# The Metabolic Effects of 4-Aminopteroylglutamic Acid in the Guinea-Pig*

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(*Received* 4 *July* 1950)

The announcement of the synthesis of pteroylglutamic acid by Angier, Boothe, Hutchings, Mowat, Semb, Stokstad, SubbaRow, Waller, Cosulich, Fahrenbach, Hultquist, Kuh, Northey, Seeger, Sickels & Smith (1945) and reports of its effectiveness in the treatment of the haematological changes of pernicious anaemia were followed by the production of numerous synthetic analogues and derivatives, some of which were shown to be of value in the treatment of leukaemia and allied disorders. The synthesis of a potent substance of this nature, 4-aminopteroylglutamic acid (aminopterin), was announced by Seeger, Smith & Hultquist (1947).

Attempts to produce anaemia in animals by inducing folic-acid deficiency in various ways have been reviewed by Girdwood (1950), who refers to the use of 4-aminopteroylglutamic acid for this purpose in mice, rats, chicks, dogs and guinea-pigs. The reports suggest that in animals other than the guinea-pig the substance produces anaemia and leucopenia, and that these changes can be reversed with difficulty by high dosage of pteroylglutamic acid. Minnich & Moore (1948) produced a hypoplastic anaemia and agranulocytosis in guinea-pigs using 4-aminopteroylglutamic acid, but these changes could not be prevented by the simultaneous administration of liver extract or of pteroylglutamic acid.

The main purpose of the present investigation was to explore further the possibility of reversing the haematological changes induced by 4-aminopteroylglutamic acid in the guinea-pig, and to assay the livers of the animals for their content of pteroylglutamic acid and of vitamin $B_{12}$.

## EXPERIMENTAL

*Methods employed.* Various tests were carried out on groups of at least five male guinea-pigs of about equal weight. Each test was controlled by the use of a group of untreated animals. All animals, including the controls, received 20 mg. ascorbic acid daily in solution, given by intraperitoneal injection. When 4-aminopteroylglutamic acid was administered it was given by intraperitoneal injection. All other injections were given intraperitoneally, with the exception of injections of *p*-aminobenzoic acid which were given into the subcutaneous tissues at the back of the neck. The animals were kept in separate cages with wire-grid bottoms, and were given unlimited amounts of a commercial diet containing a negligible quantity of folic acid.

* This work was carried out at the Thomas Henry Simpson Memorial Institute for Medical Research, University of Michigan, Ann Arbor, Michigan, during the period of tenure of a Rockefeller Travelling Research Fellowship.

*Haematological observations.* Cardiac puncture was used to obtain 0·3 ml. of blood, which was then placed in a small tube containing a measured amount of a dry mixture of ammonium and potassium oxalate. White blood-cell counts and red blood-cell counts were done in duplicate, standardized pipettes being used. Haemoglobin estimations were carried out in a Klett-Summerson photoelectric instrument. Haematocrit determinations were made in Van Allen tubes, which had been centrifuged at 3000 r.p.m. for 30 min. Peripheral blood examinations were carried out on cover-slip films that had been stained with Wright's stain. Differential counts were made on 100 cells. No bone-marrow studies were carried out on living animals, but, when the animal died or had been killed with ether, cover-slip films of the marrow were made and stained with Wright's stain.

*Vitamin assays.* Assays of pteroylglutamic acid were carried out by the method of Teply & Elvehjem (1945), using *Streptococcus faecalis* R. as the test organism and measuring the growth of the organism by turbidity estimations. The livers of the animals were homogenized in a Waring Blendor in a phosphate buffer mixture without enzyme preparations at pH 7·5. The homogenized material was then incubated for 24 hr. and kept in a refrigerator at $-10°$ until tested. Repeat assays showed that storage in this way did not affect the amount in the samples either of growth factors for *Strep. faecalis* R. or for *Lactobacillus leichmannii*. Watery extracts of stools were used for assays of pteroylglutamic acid and vitamin $B_{12}$.

Vitamin $B_{12}$ assays were done by a modification of the method of Skeggs, Huff, Wright & Bosshardt (1948), using *Lb. leichmannii* 313 as the test organism. This test is less satisfactory than that for pteroylglutamic acid in that vitamin $B_{12}$ is only one of several factors that can replace each other in the growth of the organism (Kitay, McNutt & Snell, 1949; Shaw, 1949). The time of autoclaving was always exactly 7 min., and the pressure 15 lb. There was a gap of 24 hr. between the last injection of 4-aminopteroylglutamic acid, or other substances, and the killing of animals for these assay procedures.

