the DDT or in Fundulus as late as 16 days in the medium if subjected at stage 8.

5. There was no effect of DDT on the growth rate of Fundulus larvae, but after 9 days of exposure of Rana larvae they show a definite inhibition of growth.

6. The older the larvae at the time of DDT exposure, the longer the period before the onset of deleterious symptoms.

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Biochemical Studies on Livers of Chicks Receiving Graded Levels of Pteroylglutamic Acid.*

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In a recent communication[1] it was shown that the apparent xanthine oxidase activities of livers from chicks fed a purified diet were inversely related to the pteroylglutamic acid (PGA) content of the diet. The livers from the same experimental chicks were also studied for their nucleic acid and PGA content and for their apparent conjugase activity; the values so obtained are reported here.

Experimental. The diets and the methods of handling and care of the chicks are given fully in the previous report.[1] PGA was determined microbiologically by use of *Streptococcus faecalis*.[2] Conjugase was determined by the method of Laskowski, Mims, and Day[3] using Difco yeast extract as the substrate. Since there may be several conjugase inhibitors[4-7] in a mixture such as that used in these tests the absolute values may not be valid, but there seems to be no reason why such a method is not satisfactory for comparative purposes when using a single batch of substrate and the same organ from each bird.

Pentose nucleic acid and desoxypentose nucleic acid were determined on aliquots of 1:5 liver brei employing the methods outlined by Schneider.[8,9]

Results and discussion. The results of the nucleic acid determinations are given in Table I. As indicated earlier,[1] the livers of chicks were relatively larger when the diet was PGA-deficient. The data are arranged to show the nucleic acid content per gram of liver, the total nucleic acid per chick, and the amount per 100 g of chick. The values ob-

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1 Keith, C. K., Broach, W. J., Warren, D., Day, P. L., and Totter, J. R., *J. Biol. Chem.*, 1948, **176**, 1095.

2 Mitchell, H. K., and Snell, E. E., Univ. of Texas Pub., 1941, No. 4137, 36.

3 Laskowski, M., Mims, V., and Day, P. L., *J. Biol. Chem.*, 1945, **157**, 731.

4 Bird, O. D., Robbins, M., Vandenbelt, J. M., and Pfiffner, J. J., *J. Biol. Chem.*, 1946, **163**, 649.

5 Sims, E. S., and Totter, J. R., *Fed. Proc.*, 1947, **6**, 291.

6 Mims, V., Swenseid, M. E., and Bird, O. D., *J. Biol. Chem.*, 1947, **170**, 367.

7 Hodson, A. Z., *Arch. Biochem.*, 1948, **16**, 309.

8 Schneider, W. C., *J. Biol. Chem.*, 1945, **161**, 293.

9 Schneider, W. C., *Cold Spring Harbor Symposia on Quantitative Biology*, 1947, **12**, 169.

TABLE I.
Desoxypentose Nucleic Acid and Pentose Nucleic Acid in Livers of Chicks Receiving Graded Levels of Pteroylglutamic Acid.

Diet	No. of chicks	Liver desoxypentose nucleic acid				Liver pentose nucleic acid			
		Avg, mg/g	Range, mg/g	Per chick, mg	Per 100 g chick, mg	Avg, mg/g	Range, mg/g	Per chick, mg	Per 100 g chick, mg
Basal	14	2.5	2.1-3.5	14.6	9.6	6.0	3.2-7.8	38.6	27.3
" + 5 μg PGA/100 g	4	3.2	2.9-3.9	20.0	11.3	6.8	5.4-8.4	41.6	34.3
" + 10 " "	8	3.0	2.3-3.7	22.1	8.1	7.6	4.9-9.9	56.8	35.8
" + 20 " "	7	2.9	2.2-3.8	23.1	5.5	6.9	5.0-8.2	55.4	26.6
" + 40 " "	9	3.1	2.0-4.9	26.7	4.3	6.5	4.3-7.9	58.8	27.4
" + 80 " "	9	3.3	2.4-4.2	30.2	4.8	7.0	4.8-8.0	65.3	23.4
" + 200 " "	8	3.1	2.1-4.6	34.3	3.8	6.5	4.6-7.9	70.5	23.1
" + 1000 " "	8	3.3	2.9-3.7	27.5	4.6	6.9	3.9-8.5	58.6	17.8
Commercial diet*	8	3.5	2.6-3.8	28.0	4.4	6.8	4.9-8.4	56.0	17.3

