THE ACTION OF PTEROYL GLUTAMIC ACID AND NATURAL SOURCES OF FOLIC ACID ON BLOOD DYSCRASIAS INDUCED BY SULFONAMIDE DRUGS

By H. G. Petering, Ph.D., R. A. Delor, B.S., and H. C. Murray, Ph.D.

FOLIC acid or vitamin B9 deficiency has been induced in the rat by feeding a purified diet containing succinyl sulfathiazole or sulfaguanidine.1,2,3,4,5,6 This deficiency is corrected in either case by feeding free or conjugated pteroyl glutamic acid while the syndrome produced by sulfaguanidine is also corrected by para-aminobenzoic acid. The action of these drugs has been considered to be due to their inhibitory effect on the synthesis of folic acid in the intestinal tract of the animal.7

It has been known for some time that the more soluble sulfonamides widely used in medicine also cause blood dyscrasias in human patients which resemble those due to folic acid deficiency in rats fed sulfasuxidine or sulfaguanidine.8,9 Some work with experimental animals has shown that sulfanilamide, sulfathiazole, and sulfadiazine cause leukopenia, granulocytopenia, and even anemia, in addition to other toxic manifestations upon prolonged administration.10,11,12 Kornberg, Daft, and Sebrell12 have reported that the dyscrasias in the rat due to soluble sulfonamide can be alleviated by treatment with Wilson liver fraction L and brewers' yeast.

The importance of the soluble sulfonamides in medical practice and the fact that some investigators consider that these drugs are retained in the intestinal tract in sufficient quantity to produce some bacteriostasis13,14,15 which might cause the appearance of folic acid deficiency have prompted us to report our studies on the effect of crystalline folic acid, liver extract powder,16 yeast extract, and para-aminobenzoic acid in the prevention of blood dyscrasias induced by prolonged administration of sulfanilamide, sulfathiazole, and sulfadiazine. Chronic toxicity studies reported here were undertaken, since some investigators believe they are more applicable to medical therapeutics than are acute toxicity studies.17

Experimental

Twenty-eight day old weanling white rats of random sex, 45-50 Gm. in weight, were placed on the basal purified diet8 to which in most instances was added 1 per cent of the sulfonamide drug being studied. The drug was added at the expense of the sucrose. Supplements were fed from the first day, since the object of the study was to determine the effect of certain nutritional substances on the prevention of blood dyscrasia.

Growth curves were obtained in all cases by semiweekly weighings. At the end

* The basal diet was composed of 72 per cent sucrose, 18 per cent casein (Labco, vitamin-free), 3 per cent cottonseed oil, 1 per cent cod liver oil, 4 per cent salt (Sure), 0.1 per cent choline chloride, 0.1 per cent inositol and 100 γ per cent of vitamin K (dissolved in the cod liver oil). Daily supplements of crystalline vitamins included: thiamine hydrochloride, 100 γ; riboflavin, 100 γ; pyridoxine hydrochloride, 100 γ; calcium d-pantothenate, 100 γ; nicotinamide, 500 γ; and biotin, 17 γ.

From the Department of Nutrition, The Upjohn Co., Kalamazoo, Michigan.
of about the fifth and eighth weeks, complete peripheral blood analyses were made on all surviving rats, using conventional hematological technics.

The antagonistic effects of the supplements on the antibacterial action of the sulfonamides was studied in vitro with Str. haemolyticus (B group Lancefield, 090 R, University of Kansas). The medium which was used was composed of edamin (protein hydrolysate) and dextrose supplemented with Speakman salts A and B, nicotinic acid, thiamine, uracil, guanine, and adenine, and adjusted to pH 6.9. Growth effects of various substances and drugs, either inhibitory or stimulatory, were measured turbidimetrically by comparison with the growth of the organism on the basal medium.

The supplements whose effects were tested in the work reported here were (1) pteroyl glutamic acid or folic acid,* (2) liver extract powder 1:20 (L.E.P.), (3) dried yeast extract (D.Y.E.), and (4) para-aminobenzoic acid (PABA). The liver extract powder was found to contain about 50% of total folic acid per gram (42–59γ range), of which about 40% per cent was free folic acid, while the dried yeast extract contained about 85% of total folic acid per gram (range 60–110γ), of which about 90% per cent was in the conjugated form. Some difficulty was experienced in accurately assaying the dried yeast extract by microbiological technique due to the variability of the enzymatic digestion step.†

**RESULTS**

The hematological data are summarized in tables 1 and 2, and representative growth curves of the rats are given in figure 1. An interpretation of these results is given below.

