TABLE I. Rabbits Immunized with DE Rabies Vaccine and Challenged Intracerebrally with Street Virus.

| Intracer. challenge dose of 0.25 ml of street virus diluted: | Rabbits immunized with DE rabies vaccine: Antibody titer | Result | Normal rabbits: Result | |
|---|---|---|---|---|
| 1:200 | 85 | Rabies, day 17 | Rabies, day | 15 |
| | 48 | S | *Idem* | 15 |
| 1:400 | 25 | S | " | 17 |
| | 35 | S | " | 15 |
| 1:800 | 14 | Rabies, day 19 | " | 15 |
| | 26 | S | " | 15 |
| 1:1600 | 6 | S | " | 12 |
| | 27 | S | " | 13 |
| 1:3200 | Not done | | " | 15 |
| | | | " | 15 |
| 1:6400 | *Idem* | | " | 15 |
| | | | S | |

Street virus used was mouse brain passage from a fatal human case. Antibody titers are reciprocals of dilutions of serum computed to save half the mice from 100 $LD_{50}$ of CVS virus/mouse.
S = surviving and normal at 30 days.

*Discussion.* Our DE vaccine had a "potency" (times better than N.I.H. standard vaccine No. 159A in mouse tests) of 1.27 when first made. Potency of a sample after heating 30 days at 37°C was 1.54, which indicates no decrease had taken place, and these figures are representative of the product (2). Degree of activity of this vaccine in rabbits is sufficient to cause these animals to resist from one to several fatal doses of street virus injected intracerebrally. This showing in light of Semple's results and those of many subsequent investigators using intracerebral street virus challenge is proof of direct capacity as an antirabies prophylactic. Human sera following use of DE and Semple vaccines respectively neutralize street virus as well as other fixed viruses in mouse tests. Titers of both kinds of sera, especially against street virus, are lower than when CVS fixed virus is used, however this would be anticipated by nearly all workers with these viruses. Such differences appear to be due to differences in sensitivity of virus to antibody.

TABLE II. Antibody Titers of Human Sera Measured against Various Rabies Viruses.

| Serum following use of DE and Semple vaccine | | Fixed virus strains: CVS | Phillips | Park | Street virus |
|---|---|---|---|---|---|
| (DE) | 1-31-59 | 1010 | | | 128 |
| " | 1-14 | 1350 | 730 | 560 | |
| " | 1-29 | 1600 | 790 | 670 | |
| (Semple) | 3-26 | 1650 | | 512 | 142 |
| " | 1-26 | 2450 | 850 | | |

The above sera were of sufficient volume to allow only the number of tests shown. Antibody titers are expressed as in Table I.

*Summary.* Treatment of rabbits with DE rabies vaccine causes them to withstand otherwise fatal doses of street virus administered intracerebrally. These results with 1 year old DE vaccine parallel Semple's original experience with freshy prepared phenolyzed vaccine. Furthermore, human serum following treatment with these vaccines neutralizes different fixed viruses and also street virus as tested in white mice.

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## Metabolism of Folic Acid in Folic Acid and Biotin Deficient Rat. (25102)

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In the course of a vitamin balance study with germ-free rats fed purified diets, Luckey *et al.*(1) observed that biotin administration to a rat reared on diet free of biotin and folic acid (pteroylglutamic acid, PGA) caused a 50-fold increase in PGA excretion over that

ingested each day. The observation, although limited to one rat, was interpreted as suggestive of tissue synthesis of PGA and its dependence on biotin. On the other hand, Halevy and Guggenheim(2) reported a study of PGA metabolism in certain vitamin deficiencies in the rat, that dietary biotin had no effect on hepatic levels of PGA and citrovorum factor ($N^5$-formyl tetrahydroPGA, CF) nor on conversion *in vivo* of PGA to CF. The present work was undertaken to study conditions under which biotin may influence PGA synthesis in the PGA-deficient rat.