* Purina "Startena," containing 175 μg of total PGA per 100 g, as shown by assay with *S. faecalis* and suitable conjugases.

tained agree satisfactorily with those obtained by Schneider on rat liver.[6]

The possibility that pteroylglutamic acid may be necessary for thymine synthesis and presumably, therefore, for synthesis of thymine-containing nucleic acids is emphasized in current theories concerning mechanism of action of this vitamin.[10-12] It is of special interest therefore to compare the thymonucleic (desoxypentose nucleic) acid contents of the livers of chicks on the basal diet with those receiving adequate amounts of PGA. As seen from Table I, there is a somewhat lowered desoxypentose nucleic acid content per gram of deficient chick liver as compared with livers from the other groups. However, groups receiving amounts of PGA entirely inadequate (5 μg, 10 μg) for prevention of deficiency symptoms nevertheless have values of desoxypentose acid per gram of liver equal to or greater than those receiving more than enough of the vitamin. When calculated on the basis of total liver nucleic acid per 100 g of chick, those on the basal diet have greater amounts than the ones receiving excess of PGA (200 μg, 1000 μg). If it may be assumed that the method used is specific for thymine-containing nucleic acids, the data do not offer strong support to the view that PGA deficiency blocks the synthesis of thymine. In this connection it may be recalled that Davidson[13] has found that both types of nucleic acid are elevated in the bone marrow of untreated pernicious anemia patients. Successful treatment of his patients was followed by a reduction to normal of their marrow nucleic acids.[13] It is difficult to reconcile these results with the hypothesis that the sole action of PGA is to promote thymine synthesis.

The pentose nucleic acid content was more uniform than the desoxypentose nucleic acid content. The level of pentose nucleic acid in the livers of the totally deficient groups was

[10] Stokes, J. L., *J. Bact.*, 1944, **47**, 433.

[11] Spies, T. D., Vilter, C. F., Cline, J. K., and Frommeyer, W. B., *Southern Med. J.*, 1946, **39**, 269.

[12] Wright, L. D., Skeggs, H. R., and Huff, J. W., *J. Biol. Chem.*, 1948, **175**, 475.

[13] Davidson, J. N., *Cold Spring Harbor Symposia on Quantitative Biology*, 1947, **12**, 50.

TABLE II.
Average Contents of Pteroylglutamic Acid and Conjugase in the Livers of Chicks Receiving Graded Levels of Pteroylglutamic Acid.

			No. of chicks	Free PGA autolyzed at pH 4.5 μg/g	Free PGA autolyzed at pH 7.0 μg/g	Total PGA: Hog kidney conjugase pH 4.5 μg/g	Total PGA: Chicken pancreas conjugase pH 7.0 μg/g	Apparent liver conjugase activity*: pH 4.5 units/g	Apparent liver conjugase activity*: pH 7.0 units/g
Basal			12	2.0	1.5	2.1	1.9	3.1	6.4
,,	+	5 μg PGA/100 g	4	1.3	1.5	1.1	1.5	2.9	7.4
,,	+	10 ,, ,,	8	1.3	2.0	2.5	3.1	4.2	12.3
,,	+	20 ,, ,,	7	2.2	2.2	3.2	3.0	3.7	10.6
,,	+	40 ,, ,,	9	2.9	2.3	2.9	3.2	2.7	6.3
,,	+	80 ,, ,,	9	8.5	8.7	9.9	8.6	4.1	8.9
,,	+	200 ,, ,,	9	5.0	5.1	6.0	6.3	5.1	12.5
,,	+	1000 ,, ,,	8	7.7	7.3	7.8	8.9	3.3	8.2
Commercial diet†			8	6.7	5.3	7.3	7.1	2.1	6.2

* A unit of conjugase activity is expressed here as the μg of PGA liberated in 4 hours by 0.2 ml of 1:20 liver brei acting on 80 mg of Difco yeast extract in a total volume of 2 ml.
† Purina "Startena," containing 175 μg of total PGA per 100 g, as shown by assay with *S. faecalis* and suitable conjugases.

only slightly lowered when compared with the other groups; the difference is probably not statistically significant.