## RESULTS

### *The production of anaemia with 4-aminopteroylglutamic acid. Failure of attempts to prevent or reverse its action by administration of folic acid, liver, thymine or* p-*aminobenzoic acid*

#### *Effects of 4-aminopteroylglutamic acid alone*

Fifteen animals of about 550 g. weight received 0·25 mg. 4-aminopteroylglutamic acid daily for 4 weeks. Anaemia was evident at the end of the 1st week, and after 4 weeks the mean red-cell count had fallen from 5,320,000 to 4,070,000/cu.mm. The haemoglobin level fell from 14·1 to 11·1 g./100 ml., and the mean corpuscular volume rose from 84·6 to 92·2 cu.$\mu$. Leucopenia and thrombocytopenia did not develop. A low, continued reticulocytosis was seen (usually 2–7 %); this commenced about the 3rd week and was accompanied by the appearance of a few normoblasts in the peripheral blood.

When these animals were killed, the bone marrow was found to be cellular. It contained an increase of haemocytoblasts, proerythroblasts and early normoblasts,

but no cells that appeared to be comparable to the early, intermediate or late megaloblasts in man. As in experiments described by Innes, Innes & Moore (1949), an increase of reticulum cells was seen, and early forms of the white-cell series were numerous. The general condition of the animals remained good, and no marked naked eye or microscopic changes were found in the viscera.

A further five animals of 431 g. mean weight were given 0·25 mg. 4-aminopteroylglutamic acid daily for 7 weeks. The anaemia continued and, although it varied in degree from week to week, the mean red-cell count at 7 weeks was 4,280,000/cu.mm. and at no time was it less than 3,900,000/cu.mm.

*Effects of various supplements*

Six groups of guinea-pigs of about 550 g. weight received 0·25 mg. 4-aminopteroylglutamic acid daily with the following supplements.

*Pteroylglutamic acid.* Ten animals were given daily intraperitoneally 2·5 mg., and five 5·0 mg. pteroylglutamic acid.

*Thymine.* Five animals received approximately 0·45 g. thymine daily, some of it by mouth, some by means of a stomach tube, and the rest of it as an addition to the food.

p-*Aminobenzoic acid.* Five animals were given daily subcutaneously 2 ml. of a 10 % solution of a mixed sodium and potassium salt of *p*-aminobenzoic acid and had the same salt added as a 2 % supplement to the diet.

*Liver injections.* Five animals received daily intraperitoneally 4 U.S.P. units of a crude liver extract.

*Liver by mouth.* Five animals had a liver preparation in powder form added to the diet in the proportion of 5 %.

As compared with guinea-pigs receiving 4-aminopteroylglutamic acid alone, none of these groups of animals showed any significant alteration in the extent of the anaemia, in the bone-marrow changes or in general condition.

*Effects of increasing the amounts of 4-aminopteroylglutamic acid and of pteroylglutamic acid*

Five animals of 518 g. mean weight were given daily 0·5 mg. 4-aminopteroylglutamic acid and also 5 mg. pteroylglutamic acid intraperitoneally. These guinea-pigs became severely anaemic, the mean red-cell count falling at the end of the 5th week from 5,410,000 to 2,770,000/cu.mm. and the haemoglobin from 13·2 to 7·6 g./100 ml. The animals became lethargic and their hair roughened, but growth continued, although less satisfactorily than in the controls. The polymorphonuclear leucocyte count fell from 4950 to 630/cu.mm. at the end of the 1st week, but rose again to 1250/cu.mm. at the end of 5 weeks.

Five animals that received daily 50 mg. pteroylglutamic acid together with 0·5 mg. 4-aminopteroylglutamic acid, however, became very ill. They rapidly became emaciated, lethargic, and dehydrated. Four of the animals died, at 9, 11, 14 and 21 days, respectively, after the commencement of the injections. The urines contained much

albumin, but no crystals. At autopsy the livers showed marked passive congestion and degenerative fatty infiltration. The renal tubules of three animals contained eosinophilic material infiltrated with polymorphonuclear leucocytes. The bone marrow was cellular and did not differ from that of the animals previously described. Reticulocytosis did not occur.

