importance of finding a method for preventing the development of these blood dyscrasias is obvious. It appeared that there might be an analogous situation in the occurrence of granulocytopenia in rats fed sulfonamides in purified diets[3,4] and the correction of this dyscrasia with *L. casei* factor.[5] It was decided, therefore, to study the effect on rats of administering thiourea in a similar purified diet with and without the simultaneous administration of thyroxin or thyroid powder. If granulocytopenia developed it would then be possible to test the therapeutic value of *L. casei* factor in these animals.

Anemia without granulocytopenia has been encountered in many of the animals receiving thiourea without thyroxin or thyroid powder. Granulocytopenia without anemia has occurred in high incidence in animals receiving both thiourea and thyroxin or thyroid powder and the granulocytopenic animals have been treated successfully with *L. casei* factor.

Experimental. The basal (control) diet used in these investigations consisted of leached and alcohol-extracted casein 18%, Crisco 8%, modified Osborne and Mendel salt mixture[3] 4%, $FeSO_4.7H_2O$ 0.18%, $CuSO_4.5H_2O$ 0.02% and anhydrous dextrose (Merck's U.S.P.) 69.8%. Into each 100 g of diet were incorporated 1 mg of thiamine hydrochloride, 2 mg of riboflavin, 2 mg of calcium pantothenate, 1 mg of pyridoxine hydrochloride and 200 mg of choline chloride. Each rat received a supplement twice weekly of 0.25 cc of corn oil containing 2000 units of vitamin A and 200 units of vitamin D (Natola). The experimental diets differed only in that specified ingredients were substituted for equivalent amounts of dextrose. Diet No. 985 contained 1% of thiourea; diet No. 1053 contained 1% of thiourea and

1 Astwood, E. B., *J. Am. Med. Assn.*, 1943, **122**, 78.

2 Mixner, J. P., Reineke, E. P., and Turner, C. W., *Endocrinology*, 1944, **34**, 168.

3 Spicer, S. S., Daft, F. S., Sebrell, W. H., and Ashburn, L. L., *Pub. Health Rep.*, 1942, **57**, 1559.

4 Kornberg, A., Daft, F. S., and Sebrell, W. H., *Science*, 1943, **98**, 20.

5 Daft, F. S., and Sebrell, W. H., *Pub. Health Rep.*, 1943, **58**, 1542.

TABLE I.
Development of Blood Dyscrasias in Rats Given Thiourea in Purified Diets With and Without the Simultaneous Administration of Thyroxin or Thyroid Powder.

Diet	No. of rats	No. with granulocytopenia*	No. with leucopenia†	No. with anemia‡
Basal control	21	0§	0§	0
Containing thiourea (No. 985)	28	1	7	14
Basal diet and thyroxin	16	1	0	0
Diet containing thiourea (No. 985) and thyroxin	16	10	10	1
Diet containing thiourea and thyroid powder (No. 1053)	30	18	20	2

* 300 or fewer polymorphonuclear granulocytes per cu mm.
† 4000 or fewer total leucocytes per cu mm.
‡ Hematocrit value of 35 volumes % or less.
§ White cell counts were obtained on only 9 of the 21 control animals.

0.25% of thyroid powder (Armour U.S.P.).

The study consisted of 2 parts; (a) a series of experiments designed to study the development of blood dyscrasias under various conditions and (b) a test of the value of *L. casei* factor in correcting granulocytopenia. For each experiment in the first part of the study weanling male albino rats of the Osborne and Mendel strain were divided into 2 (or 4) groups on the basis of weight and litter. One group was given the basal diet and another the experimental diet containing thiourea (No. 985); when 4 groups were employed the 2 additional ones received these identical diets and in addition were given thyroxin injections. The thyroxin (crystalline, Squibb) was administered subcutaneously 6 times weekly at a level of 1 μg per gram of body weight. In the second part of the study, diet No. 1053, containing thiourea and thyroid powder, was given to all animals throughout the entire experiment. A number of the rats which became granulocytopenic were treated with *L. casei* factor, diet No. 1053 being continued.