Experiment I illustrates the response obtained under our conditions on a preventive experiment with 1 per cent sulfasuxidine. It can readily be seen that 100 mg. or more of dried yeast extract daily, furnishing about 8.5γ of “total” folic acid, kept the animals in almost normal condition as judged by the level of blood components, but normal growth was not attained until 200–300 mg. of D.Y.E. were fed daily. This experiment also illustrates the severe leukopenia, granulocytopenia, anemia, and growth depression produced in the rat by feeding 1 per cent sulfasuxidine in the purified basal diet, which otherwise maintains the animals in as good condition as does the stock diet. In this experiment a severe anemia was experienced by the animals receiving the drug alone, the appearance of which is variable (cf. exp. II).

Experiment II (table I) was carried out to illustrate the relative effects of sulfasuxidine and sulfathiazole in the basal diet as compared with basal and stock diets in litter mate rats. It is our experience that 2 per cent sulfasuxidine frequently is necessary to produce severe symptoms of growth depression and blood dyscrasias, which accounts for that level being used here. The data show that both drugs cause severe growth retardation, leukopenia, and granulocytopenia, although 2 per cent

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* Crystalline pteroyl glutamic acid or folic acid, supplied as Folvite 5 mg. tablets of Lederle Laboratories, suspended in water containing a trace of ammonium hydroxide.

† Suarez et al.²⁸ have reported that J. R. Totter has found a polypeptide of para-aminobenzoic acid in yeast to be a specific inhibitor of the enzyme conjugase.
sulfasuxidine appears to be somewhat more drastic in its effects on the white cell picture.

The action of 1 per cent sulfanilamide is shown in the data from experiment III, table 2. These results indicate that sulfanilamide probably causes as severe a growth depression as does sulfasuxidine, but the effect on the blood is less severe although still quite pronounced. Synthetic folic acid (pteroyl glutamic acid), crude liver extract, and para-aminobenzoic acid all prevent the growth retardation to the same extent, though completely normal growth is not attained with the levels of supplements used.

Folic acid and liver extract in equivalent amounts completely prevent the appearance of leukopenia or granulocytopenia, although it appears that a slight anemia
is present in animals receiving these supplements. Para-aminobenzoic acid at the level used shows some beneficial effect, but permits the appearance of mild symptoms of leukopenia, granulocytopenia, and anemia.

Growth retardation by 1 per cent sulfanilamide therefore appears to be due in part only to a folic acid deficiency, since it is not so completely prevented as is that due to sulfasuxidine, even though the blood dyscrasia due to sulfanilamide can be completely eliminated by 50 or more of synthetic folic acid or an equivalent amount of liver extract powder.

The effects of 1 per cent sulfadiazine in the absence and presence of folic acid, liver extract powder, and para-aminobenzoic acid are shown in the data of experiment IV, table 2. These results show that 1 per cent sulfadiazine also produces a

<table>
<thead>
<tr>
<th>Experiment I. Basal diet 1% sulfasuxidine</th>
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<tbody>
<tr>
<td>Group</td>
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</tr>
<tr>
<td>1</td>
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<td>2</td>
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<tr>
<td>4</td>
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<td>5</td>
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<th>Experiment II. Comparison of sulfasuxidine and sulfathiazole</th>
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<tr>
<td>2</td>
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<td>3</td>
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severe retardation of growth and pronounced blood dyscrasias. The growth is not greatly affected by liver extract, folic acid, or para-aminobenzoic acid, but the leukopenia and granulocytopenia are eliminated by 100 mg. of liver extract powder and 50 of folic acid, the former having a more pronounced effect than the latter. Two mg. of para-aminobenzoic acid permits the appearance of mild leukopenia and severe granulocytopenia. All supplements seem to retard the development of anemia to a significant degree.

One per cent sulfathiazole in the diet appears to be more toxic than 1 per cent sulfanilamide or 1 per cent sulfadiazine, as judged by the data shown in table 2, experiments V and VI. The anemia, leukopenia, and granulocytopenia appear to be more severe with this drug than with either sulfanilamide or sulfadiazine. Mortality is greater than was shown in experiments with either sulfanilamide or sulfadiazine.
### Table 1: Hematological Data Showing the Effect of Various Supplements on the Course of Blood Dyscrasias Due to Soluble Sulfonamide Drugs