Little stress can be laid on the blood counts in these guinea-pigs, owing to their very bad general condition and the fact that they became unable to drink water or to eat.

Four animals were given 50 mg. pteroylglutamic acid alone by injection. Some loss of weight occurred, but the animals did not become severely ill like those just described.

*Effects of succinylsulphathiazole in addition to 4-aminopteroylglutamic acid*

Further experiments were carried out to assess the effect of adding succinylsulphathiazole in powder form to the diet in the proportion of 2 %.

Succinylsulphathiazole alone was administered to eight animals for a period of 5 weeks and did not produce anaemia or other adverse effect.

Five animals were made anaemic by giving 0·25 mg. 4-aminopteroylglutamic acid daily for 5 weeks and, when there appeared to be some recovery from this anaemia, succinylsulphathiazole (2 %) was added to the diet. The reason for the recovery is unexplained, but the phenonemon has been noted by several workers using guinea-pigs. As will be seen from Table 1, the anaemia became more severe.

Table 1. *Effects on blood picture of adding 2 % succinylsulphathiazole to the diet of guinea-pigs already receiving 0·25 mg. 4-aminopteroylglutamic acid daily*

| Time (weeks) | Weight (g.)* | Haemoglobin (g./100 ml.) | Red blood cells (millions/cu.mm.) | White blood cells (/cu.mm.) | Polymorphs (/cu.mm.) | Packed cell volume (%) | Mean corpuscular volume (cu.μ.) |
|---|---|---|---|---|---|---|---|
| 4-Aminopteroylglutamic acid given alone | | | | | | | |
| 0 | 576 (5) | 13·0 | 5·04 | 6030 | 1780 | 41·8 | 83·1 |
| 1 | 581 (5) | 12·1 | 4·30 | 5730 | 1780 | 37·9 | 88·5 |
| 2 | 590 (5) | 11·1 | 4·06 | 5670 | 1910 | 36·8 | 91·6 |
| 3 | 555 (5) | 10·7 | 3·96 | 4010 | 1240 | 35·1 | 89·3 |
| 4 | 540 (5) | 8·7 | 3·32 | 2450 | 560 | 30·2 | 91·6 |
| 5 | 543 (5) | 9·8 | 3·51 | 4310 | 1350 | 31·3 | 90·4 |
| Succinylsulphathiazole added to diet | | | | | | | |
| 6 | 497 (5) | 8·4 | 3·17 | 2810 | 1150 | 28·8 | 91·8 |
| 7 | 484 (4) | 7·5 | 2·46 | 5180 | 2260 | 26·0 | 106·8 |
| 8 | 590 (3) | 7·1 | 2·45 | 8530 | 4280 | 26·8 | 108·6 |
| 9 | 599 (3) | 7·5 | 2·85 | 7010 | 2730 | 28·0 | 100·8 |
| 10 | 627 (3) | 8·1 | 3·23 | 7350 | 2930 | 30·8 | 97·1 |
| 11 | 629 (3) | 8·6 | 3·38 | 6280 | 3600 | 31·2 | 96·2 |
| 12 | 645 (3) | 6·9 | 2·40 | 3820 | 1330 | 25·6 | 109·6 |

* Figures in parentheses indicate the number of animals alive at this time.

It is unfortunate that the animals selected for this study had lost weight before succinylsulphathiazole had been added to the diet, but they appeared to be recovering from this weight loss before the addition was made. The animals did not appear to be

unhealthy and there was no evidence of intestinal or other infection. Accordingly, the experiment was continued as planned.

Within a few days of the addition of the succinylsuphathiazole the animals, which had previously appeared to be healthy, became lethargic, refused to eat, and began to lose weight rapidly. The hair became rough and marked generalized oedema developed. There was individual variation in the response, however. One died in 7 days and another in 14 days.