According to Mims *et al.*,[6] desoxypentose nucleic acid is a potent inhibitor of conjugase activity. It might therefore be supposed that any alterations in the level of this nucleic acid would be reflected inversely by changes in the conjugase activity of the liver samples. In Table II are shown the apparent conjugase values at pH 7 and pH 4.5 as well as the free and total PGA content of the livers; the total PGA was determined with chicken pancreas conjugase at pH 7 and hog kidney conjugase at pH 4.5. There seems to be no particular relation between the conjugase activity and either the PGA intake or the desoxypentose nucleic acid content of the livers. It is interesting that the only group of chicks receiving conjugated PGA, those on the commercial diet, exhibited the lowest liver conjugase activity. The degree of reduction, however, is of doubtful significance.

The free PGA content of the livers appears to be about equal to the total PGA, *i.e.* little additional vitamin was liberated by treatment with added conjugase from either chicken pancreas or hog kidney. In a few cases the free is shown as greater than total PGA; these occurrences are undoubtedly due to cumulative errors in the assays.

The average contents of PGA in the livers of the various groups provide evidence from which the minimum requirement for the vitamin may be estimated. It is interesting that the liver contents of PGA in the chicks up to the group receiving 40 μg per 100 g of diet show little change, while the 80 μg group and those receiving more than 80 μg are 2 to 3 times higher than the deficient groups in this respect. From this it appears that the minimum requirement for protection from deficiency should be set at a value not lower than 40 μg per 100 g of diet. This is in agreement with the estimate from the growth curve of these chicks published earlier.[1] Estimation from blood cell numbers, however, indicates the minimum value to be somewhat lower when calculated for red cell values. On the contrary, white cell (chiefly lymphocytes)[14,15] numbers and thymus weights[15]

increased logarithmically with increasing levels of PGA to as high as 200 μg per 100 g of diet.

The concentrations of PGA in the livers of the chicks also provide some evidence concerning the mode of action of the vitamin in reducing the apparent liver xanthine oxidase activity. *In vitro* tests of the sample of PGA used in these experiments indicated that a concentration of approximately 40 μg per ml would be needed to reduce the xanthine oxidase activity to one-half its initial value.† The relatively low levels of PGA encountered in all of the liver samples indicate that either the impurity probably responsible for the *in vitro* anti-xanthine oxidase activity[16] is better retained than is PGA, or that some other mechanism than the accumulation of this substance is responsible for the reduced activity found in our previously published experiments.[1] It is conceivable that the xanthine oxidase is inhibited or reduced by a metabolic product of PGA normally produced in relatively large quantity.

Summary. Determinations of desoxypentose nucleic acid and pentose nucleic acid, pteroylglutamic acid (PGA), and conjugase have been made on the livers of chicks receiving 0, 5, 10, 20, 40, 80, 200, and 1000 μg of PGA per 100 g of diet and on controls receiving a commercial diet. Desoxypentose nucleic acid was found to be somewhat low in the livers of the negative control group of chicks but differences between all other groups were of doubtful significance.

The liver conjugase levels of the various groups, whether determined at pH 4.5 or at pH 7.0, were found to be unrelated to the dietary intake of PGA and to the nucleic acid content of the livers.

The liver PGA freed by autolysis was approximately the same as that determinable after treatment with either chick pancreas conjugase or hog kidney conjugase. The levels of PGA found in the chick livers indicate that storage of excess vitamin did not take place until the dietary level exceeded 40 μg per 100 g.

The bearing that the findings have on the mode of action of dietary PGA in reducing liver xanthine oxidase is briefly discussed.

[14] Campbell, C. J., McCabe, M. M., Brown, R. A., and Emmett, A. D., *Am. J. Physiol.*, 1945, **144**, 348.

[15] Unpublished data from this laboratory.

† Experiments conducted *in vitro* indicate that 2 mols of the xanthine oxidase inhibitor react with one mol of chick liver xanthine oxidase while only one mol reacts per mol of rat liver xanthine oxidase. That is, the degree of inhibition using chick liver is proportional to the square of the concentration of added PGA while it is proportional to the logarithm of the PGA concentration when using rat liver as the source of xanthine oxidase.

[16] Kalekar, H. M., Kjelgaard, N. O., Klenow, H., *J. Biol. Chem.*, 1948, **174**, 771.

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Influence of Pteroylglutamic Acid Administration Upon Fecal Riboflavin Values in Human Subjects.*

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In the course of investigations in this laboratory on the metabolism of riboflavin in human subjects on weighed, uniform diets of

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