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Daily supplement</th>
<th>Experimental day</th>
<th>W.B.C. 10^9/ (mm.)</th>
<th>Granulocytes 10^9/ (mm.)</th>
<th>R.B.C. 10^9/ (mm.)</th>
<th>Hemoglobin Gm./100 ml.</th>
<th>Hematocrit % vol.</th>
<th>Animals surviving Animals started</th>
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<td><strong>Experiment III. Basal diet 1% sulfanilamide</strong></td>
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<td></td>
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<tr>
<td>1</td>
<td>None</td>
<td>35</td>
<td>10.0</td>
<td>0.80</td>
<td>5.9</td>
<td>9.3</td>
<td>33</td>
<td>9/10</td>
</tr>
<tr>
<td>2</td>
<td>5 γ folic acid</td>
<td>35</td>
<td>16.6</td>
<td>1.49</td>
<td>6.8</td>
<td>10.5</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>100 mg. liver powder 1:20</td>
<td>35</td>
<td>18.4</td>
<td>3.31</td>
<td>7.1</td>
<td>10.8</td>
<td>37</td>
<td></td>
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<tr>
<td>4</td>
<td>2 mg. PABA</td>
<td>35</td>
<td>10.8</td>
<td>1.30</td>
<td>6.2</td>
<td>10.2</td>
<td>35</td>
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<td><strong>Experiment IV. Basal diet 2% sulfadiazine</strong></td>
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<tr>
<td>1</td>
<td>None</td>
<td>36</td>
<td>10.8</td>
<td>0.32</td>
<td>7.2</td>
<td>11.0</td>
<td>39</td>
<td>10/10</td>
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<tr>
<td>2</td>
<td>5 γ folic acid</td>
<td>36</td>
<td>11.1</td>
<td>1.10</td>
<td>7.6</td>
<td>11.7</td>
<td>40</td>
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<tr>
<td>3</td>
<td>100 mg. liver powder 1:20</td>
<td>36</td>
<td>12.5</td>
<td>1.06</td>
<td>7.6</td>
<td>11.7</td>
<td>41</td>
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<tr>
<td>4</td>
<td>2 mg. PABA</td>
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<td>11.9</td>
<td>0.83</td>
<td>7.4</td>
<td>11.0</td>
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<td><strong>Experiment V. Basal diet 1% sulfathiazole</strong></td>
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<td></td>
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<tr>
<td>1</td>
<td>None</td>
<td>35</td>
<td>10.1</td>
<td>0.10</td>
<td>7.4</td>
<td>12.5</td>
<td>36</td>
<td>9/10</td>
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<tr>
<td>2</td>
<td>10 γ folic acid</td>
<td>35</td>
<td>16.7</td>
<td>1.84</td>
<td>8.2</td>
<td>13.8</td>
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<td>8/10</td>
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<tr>
<td>3</td>
<td>100 mg. liver powder 1:20</td>
<td>35</td>
<td>15.2</td>
<td>1.72</td>
<td>7.1</td>
<td>14.3</td>
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<td>4</td>
<td>100 mg. D.Y.E.</td>
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<td>14.6</td>
<td>1.15</td>
<td>7.0</td>
<td>14.1</td>
<td>40</td>
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<td><strong>Experiment VI. Basal diet 1% sulfathiazole</strong></td>
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</tr>
<tr>
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<td>None</td>
<td>36</td>
<td>7.5</td>
<td>0.08</td>
<td>7.3</td>
<td>11.9</td>
<td>41</td>
<td>7/10</td>
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<tr>
<td>2</td>
<td>10 γ folic acid</td>
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<td>14.3</td>
<td>1.86</td>
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<td>15.2</td>
<td>1.22</td>
<td>7.6</td>
<td>12.6</td>
<td>43</td>
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<tr>
<td>4</td>
<td>2 mg. PABA</td>
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<td>9.4</td>
<td>0.75</td>
<td>6.6</td>
<td>11.2</td>
<td>38</td>
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All supplements have a small beneficial effect in preventing the serious growth retardation due to the drug, but 200 mg. of liver extract powder equivalent to about 10 γ of folic acid seems definitely to be the best in this respect. Synthetic folic acid,
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Liver extract powder, and dried yeast extract effectively prevent the onset of the severe anemia shown in the negative group. Para-aminobenzoic acid has a lesser effect on the anemia.

All supplements prevent the development of the severe leukopenia and granulocytopenia found in the negative control animals, although para-aminobenzoic acid again seems much less effective than the other substances.

In an experiment not shown in the tables, the addition of high potency antipernicious anemia extract (10 units) or 2 mg. of para-aminobenzoic acid to folic acid has no significant effect on the course of the blood dyscrasias or growth due to 1 per cent sulfathiazole, but the liver extract seems to increase survival. This decrease in mortality seems also to be evidenced in other groups receiving liver extract powder (cf. table 2).