At various times, total white blood cell counts, polymorphonuclear granulocyte counts and hematocrit determinations were made on the tail blood of these animals by technics which have been described.[6]

For this report, rats with counts of no more than 300 polymorphonuclear granulocytes per cu mm were considered to be granulocytopenic, those with counts of 4000 or fewer total white cells per cu mm were considered to be leucopenic, and those with hematocrit values of 35 volumes % or less were considered to be anemic. The incidence of granulocytopenia, leucopenia and anemia in the animals subjected to the different experimental conditions is given in Table I. The lowest values observed have been used in the preparation of this summary.

Of 28 animals which were given thiourea but no thyroxin or thyroid powder, only 1 became granulocytopenic while 7 became leucopenic and 14, or 50%, became anemic. Eight of these 14 had hematocrit values of 30 volumes % or less. Results at hand suggest that the anemia cannot be prevented by the inclusion of *L. casei* factor or a crude liver fraction in the diet but the data are not sufficiently extensive to permit a definite conclusion. Animals which received thiourea but also received thyroxin injections or thyroid powder in the diet showed a low incidence of anemia (3 of 46) but a high incidence of granulocytopenia (28 of 46) and leucopenia (30 of 46). Control animals which received no thiourea showed almost no blood dyscrasias (1 of 37) whether or not they were given thyroxin injections.

As a rule, the rats developed the blood dyscrasias after only a few weeks on the experimental diets. The anemia usually appeared somewhat earlier than the granulocytopenia. As noted above 8 rats given the

[6] Daft, F. S., Kornberg, A., Ashburn, L. L., and Sebrell, W. H., *Pub. Health Rep.*, 1945, **60**, 1201.

TABLE II
Treatment of 12* Granulocytopenic Rats with *L. casei* Factor (100 μg Per Rat Per Day) for 4 and 10 Days.

	Total white blood cells per cu mm		Total polymorphonuclear granulocytes per cu mm	
	Avg.	Range	Avg.	Range
Before treatment	4630	1300-8850	140	50-300
After 4 days	5470	1650-9250	630*	50-1100*
" 10 "	7920	2950-13,800	2030	800-4100

* A total of 14 animals were treated. One died on the 7th day of treatment and one responded sufficiently well in 4 days that treatment was discontinued. For the sake of uniformity the data for these 2 animals are not included in the table. The animal which responded in 4 days did so on 2 occasions; once from 200 up to 6700 and again from 250 up to 1400 polymorphonuclear granulocytes per cu mm.

thiourea-containing diet (No. 985) became rather severely anemic (hematocrit values of 30 volumes % or less). This degree of anemia developed in these 8 animals usually after about 1 month on experiment (22 to 33 days for 7 animals, average 26 days; 181 days for the eighth animal). The 28 rats which became granulocytopenic while receiving thiourea plus thyroxin or thyroid powder developed this dyscrasia after 25 to 104 days on experiment (25 to 55 days for 25 animals, average 38 days; 75, 81 and 104 days, respectively, for the other animals).

Granulocytopenic rats fed diet No. 1053, containing thiourea and thyroid powder, were used in tests of the therapeutic value of *L. casei* factor.* In preliminary trials it was found that treatment of such animals for 4 days with 25 μg of *L. casei* factor daily did not routinely correct the dyscrasia. The therapeutic dose was increased to 100 μg daily but as indicated in Table II the response even to this amount was not spectacular. Only 2 of 14 animals reached a level of polymorphonuclear granulocytes of 1000 cells per cu mm in 4 days. Treatment was continued in 12 of these animals for 6 additional days and at the end of this time 10 of the 12 animals had 1000 or more granulocytes per cu mm of circulating blood; the remaining 2 had levels of 800 and 950 cells per cu mm respectively.

