TABLE I. Recovery of Added Vitamin A from Stool.

Human subject	Vit. A, μg/g stool: Before addition of vit. A	Mean	Vit. A added	After addition of vit. A	Mean	% recovery of vit. A
T.	2.05 2.28	2.17	3.5	6.30 6.12	6.19	109.1
V.	1.80 1.76	1.78	2.26	4.00 4.00	4.00	101
C.	1.59 1.65	1.62	2.81	4.15 4.6	4.36	101.4
J.S.	5.2 5.9	5.5	22.2	30.8 34.5	32.7	118
P.	5.8 6.9	6.35	16.7	23.9 26.9	25.4	110
H.	5.8 5.1	5.45	7.85	14.3 13.6	12.5	99
F.T.	.75 .90	.83	5.00	5.2 5.9	5.6	96
J.C.	2.26 2.00	2.13	28.6	32.4 29.8	31.1	101.3
M.	0 0	0	14.9	16.1 17.6	16.9	113.4
S.M.	3.3 2.9	3.1	15.6	17.3 18.8	18.1	96.7

stool and sampling of the dry powdered mixture is not satisfactory since drying of stool requires considerable time, and since vit. A is not stable to heat and oxygen. Preparation of a homogeneous sample from fresh stool was a difficult problem until it was solved by using a magnetic stirrer as described above. The size of the stirring magnet can be varied according to the amount of stool so that even small quantities may be used. Thus well blended mixtures can be prepared from which uniform samples can be taken.

Summary. 1. A method is described for the quantitative determination of vit. A in stool. 2. Alcoholic alkali is added to fresh stool and a uniform mixture prepared by using a magnetic stirrer. The method of extraction is described in detail. The standard percentage deviation of the difference between 2 determinations on the same stool was shown to be 5.2% and the standard deviation of the per cent recovery of added vit. A is 7.5%.

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Received April 17, 1952. P.S.E.B.M., 1952, v80.

Protogen and its Relation to Ketoacids in Metabolism of Tetrahymena.* (19604)

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The function of protogen (thioctic acid, Factor II, acetate factor, pyruvate oxidation factor, α-lipoic acid(1-6)) in the metabolism of lactic acid bacteria(4,5,7) appears to be concerned solely with the oxidation of pyruvate with the simultaneous production of acetate. In the case of *Tetrahymena geleii*, however, it has been shown that acetate

* Aided by a grant from the Research Corporation.
† Generously supplied by Dr. E. L. R. Stokstad of the Lederle Laboratories.

merely spares and does not replace protogen (8).

Cultures of *Tetrahymena* grown in acetate-free protogen deficient media showed only limited growth followed by death and accompanied by a drop in pH (to 4.0 or below). A similar observation has recently been made by Slater(9) on another strain of this organism. The object of this report is to identify the acids accumulated in such cultures.

Methods. The organism used was *Tetrahymena geleii* strain W. Cultures were grown in Medium A80(10). The concentrations of protogen[+] and acetate were varied in different experiments. The effect of the addition of various acids of the tricarboxylic acid cycle and of various fatty acids was also studied. The presence of carbonyl compounds in the cultures was demonstrated by preparation of the 2,4-dinitrophenyl hydrazones by the usual procedures after deproteinization with trichloracetic acid. The dinitrophenyl hydrazones (DNPH) were extracted with either ethyl acetate, if to be used for paper chromatography, or with xylene. Tentative identification of these compounds was made on paper chromatograms by comparing them to the DNPH of commercial samples of pyruvic, α-ketoglutaric, oxalacetic(11) and acetoacetic acids, using the *n*-butanol-ammonia solvent of Cavallini *et al.*(12). The materials were further characterized by differential solubility (13) in xylene and by their absorption spectra.

Results. Table I shows the R_f values obtained from paper chromatograms of the DNPH of various known compounds as well as those for a culture deficient in protogen. It will be noted that all of the known compounds, except acetoacetate, give 2 spots in this system(14). Deficient cultures give spots corresponding closely to those of pyruvic acid DNPH as well as spots which might be those of either oxalacetic or α-ketoglutaric acid DNPH. Other solvents failed to differentiate these 2 compounds. There is in addition another spot moving close to the solvent front, as does that of acetoacetic acid DNPH. No attempt has been made to identify these 2 compounds, however.

In order to determine the nature of the material showing spots with R_f values of 0.07 and 0.72, the DNPH of deficient cultures were extracted with xylene to remove the DNPH of pyruvic acid. The residue was then made alkaline with 2 N NaOH and the absorption spectrum determined. As may be seen from Table II, the ratios of the absorptions at 430 mμ and 500 mμ show better agreement between the material obtained from the cultures and α-ketoglutaric acid than between the former and oxalacetic acid. The same is true of the absorption maximum. It is probable that more than one compound is present in the residual fraction.