Severe anaemia developed in all five animals, the red-cell count falling below 2,000,000/cu.mm. at some stage in the three animals that survived the experiment. Two animals then showed definite haematological improvement, but this was not maintained. The three animals that survived the first general effects of the addition of succinylsulphathiazole to their diet, while the administration of 4-aminopteroylglutamic acid was continued, then showed marked improvement, becoming active again, eating well, gaining weight, and looking like normal animals. One such animal had a fall in the red-cell count to 790,000/cu.mm., but only the pallor of the mucous membranes indicated that the animal was abnormal. This was in marked contrast to the condition of animals given larger doses of 4-aminopteroylglutamic acid in an effort to produce severe anaemia with it alone. There was no obvious relationship between the blood-cell counts and the general condition. The serum was not bile stained even when the anaemia was severe.

Improvement in the blood counts of these animals, when it occurred, was temporary, as has already been stated. Such improvement was preceded by a reticulocytosis and the appearance of normoblasts in the peripheral blood. Before such a development, the blood of these guinea-pigs differed from that of the previous groups in that a reticulocytosis did not accompany the anaemia. In one animal, the red-cell count was 1,815,000/cu.mm., and the haemoglobin 5·1 g./100 ml. 28 days after the commencement of the administration of succinylsulphathiazole. The reticulocyte count suddenly rose at this point from 0·4 to 38 %, and normoblasts appeared to the extent of 165/100 white cells. The serum was not bile stained. Seven days later, the red-cell count was 3,170,000/cu.mm., and the haemoglobin 6·9 g./100 ml. When the anaemia was most severe in all these animals, the polymorphonuclear leucocytes showed signs of toxic action. It will be seen that the mean corpuscular volume reached a higher value in this group than in any other animals.

Examination after death, or after the animals had been killed, showed that the marrows were cellular and contained an increase in megakaryocytes. Haemocytoblasts, proerythroblasts and early normoblasts were again seen. In addition, however, the most anaemic animal had cells that did not appear to belong to the normal red-cell series but showed some similarity to the early, intermediate and late megaloblasts found in man. The cells resembling the intermediate and late megaloblasts were very few in number. The former cells had a more open-work nucleus than the intermediate normoblast in the normal animal, but this nucleus was less oval than in the intermediate megaloblast in man. Late normoblasts were also very few, but red cells in the early stages of development were very numerous. Myeloblasts, too, were increased in number, and there were very many reticulum cells.

*Protection by pteroylglutamic acid*

This last experiment was repeated, but 2·5 mg. pteroylglutamic acid were given daily intraperitoneally in addition to 0·25 mg. 4-aminopteroylglutamic acid.

It will be seen from Table 2 that worsening of the anaemia did not occur when succinylsulphathiazole was added to the diet of this group of guinea-pigs. The general appearance of the animals at the end of the experiment was similar to that of guinea-pigs that received only 0·25 mg. 4-aminopteroylglutamic acid daily without succinylsulphathiazole.

Table 2. *Effects on blood picture of adding* 2 % *succinylsulphathiazole to the diet of five guinea-pigs already receiving* 0·25 *mg. 4-aminopteroylglutamic acid and* 2·5 *mg. pteroylglutamic acid daily. (The administration of the last two was continued throughout)*

| Time (weeks) | Weight (g.) | Haemoglobin (g./100 ml.) | Red blood cells (millions/cu.mm.) | White blood cells (/cu.mm.) | Polymorphs (/cu.mm.) | Packed cell volume (%) | Mean corpuscular volume (cu.μ.) |
|---|---|---|---|---|---|---|---|
| 4-Aminopteroylglutamic acid and pteroylglutamic acid given | | | | | | | |
| 0 | 426 | 13·4 | 5·28 | 8360 | 4620 | 46·0 | 87·6 |
| 1 | 443 | 11·3 | 4·43 | 6960 | 3880 | 39·3 | 91·0 |
| 2 | 476 | 11·6 | 4·24 | 5370 | 2760 | 39·1 | 95·4 |
| 3 | 494 | 10·7 | 4·27 | 5440 | 2510 | 37·9 | 89·6 |
| 4 | 536 | 10·3 | 3·87 | 4190 | 1400 | 33·6 | 86·8 |
| 5 | 566 | 10·1 | 4·26 | 7160 | 3710 | 35·4 | 83·6 |
| Succinylsulphathiazole added to diet | | | | | | | |
| 6 | 544 | 9·7 | 3·95 | 4840 | 940 | 33·7 | 85·6 |
| 7 | 589 | 10·1 | 3·99 | 3670 | 1240 | 31·9 | 80·6 |
| 8 | 608 | 10·0 | 4·08 | 5910 | 2220 | 32·5 | 80·0 |

In a further experiment, summarized in Table 3, two groups of five animals were given 0·25 mg. 4-aminopteroylglutamic acid and 2 % succinylsulphathiazole in the diet from the commencement. One of the groups received, in addition, 5 mg. pteroylglutamic acid daily. Again the effect of succinylsulphathiazole in increasing the anaemia was overcome by pteroylglutamic acid. At the end of 7 weeks the magnitude and character of the anaemia was similar to that in animals given 4-aminopteroylglutamic acid alone.

