the same person usually remain constant over long periods of time, although the plasma protein composition may vary considerably during this period. 3. Characteristic individual electrophoretic patterns of the plasma proteins have been observed in numerous cases of both normal individuals and patients with various diseases. The distinguishing characteristics encountered in individual electrophoretic patterns are independent of sex and age; in healthy persons they are confined mainly to the α-globulins; with only a few exceptions they are not related to the disease state in patients.

1. Gutman, A. B., The Plasma Proteins in Disease, *Adv. Protein Chem.*, 1948, v4, 155.

2. Wuhrmann, F., and Wunderly, C., *Die Bluteiweisskorper des Menschen,* Basle, 1952.

3. Longsworth, L. G., and McInnes, D. A., *J. Am. Chem. Soc.,* 1940, v62, 705.

4. Wiedemann, E., *Helv. Chim. Acta,* 1947, v30, 168.

5. Tiselius, A., and Kabat, E. A., *J. Exp. Med.,* 1939, v69, 119.

6. Bernfeld, P., Bonner, C. D., and Homburger, F., *Proc. of the 2nd Clin. ACTH Conf.,* Blakiston Co., 1951, v1, 282.

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Growth Promotion in Carnivorous Protozoa by Substituted Purines.* (20380)

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(Introduced by George W. Kidder.)

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Tetrahymena pyriformis(1) has been extensively used as a favorable animal microorganism for nutritional and biochemical studies(2-4). When *Tetrahymena* has been used as food for carnivorous protozoa an interference with their normal growth and reproduction has been noted. By the addition of small concentrations of naturally occurring substances to the culture medium, however, (together with the food organism) it has been possible to obtain satisfactory growth of several species of carnivorous protozoa. In the case of the hypotrichous ciliate, *Stylonychia pustulata,* in bacteria-free cultures growth was proportional to the number of *Tetrahymena* supplied as food, but failed immediately if a supplement, extracted from yeast or other plant materials, was omitted from the medium. The supplement was never obtained from animal tissues and attempts to substitute known vitamins were unsuccessful(5-7). Chemical properties of the supplementary factor, such as solubility in aqueous, but not in organic solvents, thermostability and photosensitivity were established. By chromatographic methods concentrates were produced which were active in dilutions as low as 0.1 μg per ml(8,9). In the case of the suctorians, *Tokophrya infusionum* and *Podophrya collini*† the interference with growth was manifested differently. The adult suctorians captured *Tetrahymena* readily with their tentacles and sucked out the cytoplasm of the ciliate. There was apparently no immediate need for a supplement in the medium as long as the number of food ciliates was limited. Thus Rudzinska was able to maintain *Tokophrya* for several years by carefully controlling the supply of *Tetrahymena*(10). It has long been known, however, that after abundant feeding on ciliates a large percentage of suctorians changed into giant forms which failed to reproduce normally(11). Under such conditions there was invariably a decline in the growth rate with eventual loss of the cultures. In bacteria-free cultures of suctorians feeding on *Tetrahymena* small concentrations of yeast

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extract were found beneficial in reducing the percentage of giantism and in maintaining cultures(12,13). In cultures of *Tokophrya* yeast nucleic acid was equally effective, but highly active concentrates of the supplementary factor required by *Stylonychia* were without effect on this suctorian. Conversely, it had previously been determined that yeast nucleic acid did not contain the *Stylonychia* factor. After a chemically defined medium for *Tetrahymena* had been developed(3), the mixture of purines and pyrimidines used in that medium was tested on *Tokophrya* with even better results than those obtained with nucleic acid. A combination of guanylic acid and uracil was also effective, but neither alone had a beneficial effect(14). These results were interpreted as indicating that the purine and pyrimidine requirement of *Tokophrya* was qualitatively similar to that of *Tetrahymena* but that these compounds were not available to the suctorian in proper quantities or proportions from the cytoplasm of the ciliate(15). It had been observed in this laboratory and elsewhere‡ that the nucleus of *Tetrahymena* was not taken in by *Tokophrya*.

The present report resulted from a study of the biological activity of purines in the carnivorous protozoa by methods similar to those used in *Tetrahymena*(16). In the first experiments on *Tokophrya* the triazolo purine analog, 8-azaguanine, acted as it did in *Tetrahymena*, as a metabolic inhibitor. When, however, tests were extended to the other suctorian, *Podophrya*, a growth promoting effect was noted. Recent trials on *Stylonychia* indicate that 8-azaguanine is also growth promoting for this ciliate and replaces the naturally occurring supplement previously found necessary.