Pathologic Findings. The heart, lung, liver, kidney, spleen, adrenal and bone marrow of 26 rats fed thiourea were sectioned and examined microscopically. Of these organs only the thyroid glands, adrenal glands, bone marrow and spleen showed significant lesions. The thyroid hyperplasia and colloid depletion found have been previously described. Grossly the adrenal glands did not appear to be enlarged but were cherry red in color. Microscopically, the pathologic alterations present in these glands were hyperemia, often of extreme degree, hemorrhage, necrosis and lipid depletion. The hyperemia and hemorrhage, while commonly present in the inner cortex (reticularis), were not limited to this area. In a few adrenals, hyperemia or hemorrhage or both were present in the outer half of cortex and absent from the reticularis. Necrosis of adrenal cortical cells occurred in about one-half of the animals. The involved cells were scattered, less often grouped, in the outer one-third of the fascicularis. Rarely were they seen deeper in the cortex. Lipid depletion of moderate or marked degree was seen in all except 2 animals. This was studied in frozen sections stained with Sudan IV. In the majority, the depletion was usually greatest in, and often limited to, the outer half of fascicular and glomerular layers. Although measurements were not made, the cortex in a few adrenals was obviously reduced in thickness. The spleen of a number of rats showed marked hyperemia and lymphoid atrophy. The bone

* The crystalline product employed had been isolated from a fermentation residue[7] and was furnished through the courtesy of Drs. E. L. R. Stokstad and B. L. Hutchings of Lederle Laboratories, Inc.

[7] Hutchings, B. L., Stokstad, E. L. R., Bohonos, N., and Slobodkin, N. H., *Science*, 1944, **99**, 371.

marrow studied in paraffin sections showed in many rats striking increase in erythropoietic activity and some decrease in number of granulocytes. The majority of the nucleated red cells were normoblasts. The only abnormal finding in the 13 control rats was lipid depletion of the adrenal glands of 4 rats; slight in 3, moderate in 1.

The adrenal hemorrhage and necrosis are almost completely prevented by thyroxin injections or by thyroid powder in the diet. Of 26 animals examined which had received thiourea, but no thyroxin or thyroid powder, 15 showed hemorrhage or necrosis or both of the adrenal. Of 15 animals which had received thyroxin injections in addition, and of 8 which had received thiourea and thyroid powder, 2 (one in each group) showed similar lesions. It would appear, therefore, that this damage to the adrenal glands is mediated in some way through the thyroid. The lipid depletion is not prevented by the injection of thyroxin or feeding thyroid powder.

Discussion. During the course of this work there appeared a report[8] describing the occurrence of granulocytopenia in rats fed a standard laboratory ration containing 0.5% of thiourea. The animals apparently developed neither leucopenia nor anemia but the average neutrophil percentage dropped from 32.7 to 5.4. It was reported that this granulocytopenia could be prevented by the inclusion of 5% of a crude liver fraction in the diet.

The anemia observed in our rats ingesting thiourea but receiving neither thyroxin nor thyroid powder apparently resulted in some way from the interference of the drug with thyroid function. This is suggested very strongly by the low incidence of anemia in the animals receiving thyroxin or thyroid powder in addition to the thiourea. These findings are in accord with the reported occurrence of anemia in clinical hypothyroidism and in thyroidectomized rats.[9]

It should be noted that the granulocytopenia in these experiments has been, comparatively, very difficult to correct. Rats with granulocytopenia induced by other methods have responded spectacularly and with considerable regularity to treatment for shorter periods and with smaller amounts of the vitamin. For example, in a recent study of the treatment of sulfasuxidine-induced granulocytopenia,[10] 11 of 14 animals responded to the administration of 25 μg of *L. casei* factor daily for 4 days. In these 11 animals the level of circulating granulocytes increased in 4 days from an initial average of 10 cells per cu mm to an average of 4530 cells per cu mm. Similar responses to identical therapy were obtained in granulocytopenic animals in which the dyscrasia was induced by diet alone,[11] by a deficiency of pantothenic acid,[6] or by a deficiency of riboflavin.[12] The granulocytopenia appearing in animals ingesting thiourea and receiving thyroxin or thyroid powder may differ therefore from the granulocytopenia resulting from these other conditions. It may be that the effect of the thyroxin and thyroid powder is to increase the animals' requirements for *L. casei* factor. A similar effect of thyroxin has been demonstrated for other vitamins of the B-complex.[13] The effect of the thiourea is conceivably due to a direct interference with some metabolic activity in the animal body.

It is of particular interest that the production of granulocytopenia by thiourea in these studies required the simultaneous administration of thyroxin or thyroid powder, each of which was given at a high level. These conditions are perhaps somewhat similar to those under which clinical agranulocytosis follows the use of thiourea.