As before, there were individual variations in response to administration of succinylsulphathiazole and 4-aminopteroylglutamic acid alone. At the end of 7 weeks, three animals with low reticulocyte counts had red-cell values of less than 1,500,000/cu.mm., whereas another guinea-pig of similar weight had a count of 4,205,000/cu.mm. associated with a reticulocytosis of 23·5 %. The general features and bone-marrow changes were as before. The two most anaemic animals received daily by injection 5 mg. pteroylglutamic acid at the end of the 7th week. This was followed by reticulocytosis and a rise in the red-cell count.

*Pteroylglutamic acid and vitamin $B_{12}$ content of the livers and stools*

The results are shown in Tables 4 and 5. The results of the vitamin $B_{12}$ estimation showed a wide scatter, but the livers of the control animals, and of those receiving

Table 3. *Effects on blood picture of administration to groups of five guinea-pigs of* 0·25 *mg. 4-aminopteroylglutamic acid daily together with succinylsulphathiazole with and without pteroylglutamic acid*

| Time (weeks) | Weight (g.) | Haemoglobin (g./100 ml.) | Red blood cells (millions/cu.mm.) | White blood cells (/cu.mm.) | Polymorphs (/cu.mm.) | Packed cell volume (%) | Mean corpuscular volume (cu.$\mu$.) |
|---|---|---|---|---|---|---|---|
| | | | 4-Aminopteroylglutamic acid given | | | | |
| 0 | 408 | 13·5 | 5·32 | 8920 | 5030 | 46·3 | 87·3 |
| 1 | 432 | 12·9 | 4·61 | 7500 | 4230 | 38·8 | 84·8 |
| 2 | 446 | 9·1 | 4·26 | 5860 | 2620 | 39·1 | 92·2 |
| 3 | 474 | 10·9 | 4·21 | 5550 | 3100 | 36·8 | 91·1 |
| 4 | 487 | 10·4 | 4·24 | 7230 | 2100 | 37·1 | 88·5 |
| 5 | 518 | 11·2 | 4·51 | 8430 | 4500 | 39·3 | 80·7 |
| 6 | 522 | 10·2 | 3·72 | 4710 | 2530 | 33·2 | 88·7 |
| 7 | 529 | 9·9 | 4·14 | 4880 | 2870 | 35·3 | 86·1 |
| | | | 4-Aminopteroylglutamic acid and succinylsulphathiazole given | | | | |
| 0 | 385 | 12·2 | 4·95 | 6970 | 3550 | 44·3 | 89·7 |
| 1 | 408 | 12·0 | 4·52 | 5640 | 2870 | 40·9 | 91·1 |
| 2 | 393 | 11·3 | 4·38 | 5840 | 1750 | 38·0 | 87·0 |
| 3 | 410 | 8·9 | 3·15 | 5860 | 2060 | 30·8 | 100·6 |
| 4 | 435 | 8·3 | 3·01 | 4420 | 1440 | 26·6 | 89·8 |
| 5 | 451 | 7·0 | 2·49 | 3960 | 600 | 23·5 | 95·7 |
| 6 | 473 | 6·8 | 2·34 | 3010 | 890 | 23·3 | 100·2 |
| 7 | 462 | 6·4 | 2·10 | 2450 | 490 | 21·3 | 101·6 |
| | | | 4-Aminopteroylglutamic acid, pteroylglutamic acid and succinylsulphathiazole given | | | | |
| 0 | 385 | 12·7 | 5·11 | 5900 | 2340 | 42·9 | 84·1 |
| 1 | 389 | 11·2 | 4·28 | 4330 | 2060 | 37·9 | 89·9 |
| 2 | 395 | 10·6 | 3·92 | 5670 | 1830 | 35·0 | 89·7 |
| 3 | 399 | 10·1 | 3·67 | 4350 | 1170 | 33·9 | 93·7 |
| 4 | 408 | 10·1 | 3·44 | 6870 | 3100 | 32·4 | 95·1 |
| 5 | 437 | 9·5 | 3·43 | 4480 | 1150 | 32·1 | 95·1 |
| 6 | 469 | 9·7 | 3·51 | 5160 | 1760 | 33·6 | 96·9 |
| 7 | 486 | 9·1 | 3·78 | 5260 | 1650 | 31·9 | 82·5 |

