A Protein Factor in the Nutrition of *Paramecium caudatum*

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SUMMARY

The ciliate *Paramecium caudatum* was cultivated in a medium consisting principally of known chemical compounds, including 17 amino acids, guanylic, adenylic, cytidylic and uridylic acids, sodium acetate and sodium pyruvate, linoleic and oleic acids, a mixture of B vitamins and several inorganic salts. In addition it was necessary to add microgram quantities of a protein concentrate, first obtained from autolysed yeast, but recently by an improved method from dried green peas. Lipids were first extracted from the crude material and the protein was then dissolved and precipitated with 10% trichloroacetic acid. This protein was further purified by paper chromatography to yield a concentrate active in dilutions as low as 10 µg./ml. When the protein was hydrolysed enzymically or by acid or alkali, the hydrolysates were inactive. Sixteen amino acids were qualitatively identified in the hydrolysate. The nutritional role of a protein effective in such small concentrations has not yet been satisfactorily explained.

INTRODUCTION

The ciliate *Paramecium caudatum* has many advantages for microbiological experiments, but a serious limitation on its usefulness has been the difficulty involved in maintaining pure cultures. This became apparent when Glaser & Coria (1933) first succeeded in establishing bacteria-free cultures of this species in a medium containing dead yeast cells, liver extract and kidney tissue. Shortly afterwards the rapid progress in the cultivation of smaller ciliates, particularly *Tetrahymena pyriformis*, led to comparative neglect of the genus *Paramecium* until Johnson & Baker (1942) reported the growth of pure cultures of *P. multimicronucleatum* in a special yeast medium. Van Wagten-donk & Hackett (1949) were successful with cultures of *P. aurelia* in another medium containing yeast. Burbank (1950) showed that *P. caudatum* was similar in its growth requirements, although his medium contained killed bacteria as well as autolysed yeast. After the development of a chemically defined medium for *Tetrahymena* by Kidder & Dewey (1951) modifications of that medium were adapted to the requirements of *Paramecium* by Van Wagten-donk and his students (1953) and by Johnson & Miller (1956), but it was still necessary to add the unknown factor from yeast.

In this laboratory Sterbenz (1956) confirmed the expectation that the media developed for related species would also support growth of *Paramecium caudatum*. It appeared that the three species had a common requirement for the special factor obtained from yeast. Ordinary yeast extracts were not satisfactory and the most important part of the preparation involved a period of autolysis for several

hours; when this step was omitted no growth was obtained. After such autolysis, however, the medium could be autoclaved without loss of growth-promoting activity. Although some progress had been made in purifying the unknown factor from yeast a search for another source of the factor appeared worth while. Earlier Van Wagtendonk & Hackett (1949) had reported that a lettuce infusion inoculated with *Aerobacter aerogenes* and incubated for 24 hr. before sterilization would support the growth of *P. aurelia*. A preliminary search was, therefore, made for various plant infusions which when inoculated with bacteria would yield the factor. The first significant advance was made when it was found that an infusion of split green peas could be used as a source of the factor and that under certain conditions the inoculation with bacteria was unnecessary. Thus it became possible to obtain the factor directly from peas and to prepare the concentrates more active than those previously obtained from the yeast autolysate (Lilly, Klosek & Hartig, 1958). This present report is concerned with attempts at further purification of the factor required by *P. caudatum* and improvement of the test medium in which it is used. While the unknown factor itself still has not been characterized chemically, highly active concentrates have been prepared and additional information has been obtained about its properties.