It should be noted that in the two experiments with 1 per cent sulfathiazole the relative effectiveness of synthetic folic acid, the mixture of free acid and combined folic acid in a natural source such as liver extract, and conjugated folic acid, as contained almost entirely in yeast extract, was investigated. It seems certain that both free and conjugated folic acid are effective with sulfathiazole as well as with sulfasuxidine. Any increased value of liver extract over its folic acid content must be ascribed to other materials present.

Fig. 1. In vitro microbiological data of antagonism of various supplements to sulfadiazine, using Str. haemolyticus, B. Lancefield (o9o R, University of Kansas). Curves 1, 3, 7 are folic acid vs. 0, 10, and 100 γ sulfadiazine per ml. respectively; curves 4, 5, 6 are PABA vs. 0, 10, and 100 γ sulfadiazine per ml. respectively; curves 7, 8, 9 are L. E. P. (1:120) vs. 0, 10, and 100 γ of sulfadiazine per ml. respectively.
In view of the striking effect of folic acid, liver extract powder, and dried yeast extract in preventing blood dyscrasias due to soluble sulfonamides as well as in alleviating toxicity as manifested in growth and mortality and on the basis of the reports of Lampen and Jones that pteroyl glutamic acid was not antagonistic in most cases to the antibacterial action of sulfadiazine, in vitro studies of the supplements used in the animal experiments reported above were carried out, using an enterococcus which on the medium used does not require any of the supplements for good growth.

The data of these studies are shown in figures 2 and 3. They show that in very large concentration liver extract and possibly folic acid have some stimulatory effect, although these are not needed for good growth on the medium used; para-aminobenzoic acid and dried yeast extract have no stimulatory effect. Furthermore they indicate that sulfadiazine and sulfathiazole have the expected antibacterial effect, which is counteracted in stoichiometric proportion by para-aminobenzoic acid. From the same data it can be seen that relatively large amounts of liver extract, yeast extract, or folic acid have no significant antagonism to the drugs used. The effect of liver extract at large concentration is considered to be due to artefacts of color and turbidity caused by the liver extract itself and to a nonspecific stimulation, since the controls without drug show stimulatory growth effect at the same

![Graph](image-url)
concentration, and even at these high concentrations of supplements definite bacteriostasis due to the sulfonamide drugs occurs.

Thus these data confirm those of Lampen and Jones and extend it to include such natural sources of folic acid as are quite devoid of 'effective' para-aminobenzoic acid.*

**DISCUSSION**

The blood dyscrasias due to the soluble (or absorbable) sulfonamides have received very little attention from the experimental point of view, at least in comparison with the attention given the insoluble sulfonamides. This is understandable in view of the value of these latter drugs in producing clear-cut nutritional deficiencies in animals not otherwise susceptible to them, and in view of a rather simple explanation of the action of these insoluble intestinal bacteriostatic agents, namely, the interference with alimentary synthesis of vitamins by bacteria. However, the action of the soluble drugs and especially their toxic action is of the highest importance, since these are the ones most widely used in medicine.

Although it has been shown15, 16, 17 that significant amounts of the soluble sulfonamides remain in the gut, especially on prolonged administration, to cause some bacteriostasis, the work of Light et al.13 in rats seems to show that appreciable if not adequate synthesis of folic acid occurs in rats fed 0.5 per cent sulfadiazine or sulfanilamide in a purified diet. The same authors showed that this was not true for rats fed 0.5 per cent sulfaguanidine in a similar diet. The beneficial action of folic acid, liver extract, and dried yeast extract under our conditions may be due to the fact that the folic acid not synthesized in the gut is supplied in the diet, but some unpublished data seem to indicate that the soluble sulfonamides may actually raise the animals' requirement for folic acid.

The action of para-aminobenzoic acid obviously is due to antagonism of the sulfonamide drugs. The fact that 2 mg. of PABA† daily, which was probably adequate for completely blocking sulfonamide action, was never as beneficial as the other supplements, regardless of the drug used, lends some credence to the assumption that folic acid may be required in larger than normal amounts for tissue metabolism or for normal bone marrow integrity in the presence of soluble sulfonamide drugs.

Our data tend to confirm the observations of Kornberg, Daft, and Sebrell19 that the severity of the dyscrasias due to sulfathiazole and sulfadiazine is greater and less easily prevented than that due to sulfanilamide. Mortality is also greater with sulfathiazole than it is with the other drugs in the absence of nutritional supplements.