TABLE I. R_f Values Obtained from Paper Chromatograms of the 2,4-dinitrophenyl Hydrazones of Keto Acids. (Solvent: Butanol-ammonia; ascending.)

Acids	R_f values
Pyruvic acid	.36; .59
Oxalacetic "	.10; .70
α-ketoglutaric "	.09; .70
Acetoacetic "	.96
Protogen deficient culture	.07; .38; .58; .72; .92

TABLE II. Spectral Absorption Data of the 2,4-dinitrophenyl Hydrazones of Keto Acids.

Acids	Ratio, 430 mμ:500 mμ	Max mμ
Oxalacetic acid	1.39	442
Pyruvic "	1.55	438
α-ketoglutaric "	1.98	428
Protogen deficient culture Non-pyruvic acid fraction	1.79	428–430

Relative concentrations of the xylene-soluble and the xylene-insoluble fractions of cultures containing varying amounts of protogen with or without added acetate or fatty acid were also followed. Fig. 1 and 2 show the relationship between growth, pH and the accumulation of carbonyl compounds in such cultures. It is to be noted that in cultures containing no acetate the greatest rise in the non-pyruvic acid carbonyl compounds occurs at protogen concentrations in which the pyruvic acid has begun to decline (Fig. 1), while in cultures containing acetate or fatty acid (pentadecylate, Fig. 2) the 2 are more or less parallel. All carbonyl compounds fall to a low level as the amount of protogen approaches an amount adequate for growth (1 unit per ml). Omission of glucose from

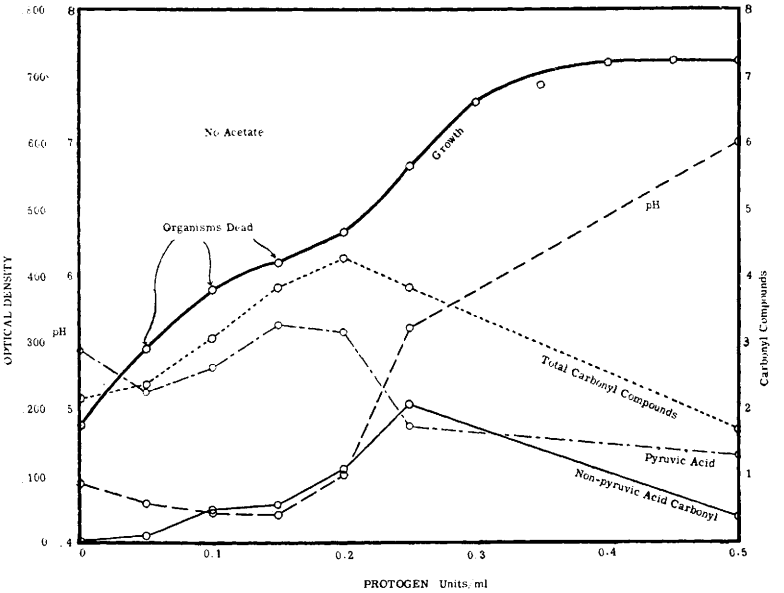

FIG. 1. Relationship between growth, final pH of medium and accumulation of carbonyl compounds as protogen content of medium is increased. Growth (optical density) and pH on left hand scale. Concentration of carbonyl compounds in arbitrary units on right hand scale.