Materials and methods. *Tetrahymena pyriformis* W was supplied to the carnivorous protozoa by two methods. Originally the food ciliates were cultured separately on slants containing 2% proteose peptone, 0.5% dextrose and 1.4% agar. After 4 days' growth the ciliates were suspended in sterile distilled water for addition to the experimental cultures. This method was continued for the studies on the *Stylonychia* supplementary factor and was used for the preliminary studies on the suctorians. Recently a chemically defined medium has been developed based on Medium A for *Tetrahymena*(3). The amino acids, purines and pyrimidines were diluted 10 times, dextrose and Tween were omitted, and a chelating agent (ethylenediamine tetraacetic acid) was added. The carnivorous protozoa were washed free of bacteria by a technic previously published(17). Cultures were maintained in tubes containing 5 ml of medium and were incubated in the dark at 26°C. When *Tetrahymena* were taken from slants relatively heavy suspensions were used as described elsewhere(6). In the chemically defined medium the food ciliates were introduced with the carnivorous protozoa, in a ratio of approximately 50 to 1, from a stock cul-

TABLE I. Effect on Giantism in Suctorians of Increasing Concentrations of Purines and Pyrimidines.

Medium	*Tokophrya infusionum*, %	*Podophrya collini*, %
Basal medium*	83	8.5
" " and 3x†	33.8	32
" " " 6x	80	50
" " " 9x	100	56

* Contains 3 γ/ml guanylic acid and 1 γ/ml uracil.

† x = guanylic acid, 30 γ/ml; adenylic acid, 20 γ/ml; cytidylic acid, 25 γ/ml; uracil, 10 u/γ/ml.

TABLE II. Effect on Giantism in Suctorians of 8-Azaguanine, Guanylic Acid and Uracil.

Medium	*Tokophrya infusionum*, %	*Podophrya collini*, %
Basal medium*	87.3	9
" " and uracil†	30	100
Basal medium and guanylic acid‡	1.3	
Basal medium, uracil and guanylic acid	20.9	100
Basal medium, uracil and 8-azaguanine§	100	
Basal medium, guanylic acid and 8-azaguanine	100	0
Basal medium, uracil, guanylic acid and 8-azaguanine	100	25
Basal medium and 8-azaguanine	100	0

* Contains 3 γ/ml guanylic acid and 1 γ/ml uracil. † 10 γ/ml. ‡ 10 γ/ml. § 0.18 γ/ml.

‡ Personal communication from Dr. Maria A. Rudzinska.

TABLE III. Effect of Substituted Purines on Growth of *Stylonychia pustulata* Feeding on *Tetrahymena*.*

μg. ml guanylic acid	Substituted purine	μg/ml substituted purine 100	10	1.0	0.1	.01	.001	0
9	8-Azaguanine		+	+	+	+	+	0
50	"		+	+	0	0	0	0
0	Theobromine	+	+	+	+	+	+	0
5	"	++	++	++	++	++	+	0
0	Theophylline	+	+	+	++	++	++	0
5	"	+	+	+	+	++	+	0
0	Caffeine	0	+	+	++	+	+	0
		400	200	100	50	25		0
1	Caffeine	0	+	+	+	+		0

* No growth without naturally occurring supplement or substituted purine.
0 = No growth; + = Significant growth; ++ = Approximately half maximum growth.

ture. Growth of *Tetrahymena* kept pace with the growth of the carnivorous organisms during the first 3 days, except when substances inhibitory for the food ciliate were added. The tubes were examined for recording results usually on the seventh day. The suctorians were counted in a zone just below the surface of the medium on the sides of the tube. Growth of suctorians was recorded in terms of numbers of organisms per low power field, 1.13 sq mm area. At the same time the percentage of giantism was determined. Giants were characterized by a size at least twice that of the normal form and by the absence of visible tentacles. Methods for recording growth of *Stylonychia* have been given previously(6) but in the work presented here only a rapid, routine grading of significant growth by plus marks was attempted.