METHODS

Strains of *Paramecium caudatum* were collected from freshwater ponds on Long Island, New York, U.S.A., but not all of these proved adaptable to artificial media. Suitable strains were obtained by selecting individuals from cultures containing the natural bacterial flora and washing them by a modification of the Parpart (1928) technique previously published (Kidder, Lilly & Claff, 1940). Bacteria-free cultures were first maintained in a two-membered system originally described by Johnson (1946) with the flagellate *Polytomella caeca* as an associate. Later, when large numbers of *Paramecium* were obtained, they were introduced to the stock culture medium and washed free of the flagellates. This method proved more dependable for the isolation of new strains than did direct attempts at cultivating single specimens under axenic conditions. Stock and experimental cultures were maintained in 5 ml. quantities of medium in screw-capped Pyrex tubes (16 mm. diameter, 125 mm. long). The usual inoculum consisted of 0.5 ml. medium containing approximately 400 organisms from a stock culture. In a few experiments growth was determined by a direct count of the number of ciliates in a sample of 0.1 ml. taken by pipette from duplicate cultures. Maximum growth of 1000–1500 organisms/ml. was attained in the stock medium after 10–14 days, depending on the strain used. This method of direct counting was not practicable for the numerous routine tests of activity of the protein fractions. Evaluation of growth in such tests was based on a visual comparison of the experimental cultures with control cultures in stock medium. Test cultures exhibiting growth comparable with the maximum concentration in the controls were graded + + + +, and growth estimated at 3/4 maximum was graded + + +. Lower concentrations were graded + +, +, or –, but these were not considered significant. Lower rates of growth were sometimes observed when the ciliates required more than 14 days to reach a maximum, but this type of growth could not be compared with controls and was considered negative. Results of experimental cultures were regarded as positive when the rate of growth and final

concentration were within 25% of that observed in controls. All tests were based on triplicate cultures carried through at least five transfers. Incubation temperature was 25°.

The first medium used for stock cultures was similar to that used by Van Wagendonk & Hackett (1949). Fleischmann's Activated Dry Yeast was suspended in four times its weight of distilled water. This was first incubated for 4 hr. at 37° with frequent stirring, and then autoclaved for 20 min. at 120°. Most of the solids were removed by centrifugation and the supernatant fluid used as a crude stock medium. Complete removal of all suspended particles resulted in a decrease of the growth rate and of the final concentration of organisms. For stock cultures the supernatant fluid was not filtered. Since older preparations kept at room temperature had less activity than those freshly prepared the unused portion of the crude medium was kept frozen until needed; in this way activity could be preserved for several months.

Table 1. *Stock culture medium*

Preparation of pea infusion

1. 10 g. of dried green split peas suspended in 100 ml. of distilled water.
2. Boil for 15 min.
3. Remove solids by centrifugation or filtration.
4. Inoculate with one loopful of *Aerobacter aerogenes*.
5. Incubate 16 hr.
6. Autoclave at 125° for 15 min.

Preparation of medium

Pea infusion (as prepared above)	50 ml.
Proteose peptone (Difco)	5.0 g.
Cytidylic acid	0.05
Guanylic acid	0.05
Glucose	5.0
Stigmasterol	0.002

Bring volume to 1 l. with distilled water. Adjust pH to 7.0

Later, by substituting the pea infusion for the yeast autolysate a simpler stock culture medium was prepared as given in Table 1. *Aerobacter aerogenes* was introduced to produce an effect comparable to the autolysis of the yeast. By referring to the work done with other species of *Paramecium* and by testing various modifications on available strains of *Paramecium caudatum* a satisfactory combination of known chemical components with a minimum amount of purified fractions from the peas was eventually developed. This is the experimental test medium as given in Table 2.

RESULTS

Chemically defined components of the medium

The amino acids used were essentially the same as those used for *Tetrahymena* with the addition of tyrosine. Minor variations of concentrations of individual amino acids similar to the combinations used by other workers for other species of *Paramecium* did not appreciably affect growth. Omission of any single one or significant lowering of the concentration of any one resulted in cessation of growth. It appeared that as long as the unknown protein component was used with these

Table 2. *Experimental test medium*All amounts given in $\mu\text{g./ml.}$ of final medium

<i>Chemically defined portion</i>			
L-Alanine	110	Linoleic acid	3.75
L-Arginine	206	Oleic acid	1.25
L-Aspartic acid	122		
Glycine	10	*Stigmasterol	2
L-Glutamic acid	233		
L-Histidine	87	*Calcium pantothenate	2
DL-Isoleucine	270	Nicotinamide	4
L-Leucine	244	Pyridoxal HCl	4
L-Lysine	272	Riboflavin	4
DL-Methionine	245	Folic acid	2
L-Phenylalanine	160	Thiamine HCl	12
L-Proline	250		
DL-Serine	394	Sodium ethylenediamine tetra-acetate	20
DL-Threonine	238		
DL-Tyrosine	100		2
L-Tryptophan	76	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	40
DL-Valine	96	$(\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$	10
		$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	0.1
Guanylic acid	75	ZnCl_2	0.02
Adenylic acid	30	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	20
Cytidylic acid	75	$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	2
Uridylic acid	20	$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	0.5
Sodium acetate	570	K_2HPO_4	570
Sodium pyruvate	570	KH_2PO_4	570