*Materials and methods.* Young rats (Wistar Strain), of 40 g initial average weight were kept individually in raised-bottom wire-mesh cages on a purified diet deficient in biotin and PGA. The diet consisted of (in g/100 g): dried raw egg white(3) 10, hot alcohol-extracted casein 8, starch 60, sucrose (vitaminized) 10, salt mixture (U.S.P. No. IV) 4, sesame oil 4, shark liver oil 2 and succinyl sulphathiazole 2. Vitamin supplements, added through sucrose, provided (mg/kg diet): thiamine hydrochloride 4, riboflavin 5, niacin 10, calcium pantothenate 20, pyridoxine hydrochloride 5, menadione 3, inositol 500 and choline chloride 500. Sesame oil was fortified to give 10 mg $\alpha$-tocopherol/kg of diet. A comparison group of PGA-deficient animals received a similar diet with devitaminized casein replacing egg white and with biotin included in the vitamin supplement at a level of 1 mg/kg diet. A group, serving as control, received supplements of biotin (1 mg/kg) and PGA (3 mg/kg) in the latter (18% casein) diet. After 8 weeks feeding, half of doubly deficient animals were each administered 100 $\mu$g of biotin intraperitoneally and placed on PGA-deficient casein diet for one week. Urine and feces were collected daily according to usual procedures during this (ninth) week for animals of the 3 deficient groups and food intake data were also recorded. All animals were sacrificed at end of 9 weeks under light ether anesthesia. Blood was obtained from portal vein and collected in citrated vials. Liver, spleen and kidney were excised with usual precautions and assayed for PGA and CF activities. The samples were homogenized in 10 volumes of ice-cold distilled water, aliquots taken in 0.1 M phosphate buffer, pH 7.4, and autolysed at 37°C for 18 hours under toluene. They were then steamed and centrifuged. PGA activity in supernatants was determined using modified medium of Mitbander and Sreenivasan(4) with synthetic PGA (Lederle) as standard and *Streptococcus faecalis* R as test organism. For CF activity determinations, the medium of Sauberlich and Baumann(5) was employed with Leucovorin (Lederle) as standard and *Leuconostoc citrovorum* (*Pediococcus cerevisiae*) as test organism. Erythrocyte and leucocyte count in blood samples were arrived at by standard hematological procedures and hemoglobin determined by the acid-hematin method. All results reported are averages of independent determinations from at least 4 animals in each group. Aliquots from urine and homogenized feces, collected for 7-day period, were autoclaved at 10 lb for 10 minutes after addition of ascorbic acid (10 mg/ml) which served to stabilize the labile reduced folate derivatives known to be excreted(6,7). Samples were then centrifuged and assayed for PGA. Amounts of PGA ingested were determined from data for food intake for each group and PGA analyses of diets fed. The results reported are daily averages determined from pooled samples collected during the week.

*Results.* Animals in the biotin and PGA-deficient group increased in weight from 40 to 140 g (averages) during 8 weeks, while PGA-deficient rats and the control group receiving all vitamins, had increased, respectively, to 170 g and 240 g (averages). Characteristic symptoms of biotin deficiency, such as alopecia and spectacle-eyes were also manifest in the former group. In ninth week of growth, deficient animals receiving biotin had increased in weight by an average of 14 g as compared to 2 g for biotin and PGA-deficient animals, 8 g for PGA-deficient animals and 12 g for control animals.

Data on blood and liver constituents are summarized in Table I. In simple PGA deficiency, marked reductions in liver and blood PGA and CF were accompanied with definite reductions in blood hemoglobin content (P

$<0.001$) and in leucocyte count ($P<0.05$). A superimposed deficiency of biotin was reflected in further reductions in liver levels of PGA ($P<0.001$) and CF ($P<0.02$), blood hemoglobin concentration ($P<0.005$) and, additionally, of blood erythrocyte count ($P<0.01$). However, there were no significant changes in blood levels of PGA ($P>0.1$) and CF ($P>0.1$). Administration of a single dose of biotin to doubly deficient animals caused in one week almost complete restoration of liver PGA ($P>0.1$) and CF ($P>0.5$) and blood hemoglobin ($P>0.1$) levels to those in the PGA deficient animals but the blood erythrocyte count was still maintained at a significantly lower level ($P<0.01$).

Observations on intake of dietary PGA and urinary and fecal excretions of the vitamin by deficient animals are recorded in Table II. The diet containing raw egg white carried more vitamin than the 18% casein diet and would account for the differences in PGA intake between groups, in spite of small differences in food intake. However, fecal excretion of PGA in biotin-fed groups was higher than in biotin-deficient animals and in fact exceeded amounts ingested. No differences were observed in urinary excretion of the vitamin which was low in all groups. The latter observation is more or less similar to blood levels of vitamin in these animals.