Results. The average number of adult suctorians per low power field was approximately doubled by the same factors which decreased giantism. The effect on the percentage of giantism, however, was much more striking and was taken as a better indication of the influence on growth in these organisms. In *Tokophrya* addition of 30 γ per ml of guanylic acid and 10 γ per ml of uracil to the basal medium reduced giantism from 82.6% to 34.8%. The influence of increasing concentrations of purines and pyrimidines was then tested on both species of suctorians with the results given in Table I. In *Tokophrya* there was a favorable effect on growth with up to 3 times the concentration of nucleic acid components usually used for *Tetrahymena* but higher concentrations were inhibitory. Concentration is not the only factor influencing giantism, however, in this species as is shown by the high incidence of giantism in the most favorable concentration tested. Table II affords better evidence that the proportion of purine to pyrimidine is more important, since the least giantism occurred in *Tokophrya* when the ratio of guanylic acid to uracil in the medium was 13 to 1. The basal medium contains some guanylic acid and uracil to permit the growth of *Tetrahymena*. The guanine analog, 8-azaguanine, effectively reverses this ratio and increases giantism to the maximum.

In contrast with the findings in *Tokophrya*, the results with the other suctorian, *Podophrya* indicate that any addition of nucleic acid components increases the percentage of giantism. Even uracil has this effect. The guanine analog, however, completely reverses the condition with total elimination of giant forms. Although not shown in the tables, giantism in this species is also prevented by other guanine antagonists, caffeine, theobromine and theophylline. Concentrations from 0.1 to 100 γ per ml of these methylpurines are growth promoting for this suctorian.

In the hypotrich *Stylonychia* there is also a growth promoting effect with the same purine antagonists which benefit *Podophrya*. It should be noted, however, that the growth thus far obtained is not equal to that observed in concentrates from natural sources. Other factors are undoubtedly involved, but the guanine antagonism, even in these preliminary experiments, is quite clearly shown to be

operative. These are the first known chemical compounds which have given positive results as substitutes for the naturally occurring supplementary factor. The indication that the growth promoting action of theobromine is slightly improved by the addition of guanylic acid suggests that a delicate purine balance is involved.

Discussion. The remarkable difference in the effects of 8-azaguanine in the two species of suctorians suggests that equally great variations in purine metabolism remain to be discovered in other organisms. *Tetrahymena pyriformis* has been unique among the ciliates in the facility with which it was first cultured in peptone solutions(2) and in its progressive yield of biochemical information on its metabolism(3). Yet the most striking peculiarity of *Tetrahymena* when compared to other animals is its guanine metabolism(16). The indication that guanine metabolism is critically involved as a condition for growth in other protozoa may explain many of the difficulties which have hindered nutritional studies with animal microorganisms other than *Tetrahymena*. It now appears that *Tokophrya infusionum* has an optimum ratio of guanine to uracil considerably higher than that afforded by its method of feeding on *Tetrahymena*. Adding guanylic acid to the medium, therefore, improves growth while adding 8-azaguanine aggravates the interference. In *Podophrya collini,* on the other hand, the optimum purine intake is apparently exceeded by feeding on *Tetrahymena*. Hence quanylic acid aggravates the interference with growth while the guanine antagonist improves growth. The fact that uracil also increases the percentage of abnormal forms, of course, requires further study. Quite different from the suctorians by any morphological or physiological comparison, the hypotrich *Stylonychia pustulata* nevertheless responds to 8-azaguanine and the methylpurines as does *Podophrya*. Hence the phenomenon transcends the suctorian group of protozoa and may apply to many ciliates. The results with *Stylonychia* suggest that in this organism there is such a critical purine balance that the ingestion of a few *Tetrahymena,* with their peculiar guanine metabolism, is sufficient to prevent growth. The guanine antagonists apparently affect the balance to the extent that growth on the *Tetrahymena* diet is possible, although the optimum has not yet been achieved. In *Stylonychia* also there is a difference between the effect of 8-azaguanine and that of the methylpurines. It has been pointed out(16) that the methylpurines inhibit more than one enzyme system. Purine is the most easily affected but there is a possible effect on pyrimidine and other non-purine systems. A close similarity is noted between theobromine and the most active preparations of the *Stylonychia* supplementary factor previously concentrated from natural sources(15).

Summary. The guanine analog, 8-azaguanine, had a growth inhibiting effect on one species of suctorian, *Tokophrya infusionum,* and a growth promoting effect on another species, *Podophrya collini.* The compound induced abnormal giant forms in the former and prevented their appearance in the latter. Guanylic acid antagonized both effects. In the hypotrichous ciliate, *Stylonychia pustulata* 8-azaguanine promoted growth and substituted for a supplementary factor previously obtained from natural sources. The methylpurines, caffeine, theobromine and theophylline were also growth promoting in *Podophrya* and *Stylonychia.* The results may be explained on the basis of a purine balance in these protozoa. Apparently guanylic acid intake in other than optimum proportions can interfere with growth in the organisms studied.