Final pH adjusted to 7.0 with 0.1 N-NaOH

Undefined portion

* Protein factor obtained from peas 50 to 10

* The stigmasterol, the mixture of B vitamins and the protein factor were each sterilized separately and added aseptically to the other components which were mixed and sterilized together.

mixtures, precise quantitative studies on amino acid requirements would not be practicable. As the amount of unknown protein introduced was decreased, however, there was no indication that the amino acid balance was any more critical than previously. Further experiments on the optimum amino acid balance for *Paramecium caudatum* were postponed pending the results of more promising work on purification of the unidentified protein component.

Some of the purine and pyrimidine components of nucleic acid were also necessary for the satisfactory growth of *Paramecium caudatum*. As reported for other species the best mixture tested contained the four nucleotides, guanylic, adenylic, cytidylic and uridylic acids. The optimum proportions of these were not readily determinable while changes were constantly being made in the protein component and, as in the case of the amino acids, it was decided to use the mixture which had given the most satisfactory results in the earlier work.

An additional carbon source was required for the best growth. Glucose was satisfactory in preliminary experiments, but later sodium acetate+sodium pyruvate gave slightly better results. A sterol requirement similar to that reported for the other species was satisfied by the inclusion of stigmasterol in the medium. The

B vitamins were also required in approximately the same concentrations as reported for the other species. A mixture of inorganic salts similar to that used for *Tetrahymena* was included. The chelating agent sodium ethylenediamine tetra-acetate, although not necessary, was useful in preparing the stock solution of inorganic salts.

The only components of the chemically defined portion of the medium that differed much from those used for the other species were the two fatty acids, linoleic and oleic acid. After some preliminary work with natural oils, particularly corn oil, it was found that these two fatty acids in a three to one ratio promoted the best growth of *Paramecium caudatum*. The fact that in the early work with all three species the yeast autolysates contained both lipids and proteins probably contributed to some of the difficulties encountered at that time. In any event, after the lipids were separated from the pea material by preliminary extraction and replaced in the final medium by the known mixture of fatty acids it was possible to use much less of the chemically undefined material as a source of the protein factor.

Concentration of the protein factor

Progress in purification of the unidentified factor has resulted mainly from several improvements over previous methods. The first promising modification was the use of trichloroacetic acid (TCA) to precipitate the protein. This was originally used with the yeast autolysate when that material was the only source of the factor. Later this protein precipitant was found to be equally effective when the green peas were used as the source. Other methods of separating protein from non-protein material yielded preparations which were active only in much higher concentrations. There was some indication that treatment with TCA actually improved the yield of active material. When some of the native protein was treated with hydrochloric acid a precipitate was obtained that had no activity. When, however, this was dissolved in distilled water and a precipitate obtained with TCA this material proved to be active. This suggested that the native protein either contained an inactive form of the factor or that some sort of inhibition was involved. Another fortunate development coincident with the use of TCA with the crude pea infusion was the finding that no period of enzymic action or autolysis was necessary as had previously been the case when yeast was used. Removal of the TCA was carried out by dialysis against running water since the active factor was non-dialysable. The resulting product was a slightly hygroscopic white powder which could be further purified. Figure 1 outlines the method of preparation. The most active concentrates tested so far were obtained from this material by descending chromatography on Whatman no. 1 paper with 1 % (v/v) ammonia (sp.gr. 0.900) in 50 % (v/v) aqueous ethanol. Under these conditions the R_F value was 0.84. Larger quantities of concentrates were obtained by use of the same system as applied to the chromatopile described by Mitchell & Haskins (1949). When used with the chemically defined portion of the medium the concentrate was active in dilutions of 10 $\mu\text{g./ml.}$

Before chromatography the preparation, even though active in dilutions of 20 $\mu\text{g./ml.}$, probably contained considerable amounts of impurities. Ultraviolet and infrared absorption spectra of this relatively crude material did not reveal any characteristic patterns of maximum absorption. After chromatography, however,

Fig. 1. Preparation of protein factor.

the active material did yield reproducible spectra showing maximum absorption at 2660 Å. in the ultraviolet and more consistent patterns in infrared spectroscopy. Apparently some of the impurities contributing to the non-specific absorption had been eliminated by the chromatography.