In a further experiment, weanling rats, reared on the PGA- and biotin-deficient diet for 8 weeks, were divided into 3 groups of 4 animals each. Each animal in one group was administered intraperitoneally 20 $\mu$g biotin daily while a similar amount of vitamin was fed orally by a catheter tube to animals of a second group. All 3 groups were continued on basal diet for a further week during which urine and feces collections were made as before. Animals were killed at end of 9 weeks for determinations in liver, spleen and kidney of total PGA. Table III shows that, while urinary excretion of PGA remained unaffected, tissue stored and, more markedly, fecal excretion of PGA increased on biotin administration. The extent of this stimulation was not significantly influenced by mode of administration.

*Discussion.* It appears that, while there

TABLE I. Effects of Biotin and Folic Acid (PGA) on Blood Picture and on PGA and Leucovorin (CF) Concentrations in Liver and Blood. Results represent mean values for at least 4 animals from each group ± stand. error of mean.

| Group* | Erythrocytes/mm³ (× million) | Leucocytes/mm³ (× 1000) | Hemoglobin, g/100 ml | Liver | | Blood | |
|---|---|---|---|---|---|---|---|
| | | | | PGA | CF | PGA | CF |
| | | | | μg/g | | μg/100 ml | |
| 1. Biotin and PGA-deficient | 6.13 ±.27 | 5.86 ±.45 | 11.88 ±.34 | .563 ±.016 | .156 ±.011 | .291 ±.017 | .077 ±.016 |
| 2. *Idem*, administered biotin | 6.08 ±.32 | 6.90 ±.36 | 12.63 ±.66 | .660 ±.017 | .201 ±.018 | .330 ±.019 | .115 ±.015 |
| 3. PGA-deficient | 8.03 ±.45 | 6.40 ±.34 | 13.68 ±.10 | .695 ±.015 | .217 ±.017 | .337 ±.020 | .117 ±.015 |
| 4. Biotin and PGA-fed | 8.13 ±.55 | 7.65 ±.32 | 15.21 ±.23 | 4.730 ±.320 | 3.210 ±.430 | 2.760 ±.130 | 2.130 ±.210 |

* Young rats were fed diets deficient in PGA (group 3) or PGA and biotin (groups 1 and 2) for 8 wk when half the doubly deficient animals (group 2) were given each a single dose, intraper., of 100 μg biotin. All animals were continued on their respective diets for another week when they were killed for determinations. A comparison group (Group 4) received both PGA and biotin.

TABLE II. Effect of Biotin on Folic Acid (PGA) Excretion in PGA-Deficient Rat. Results represent mean values for at least 4 animals from each group ± S.E.

| Group* | Food intake during 7 days, g | PGA intake | PGA excretion — Urine | PGA excretion — Feces |
|---|---|---|---|---|
| | | mμg/rat/day | | |
| 1. Biotin and PGA-deficient | 98 ± 5 | 472 ± 20 | 50 ± 12 | 267 ± 12 |
| 2. *Idem*, administered biotin | 95 ± 5 | 423 ± 27 | 53 ± 6 | 507 ± 13 |
| 3. PGA-deficient | 93 ± 9 | 415 ± 40 | 46 ± 6 | 487 ± 17 |

* For details of grouping, see Table I. Urine and feces were collected for analysis during all of 9th wk of experiment.

may be differences in rates of recovery from biotin deficiency, administered biotin increased availability of PGA to the organism. These observations, however, differ from those of Halevy and Guggenheim(2) who reported that biotin causes no significant increase in hepatic PGA or CF concentrations as well as in *in vivo* conversion of PGA to CF in the tissue.

The effect of biotin on fecal excretion of PGA is more marked than on liver levels of the vitamin; together with low values for urinary excretion and blood levels of PGA, these observations suggest that biosynthesis of PGA occurs in the intestinal tract, rather than in body tissues, through some, as yet unclear, influence of biotin. Such an effect apparently prevails even in succinyl sulphathiazole-added diets.

In a study on synthesis of PGA-active compounds by growing cells of *Lactobacillus arabinosus* 17-5 in presence of p-aminobenzoic acid, Mitbander and Sreenivasan(4) reported that biotin and xanthine, among various metabolites tested, showed marked stimulation of PGA biosynthesis. Use of Tween 80 (polyoxyethylene sorbitan monoleate) in the medium also stimulated PGA synthesis. The effects due to biotin and Tween 80 were not, however, additive. It was concluded that stimulation afforded by these compounds could be through increased surface activity and consequent cell permeability to precursors needed for PGA biosynthesis. Such an effect due to biotin may explain the observed stimulation of PGA synthesis by intestinal microorganisms.