1. Corliss, J. O., *Trans. Am. Micros. Soc.,* 1952, v71, 159.

2. Lwoff, A., *Ann. Rev. Microbiol.,* 1947, v1, 101.

3. Kidder, G. W., and Dewey, V. C., in *Biochemistry of Protozoa,* A. Lwoff, ed., Academic Press, 1951.

4. Kidder, G. W., *Ann. Rev. Microbiol.,* 1951, v5, 139.

5. Lilly, D. M., *Anat. Rec. Suppl.,* 1940, v78, 40.

6. ———, *Physiol. Zool.,* 1942, v15, 146.

7. Lilly, D. M., Pignataro, A., and Heilshorn, K., *Anat. Rec.,* 1949, v105, 512.

8. Lilly, D. M., Bulda, F. E., and Daly, J. J., *Anat. Rec.,* 1951, v111, 112.

9. Lilly, D. M., Daly, J. J., and Tarantola, V., *Proc. Soc. Protozool.,* 1952, v3, 13.

10. Rudzinska, M. A., *Science,* 1951, v113, 10.

11. Collin, B., *Arch. Zool. Exp. et Gen.*, 1912, v51, 1.

12. Guilcher, Y., Thesis, University of Paris, France, 1950.

13. Lilly, D. M., Fleming, P. J., Pedagno, C. F., and Gilmore, J. P., *Anat. Rec.*, 1951, v111, 62.

14. Lilly, D. M., and Gilmore, J. P., *Anat. Rec*, 1952, v113, 64.

15. Lilly, D. M., *Ann. N. Y. Acad. Sci.*, 1953, in press.

16. Kidder, G. W., and Dewey, V. C., *J. Biol. Chem.*, 1949, v179, 181.

17. Kidder, G. W., Lilly, D. M., and Claff, C. L., *Biol. Bull.*, 1940, v78, 9.

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III. Chemotherapy of Tumors in Rats with Certain Ethylenephosphoramides.*† (20381)

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As a continuation of our investigation of the therapeutic effectiveness of ethylenimines in the treatment of transplantable tumors in rats(1,2). we report here results obtained with N,N',N'' - Triethylenephosphoramide, (I), N,N-Diethyl-N',N''-diethylenephosphoramide, (II), N-Pentamethylene-N',N''-diethylenephosphoramide, (III), N-(3-Oxapentamethylene) - N',N'' - diethylenephosphoramide, (IV), N-Tetramethylene-N',N''-diethylenephosphoramide, (V), N,N',N''-*tris* (Tetramethylene)phosphoramide, (VI), N,N', N''-Triethylenethiophosphoramide, (VII) and N - (3 - Oxapentamethylene) - N',N'' - diethylenethiophosphoramide, (VIII).

Materials and methods. The compounds were synthesized in the Pharmaceutical Research Laboratory of The Calco Chemical Division of The American Cyanamid Co. Their structural formulas are shown in Fig. 1. They are colorless, crystalline compounds. (I) melts at 41°C, is extr. soluble in water, very soluble in alcohol, ether and acetone. (III) boils at 95°C/.15 mm Hg and is very soluble in water, benzene, toluene and ether. Both (I) and (III) are very hygroscopic. (IV) melts at 62.5-64°C, is very soluble in water and is soluble in benzene, toluene and ether. (VII) melts at 51.5°C and its solubility in water at room temperature is 19 g in 100 ml. It is soluble in benzene and ether. (VIII) melts at 75-77°C, is slightly soluble in water and very soluble in benzene, toluene and hot petroleum ether. Isotonic saline solutions of the compounds were prepared to contain the desired dose in .2 ml. At first solutions were prepared daily, but equally good results could be obtained with solutions prepared once a week, provided that solutions were kept at about 5°C. One dose was given daily by intraperitoneal injection. In certain cases the subcutaneous route was used for comparison and similar results were obtained. Treatment was given daily throughout the experiment which varied from 2 to 9 weeks. Young male rats, averaging about 150 g were used. In each experiment there were, generally, 4 groups of 6 to 8 rats each; normal controls (N.C.), drug controls (D.C.), tumor controls (T.C.), and tumor treated (T.T.). They were group pair-fed throughout period of treatment. In certain cases the W.B.C. and differential counts were made on all animals in the 4 groups, prior to implantation of the tumor, just before beginning the course of treatment and when the drug was discontinued or the experiment terminated; in certain experiments they were made, also, sometime

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