Since all the evidence indicated that the active principle was protein in nature some experiments were done to gain information about its constitution. Firstly, most of the proteolytic enzymes, including pepsin, chymotrypsin, trypsin, papain and ficin, when allowed to act for a sufficient length of time destroyed all growth-promoting activity of the material. All enzyme preparations used were obtained from the Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A. In the experiments with pepsin 3 mg. of the commercial preparation were dissolved in 3 ml. of 0.01 N-HCl. The mixture was incubated 24 hr. at 37°. The pepsin was then inactivated by boiling and the solution evaporated to dryness *in vacuo*. In the experiment with chymotrypsin 23 mg. of the commercial preparation were added to 50 ml. of a solution containing 500 mg. of the pea protein at pH 7.8. After incubation at 25° for 24 hr. the solution was brought to pH 3 by the addition of HCl and evaporated to dryness *in vacuo*. In a similar test 12 mg. of crystalline trypsin was dissolved in 12 ml. of water and added to a solution of 250 mg. of the pea protein in 25 ml. of water at pH 7.5. In this case the incubation temperature was 37°. The above methods for the three common proteolytic enzymes were based on those described by Sanger & Tuppy (1951).

In the experiments with papain and ficin the methods were based on those published by Petermann (1946), and by Kimmel & Smith (1957). A stock solution of 200 mg. of the commercial enzyme in 25 ml. of 0.9% NaCl solution was first pre-

pared. To 2.5 ml. of this stock preparation 1 ml. of 0.5 M-cysteine solution and 1.5 ml. of 0.1 M-sodium citrate buffer (pH 6) were added. Of this enzyme + cysteine solution 1 ml. was added to 120 mg. of the pea protein dissolved in 30 ml. of water and buffered with 0.3 ml. of the same sodium citrate solution used above. The mixture was incubated for 24 hr. at 37°.

When the active material was subjected to acid or alkaline hydrolysis the activity progressively decreased as the hydrolysis continued. After complete hydrolysis when the biuret test was negative, there was no growth-promoting activity. Acid hydrolysis was accomplished by refluxing one part of the protein with 5 parts of 6N-hydrochloric acid for 24 hr. The hydrochloric acid was then removed by distillation under reduced pressure. Alkaline hydrolysis was carried out by refluxing one part of the protein with five parts of 6N-barium hydroxide for 10 hr. The barium was removed with sulphuric acid.

The hydrolysates thus obtained were analysed chromatographically for their amino acid content by the two-dimensional methods described by Redfield (1953). The following were qualitatively identified: alanine, arginine, cystine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine. No evidence was obtained for the presence of either aspartic or glutamic acid either by the above methods or by the one-dimensional methods described by Hardy, Holland & Nayler (1955). Otherwise the protein involved appeared to be complete and not unusual in its amino acid composition. The possibility that more than one type of protein might have been present in the original sample of concentrate before hydrolysis was not eliminated by this preliminary study.

DISCUSSION

The fact that *Paramecium caudatum* is so common in nature suggested the extension of nutritional studies to this species. Up to the present no striking differences have been found from the known requirements of related species. The chief advance has been the discovery of a new and more convenient source of the unknown protein factor which has been the principal obstacle to formulation of a completely defined medium. Now that it is possible to obtain from dried green peas a highly active protein material by relatively simple chemical methods and without preliminary enzymic processing, more rapid progress can now be expected leading to the identification of this factor. It should be emphasized that it is by no means established that the protein obtained from the peas is necessarily identical with the material previously obtained from autolysed yeast, or that the active component of either of these artificial products enters into the nutrition of *Paramecium* under natural conditions. In the present state of knowledge it is difficult to conceive of a protein or a polypeptide having a vitamin-like activity, yet these latest preparations are active in microgram quantities. One possibility that should not be overlooked is that the difficulty in developing a defined medium may be due to some imbalance of the known components so that some type of toxicity or inhibitory effect on growth occurs. The role of the unknown component may be to detoxify the medium or to annul this inhibition. It is conceivable that several different types of compounds might have such an effect, comparable to the 'protective action of proteins' in other situations. In any event, even if one such compound could be

isolated in pure form and its structure determined this would be a step towards understanding the mode of action.

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