Since the animals were deficient in PGA, it is unlikely that the large amounts of PGA excreted in feces are of tissue origin. It is more probable that biotin, whether administered orally or intraperitoneally, becomes available to intestinal microflora, thence causing increased synthesis of PGA, part of which becomes available to the host animals.

Our experiments neither demonstrate nor exclude the possibility of tissue synthesis of PGA, but point out that biotin administration to PGA-deficient rats may augment intestinal synthesis of PGA, and, as a consequence, improve tissue levels of the vitamin.

*Summary.* A deficiency of biotin, superimposed over a simple PGA deficiency in rats resulted in further reductions in liver levels of PGA and CF, blood hemoglobin and, additionally, blood erythrocyte count. Administration of biotin to doubly-deficient animals caused almost complete restoration of liver levels of PGA and CF and of blood hemoglobin; under these conditions there was

TABLE III. Effect of Biotin on Tissue Retention of Folic Acid (PGA) and Its Excretion. Results represent mean values for at least 4 animals from each group ± S.E.

| Group* | PGA excretion — Urine | PGA excretion — Feces | Tissue content of PGA — Liver | Spleen | Kidney |
|---|---|---|---|---|---|
| | mμg/rat/day | | mμg/g | | |
| 1. Biotin-deficient | 42 ± 6 | 222 ± 22 | 550 ± 20 | 6 ± 2 | 310 ± 30 |
| 2. " -ingested | 43 ± 3 | 468 ± 12 | 620 ± 13 | 16 ± 5 | 493 ± 15 |
| 3. " -injected | 41 ± 5 | 420 ± 10 | 610 ± 21 | 12 ± 4 | 400 ± 14 |

* Weanling rats were reared on PGA and biotin-deficient diet 8 wk after which they were divided into groups receiving biotin (20 μg/rat/day) either orally (group 2) or intraper. (group 3). Group 1 served as control. Urine and feces collections were for 9th wk after which animals were killed for tissue analysis of total PGA.

marked excretion of PGA in feces, but not in urine, the amount excreted exceeding by far the tissue rise in level of vitamin. It is suggested that biotin administration may influence synthesis of PGA by intestinal microflora and, as a consequence, improve tissue levels of the vitamin.

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## Formation of Creatine from Guanidinoacetate in Pancreas.* (25103)

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Several investigators established that 2 enzymes are involved in biosynthesis of creatine (1). Arginine + glycine ⇄ guanidinoacetate + ornithine. Guanidinoacetate + S-adenosylmethionine → creatine + S-adenosylhomocysteine. The first enzyme, arginine-glycine transamidinase, has long been known to occur in mammalian kidney(2), while the second enzyme, guanidinoacetate methylferase, has been found in liver(3,4). Previously it has been assumed that under physiological conditions these 2 synthetic reactions are carried out in successive steps in different organs of the mammal. Guanidinoacetate was believed to be synthesized in the kidney from arginine plus glycine and then transported *via* the blood stream to the liver, where methylation to creatine took place. Recently, however, another mammalian organ has been implicated in creatine biosynthesis, with the discovery that pancreas contains a high concentration of arginine-glycine transamidinase (5). Since guanidinoacetate can be synthesized in pancreas, it became of interest to determine whether methylation of guanidinoacetate to form creatine might also occur in this organ. The purpose of this paper is to present evidence for occurrence of guanidinoacetate methylferase activity in mammalian pancreas.

*Methods.* The enzyme preparations were dialyzed beef or dog pancreas fractions which precipitate between 30% and 60% saturation with ammonium sulfate, prepared as described elsewhere(5). All incubation mixtures contained pancreas enzyme preparation, 24 mg; tris (hydroxymethyl) aminomethane buffer, pH 7.6, 100 μmoles; other additives as noted in individual experiments; final volume, 1 ml. Tubes were incubated 2 hr at 37°C; the reactions were terminated by adding 1 ml water and heating 3 min at 100°C. In the time-course experiment with guanidinoacetate-2-$C^{14}$, 5 μliters were removed from each reaction mixture at times indicated and spotted on paper chromatograms. Non-labelled guanidinoacetate and creatine were added to each spot as carriers. After development of the paper chromatograms with buffered phenol, the separated compounds were located with alkaline ferricyanide-nitroprusside spray. Areas of the paper containing creatine and guanidinoacetate were cut out, eluted, dried, and counted. In experiment with glycine-2-$C^{14}$, non-labelled guanidinoacetate and creatine were added as carriers to the reaction mixtures immediately prior to termination of incubation. Guani-

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