Table 4. *Liver content of growth factors for* Strep. faecalis *and* Lb. leichmannii *in various groups of guinea-pigs*

| Group | No. of animals | *Strep. faecalis* growth factors as pteroylglutamic acid ($\mu$g./g.) Mean | Range | *Lb. leichmannii* growth factors as vitamin $B_{12}$ ($\mu$g./g.) Mean | Range |
|---|---|---|---|---|---|
| Normal controls | 5 | 8·0 | (6·8–11·5) | 0·16 | (0·08–0·31) |
| Receiving 0·25 mg. 4-aminopteroylglutamic acid daily | 10 | 8·4 | (4·8–12·5) | 0·34 | (0·14–0·76) |
| Receiving 0·25 mg. 4-aminopteroylglutamic acid and 0·45 gm. thymine daily | 5 | 16·0 | (4·2–26·7) | 0·34 | (0·24–0·43) |
| Receiving 0·25 mg. 4-aminopteroylglutamic acid and 2·5 mg. pteroylglutamic acid daily | 5 | 18·6 | (13·5–28·6) | 0·49 | (0·25–0·77) |
| Receiving 0·25 mg. 4-aminopteroylglutamic acid and 2 % succinylsulphathiazole daily | 5 | 18·8 | (13·6–26·5) | 0·33 | (0·19–0·48) |

Table 5. *Stool content of growth factors for* Strep. faecalis *and* Lb. leichmannii *in various groups of guinea-pigs*

| Group | No. of animals | *Strep. faecalis* growth factors as pteroylglutamic acid (μg./g.) Mean | Range | *Lb. leichmannii* growth factors as vitamin $B_{12}$ (μg./g.) Mean | Range |
|---|---|---|---|---|---|
| Normal controls | 4 | 13·9 | (10·5–15·8) | 0·44 | (0·41–0·47) |
| Receiving 0·25 mg. 4-aminopteroylglutamic acid daily | 4 | 10·7 | (7·0–12·0) | 0·60 | (0·48–0·70) |
| Receiving 0·25 mg. 4-aminopteroylglutamic acid and succinylsulphathiazole daily | 4 | 3·9 | (3·0–4·9) | 0·54 | (0·45–0·61) |

4-aminopteroylglutamic acid, contained less pteroylglutamic acid than the livers of the animals that had also been given pteroylglutamic acid, thymine, or succinylsulphathiazole.

As might be expected, this last group of guinea-pigs had less pteroylglutamic acid in the stools than the controls.

## DISCUSSION

In guinea-pigs of suitable weight 0·25 mg. 4-aminopteroylglutamic acid daily caused moderate anaemia of macrocytic type with a cellular marrow that showed an increase in early red-cell types. The effects were not prevented by pteroylglutamic acid, thymine, *p*-aminobenzoic acid, or by liver preparations employed in the manner described.

The giving of succinylsulphathiazole in addition to 4-aminopteroylglutamic acid caused a more severe macrocytic anaemia with leucopenia and the appearance of cells in the marrow not unlike those seen in pernicious anaemia of man. Moreover, it was possible by this means to produce very severe anaemia in some of the animals without the development of toxic manifestations. This aggravation of the anaemia was prevented by giving pteroylglutamic acid, the degree of anaemia then being similar to that seen in the guinea-pigs given 4-aminopteroylglutamic acid without succinylsulphathiazole.

Other workers, using different animals, have shown that large amounts of pteroylglutamic acid are required to reverse the anaemia-producing effects of 4-aminopteroylglutamic acid (Seeger *et al.* 1947; Franklin, Stokstad & Jukes, 1948). It is of interest that in the present experiment death occurred when 50 mg. pteroylglutamic acid were given to a group of guinea-pigs in addition to 0·5 mg. 4-aminopteroylglutamic acid. It may be that it is not possible to administer sufficient pteroylglutamic acid to guinea-pigs to prevent the effects of 4-aminopteroylglutamic acid. There is also the possibility that 4-aminopteroylglutamic acid has, in this animal, an effect that is not due to its action as a folic-acid antagonist.