Summary. Rats given thiourea in a purified diet develop anemia and, in lesser incidence, leucopenia. They also develop hemorrhage

[8] Goldsmith, E. D., Gordon, A. S., Finkelstein, G., and Charipper, H. A., *J. Am. Med. Assn.*, 1944, **125**, 847.

[9] Crafts, R. C., *Endocrinology*, 1941, **29**, 596.

[10] Endicott, K. M., Daft, F. S., and Ott, M., *Arch. Path.*, 1945, **40**, 364.

[11] Kornberg, A., Daft, F. S., and Sebrell, W. H., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 46.

[12] Kornberg, A., Daft, F. S., and Sebrell, W. H., *Arch. Biochem.*, 1945, **8**, 431.

[13] Drill, V. A., and Overman, R., *Am. J. Physiol.*, 1941-42, **135**, 474.

and necrosis of the adrenals. Animals which receive, concomitantly, thyroxin injections or thyroid powder become granulocytopenic and leucopenic, while the incidence of anemia and of adrenal hemorrhage and necrosis is greatly reduced. The granulocytopenia and leucopenia of these rats may be corrected by treatment with *L. casei* factor.

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Microbiological Determination of Amino Acids. IV. Lysine, Histidine, Arginine, and Valine

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Microbiological assays for amino acids have been reported in a series of papers from this laboratory.[1-3] A considerable portion of our more recent work has been anticipated by published results from other laboratories,[4-6] and we are therefore presenting only in abbreviated form additional methods for lysine, histidine, arginine, and valine and results of their application to 6 purified proteins: casein, gelatin, ovalbumin, β-lactoglobulin, horse hemoglobin, and silk fibroin.

Assay Procedures. Samples for assay were prepared by autoclaving (at 15 lb. steam pressure) 100 mg of the protein with 2 cc of 10% hydrochloric acid in a sealed tube for 24 hours. All assays were carried out as described by McMahan and Snell.[1]

Leuconostoc mesenteroides was employed for the lysine and histidine assays. The medium of McMahan and Snell[1] was modified for *L. mesenteroides* by increasing the phosphate content (Salts A) 8-fold and the arginine content 3-fold, and by altering the vitamin supplement (Solution 2) as described by Hac *et al.*[2] †

The lysine assay was incubated 63 to 65 hours at 32 to 34° C. The standard curve was constructed from response of the test organism to graded amounts of *l* (+)-lysine in the range 5-50 γ per 2.5 cc of medium. Growth response was measured turbidimetrically after dilution of the contents of each tube with 5 cc of a saturated aqueous solution of chlorothymol. Growth response may also be measured acidimetrically‡ using the micro quinhydrone-calomel-electrode technic or with the glass-electrode assembly described by McQuarrie and Jones.[7] When the assay tubes were to be titrated, 2% glucose was used instead of 1%, to permit formation of larger quantities of acid.

For the histidine assay, graded amounts of *l*(+)-histidine in the range 1-12 γ made up the standard curve. The culture tubes were incubated at 32 to 34° C for 72 hours. Shorter periods of incubation resulted in poor recoveries and drifting assay values.

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[1] McMahan, J. R., and Snell, E. E., *J. Biol. Chem.*, 1944, **152**, 83.

[2] Hac, L. R., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, 1945, **159**, 273.

[3] Hac, L. R., and Snell, E. E., *J. Biol. Chem.*, 1945, **159**, 291.

[4] Dunn, M. S., Camien, M. N., Shankman, S., Frankl, W., and Rockland, L. B., *J. Biol. Chem.*, 1944, **156**, 715.

[5] Dunn, M. S., Camien, M. N., Shankman, S., and Rockland, L. B., *J. Biol. Chem.*, 1945, **159**, 653.

[6] Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C., *J. Biol. Chem.*, 1945, **160**, 35.

† The folic acid requirement is based on a concentrate with a potency of 40,000.

‡ The values obtained for the lysine content (uncorrected for moisture) of a casein hydrolyzate assayed turbidimetrically and acidimetrically were 7.0 and 7.1%, respectively.

[7] McQuarrie, E. B., and Konen, H. J., *Ind. Eng. Chem.*, Anal. Ed., 1944, **16**, 205.