These workers showed that liver extract and brewers' yeast had a favorable effect on the dyscrasias. Our work shows that the beneficial effects are almost

* It is not known whether the polypeptides containing PABA have an antagonistic action to sulfonamides.

† It is estimated that 2 mg. of PABA daily maintained the ratio of PABA: sulfonamide in the animal's food at 1:50, which is much higher than the in vitro ratio required for inhibition of sulfonamide bacteriostasis.
entirely due to the addition of folic acid, either free or conjugated, to the diets. It is interesting to note that no significant difference between free and conjugated folic acid (as dried yeast extract and in liver powder) could be found. There is some indication in our data that liver extract powder is more beneficial prophylactically against sulfadiazine and sulfathiazole than can be accounted for on the basis of its folic acid content. This effect is most noticeable with sulfathiazole in reducing mortality, in growth response, and in preventing leukopenia.

The anemia tends to be hypochromic in the case of all the soluble sulfonamides under the conditions used, which is similar to that produced by Higgins in rats fed promin and promizole. Higgins showed that on a diet similar to the one used here the effects of promin and promizole could be prevented and alleviated with vitamin B.

The value of para-aminobenzoic acid in the therapy is nil, since it is contraindicated during administration of sulfonamide drugs of any kind. This fact has led clinicians to be suspicious of all B vitamins as possibly interfering with sulfonamide drug action. The work of Lampen and Jones and the data reported here on the difference between the in vitro action of para-aminobenzoic acid and sources of folic acid indicate that B vitamins may well be found to be valuable during sulfonamide therapy.

It seems that the quantities of folic acid even in the form of liver extract powder or dried yeast extract needed to prevent or lessen the adverse effects of soluble sulfonamides on the blood picture do not interfere with the bacteriostatic action of these drugs. In fact, the recent reports of Wood et al. which show the importance of blood phagocytes in supplementing the bacteriostatic action of sulfapyridine in experimental pneumonia, seem to permit the extrapolation that folic acid or liver extracts and adequate B vitamin supplementation may actually be beneficial in augmenting soluble sulfonamide bacteriostasis by keeping the circulating granulocyte concentration at a high level. (Para-aminobenzoic acid is not considered to be an essential B vitamin for animals per se and hence is not included in B vitamin requirement.)

The work of Wood and co-workers has shown that in experimental pneumonia sulfapyridine acts only bacteriostatically and that the bacterial invasion is overcome by normal surface phagocytosis. Furthermore, the maintenance of a high concentration of leukocytes and granulocytes during soluble sulfonamide therapy seems to be desirable as a preventive measure against the possible bacterial invasion not readily combated with the sulfonamide being administered.

**Summary**

Sulfanilamide, sulfathiazole, and sulfadiazine have been fed at 1 per cent levels in highly purified diets and their effect on growth, mortality, and blood dyscrasias compared with that of sulfasuxidine.

The soluble drugs produce conditions which are similar to those produced by sulfasuxidine. The growth depression is alleviated in large measure in the case of

*From the in vitro work shown in figures 1 and 3 it is estimated that the free PABA content of L. E. F. and D. Y. E. is less than 27 g/m.**
sulfanilamide and to a lesser extent for sulfathiazole and sulfadiazine by folic acid, liver extract powder, and dried yeast extract as well as by para-aminobenzoic acid.

The blood dyscrasias due to sulfanilamide, sulfathiazole, and sulfadiazine are severe leukopenia, granulocytopenia, and mild-to-severe anemia. These are uniformly prevented or the severity greatly curtailed by feeding folic acid, liver extract powder, or dried yeast extract. PABA has a lesser effect in the amounts fed.

Liver extract powder seems to have a beneficial effect on growth and mortality which is not shown by the other supplements. Both free and conjugated folic acid (as yeast extract and in liver extract powder 1:20) are active in combating the dyscrasias.

Evidence from in vitro experiments with Str. haemolyticus (B Lancefield) indicates that neither folic acid, liver extract powder, nor dried yeast extract in ratios to sulfonamide which are effective in preventing the blood dyscrasias will inhibit or block the bacteriostatic action of the sulfonamide drugs in vitro.

It is suggested that the action of folic acid, liver powder, and yeast extract is not wholly explained by alleviating a folic acid deficiency caused by intestinal bacteriostasis due to the drugs, but by an increased demand of the animals for folic acid in the presence of certain sulfonamides.

ACKNOWLEDGMENTS

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REFERENCES

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