It is easy to speculate on the reason for the partial reversal of the effects of 4-aminopteroylglutamic acid given with succinylsulphathiazole by means of pteroylglutamic acid. Such speculation would serve little purpose while we lack sufficient information about the function of the *Leuconostoc citrovorum* factor (Sauberlich & Baumann, 1948; Nichol & Welch, 1950). It is usually thought that succinylsulphathiazole acts by killing bacteria able to synthesize pteroylglutamic acid. Reports on the ability of

certain micro-organisms to carry out such a synthesis have been given by Hutchings, Bohonos & Peterson (1941), Mitchell & Isbell (1942) and Thompson (1942).

In this instance, however, certain findings are not easily explained on the assumption that the effect of succinylsulphathiazole was due to such an action on intestinal bacteria, and that 4-aminopteroylglutamic acid and succinylsulphathiazole act by producing folic-acid deficiency in different ways. It has been shown by Minnich & Moore (1948) that large doses of 4-aminopteroylglutamic acid given for a relatively long period will produce anaemia. When this drug alone is used to produce severe anaemia, however, the marrow is hypoplastic, not cellular as in the present series of experiments. Moreover, as we have seen, it is possible to produce severe anaemia using 4-aminopteroylglutamic acid and succinylsulphathiazole without inducing general toxic effects, a result not found when 4-aminopteroylglutamic acid alone is used. The application of this observation to the treatment of leukaemia in man is at present being investigated.

It is not surprising that the liver content of pteroylglutamic acid was raised in animals receiving pteroylglutamic acid, or thymine which is also a growth factor for *Strep. faecalis*. On the other hand, the rise in the liver content of pteroylglutamic acid following the addition of succinylsulphathiazole to the diet is not easily explained. Further experiments of this nature, with larger groups of animals, are required.

We have seen that 5 mg. pteroylglutamic acid will not counteract the anaemia-producing effect of 0·25 mg. of the antagonist. Yet, since the animals in this experiment passed approximately 2·75 g. of stool in 24 hr., the controls passed 0·038 mg. of pteroylglutamic acid in this time, and succinylsulphathiazole eliminated 0·027 mg. from the stool over the same period. If the stool content of pteroylglutamic acid gives any indication of the amount being absorbed from the alimentary tract, it is surprising that amounts of this magnitude can so effectively prevent 0·25 mg. 4-aminopteroylglutamic acid from lowering the red-cell level below 4,000,000/cu.mm.

## SUMMARY

1. Small groups of male guinea-pigs of about equal weight developed a mild macrocytic anaemia when they were given 0·25 mg. 4-aminopteroylglutamic acid daily. This action was not prevented by simultaneous administration of pteroylglutamic acid in quantities ten or twenty times as great, or by giving thymine, *p*-aminobenzoic acid or liver by mouth or injection.

2. The addition of succinylsulphathiazole to the diet in the proportion of 2 % resulted in a more severe form of macrocytic anaemia when 4-aminopteroylglutamic acid was given simultaneously in the above dosage. This aggravation of the anaemia was prevented by giving 2·5 mg. pteroylglutamic acid daily.

3. The guinea-pigs receiving pteroylglutamic acid, thymine or succinylsulphathiazole in addition to 4-aminopteroylglutamic acid had a higher liver content of pteroylglutamic acid than those receiving 4-aminopteroylglutamic acid alone. The stools of the animals receiving succinylsulphathiazole had a lower content of pteroylglutamic acid.

4. Reasons are given for doubting that 4-aminopteroylglutamic acid and succinylsulphathiazole act merely by producing pteroylglutamic-acid deficiency in different ways.

The author wishes to thank Dr C. C. Sturgis and Dr F. H. Bethell for the facilities granted for the carrying out of this investigation and for their interest and advice, and to thank Dr M. E. Swendseid for instruction in the technique of microbiological assay work. He is also indebted to Dr S. M. Hardy of the Lederle Laboratories Division of the American Cyanamid Company for generous supplies of Folvite and aminopterin.

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