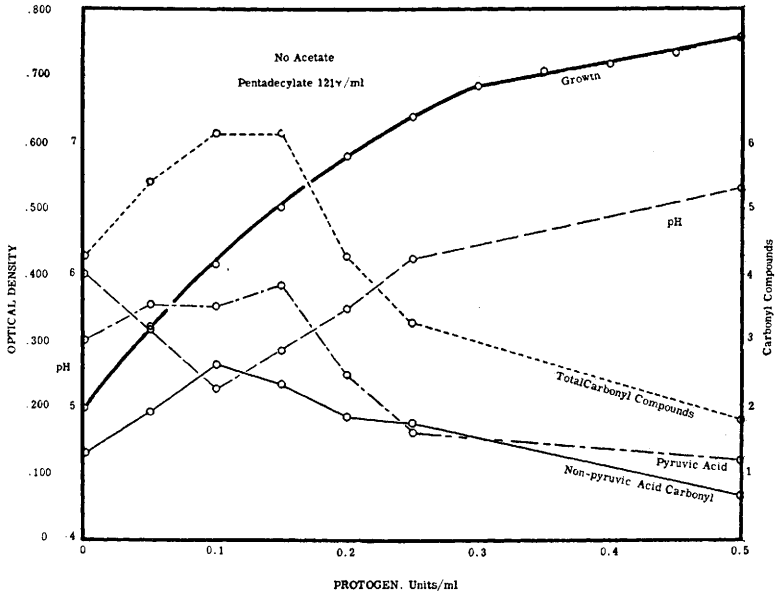

FIG. 2. Relationship between growth, final pH of medium and accumulation of carbonyl compounds as protogen content of medium is increased. Pentadecylate present, .5 micromole per ml. Growth (optical density) and pH on left hand scale. Concentration of carbonyl compounds on right hand scale (arbitrary units).

the medium prevents the accumulation of acid, while the omission of glutamic acid does not.

Since the organisms do not die when acetate is present in the medium, even though acids are accumulated to approximately the same extent in its presence or in its absence, a part of the effect of acetate must be attributed to

its buffering capacity. A part of the "buffering effect" may be due to the contribution of Na^+ to the medium as the acetate is oxidized. A similar effect is obtained if the pH of the medium is adjusted to 7.6 before inoculation and the organisms are not killed by acid accumulation. The fact that fatty acids (valerate through stearate) also have a sparing effect on protogen even though present in amounts too small to contribute significant amounts of Na^+, indicates that acetate has a real effect in and of itself, since fatty acids may be considered to be a source of "active acetate." In fact fatty acids are a more efficient source of "acetate" than is acetate itself, since maximal effects are obtained with 0.5 micromole of fatty acids of 5 or more carbons while approximately 4.0 micromoles of sodium acetate are required. Pentadecylate was chosen to replace acetate for use in these studies because the lower fatty acids (propionate and butyrate) are relatively ineffective in replacing acetate, those of intermediate chain length (caproate through laurate) cause considerable inhibition of growth and those of longer chain length (palmitate and stearate) are too insoluble for practical use. Valerate and pentadecylate represent compromises between these factors. The Krebs cycle acids tested (citric, *cis*-aconitic, succinic, malic, fumaric and oxalacetic, added as their sodium salts) were effective in preventing death of the organisms only in so far as they contribute Na^+, since the sodium salts of such unnatural materials as *trans*-aconitic acid, maleic or malonic acids were equally effective.

Discussion. Although the acids accumulated by cultures of *Tetrahymena* deficient in protogen have been identified only tentatively as consisting mainly of pyruvic and α-ketoglutaric acids, the presence of the former was suspected from the known function of protogen in the oxidation of pyruvic acid by lactic acid bacteria. The accumulation of a second acid only as pyruvate disappears and its increased concentration in the presence of acetate or a source of "acetate" suggests that the acid is a Krebs cycle intermediate requiring acetate for its formation. Since the material contains a carbonyl group, oxalsuccinic, α-ketoglutaric and oxalacetic acids are possibilities. Of these α-ketoglutaric appears to be the most probable, not only because of its greater stability, but because of the similarity of the reactions involved in its oxidative decarboxylation to those with pyruvic acid. Both reactions are known to require thiamine pyrophosphate, diphosphopyridine nucleotide and Coenzyme A, and both reactions yield a large amount of energy. This appears not to be true of the decarboxylation of oxalacetic acid. That the α-ketoglutaric acid does not represent merely a deamination product of glutamic acid is demonstrated by its presence in cultures grown in the absence of added glutamic acid and the failure of the cultures grown in the absence of glucose to accumulate acid.

In view of the ability of lactic acid bacteria to utilize acetate in the absence of protogen (acetate factor), it was hoped that a similar substitution could be made in the case of *Tetrahymena*. Consequently a mixture of acetate and succinate was supplied in cultures lacking protogen. No growth occurred. The failure to grow on this mixture may be only an indication of the impermeability of the cells to succinic acid as suggested by Seaman and Houlihan(15). This explanation seems rather improbable, however, in view of the fact that these organisms have mouths and that material is taken into food vacuoles. It therefore appears probable that protogen also functions at some point in the metabolism of *Tetrahymena* other than in the production of acetic and succinic acids.

Summary. *Tetrahymena geleii* strain W was grown in media in which the concentrations of protogen and of acetate were varied. The effect of additions of Krebs cycle intermediates and of fatty acids to such media was studied. In deficient media the organisms grow to a limited extent and then die. Death is due to the acidity of the medium. The acids accumulated have been tentatively identified as consisting mainly of pyruvic and α-ketoglutaric acids using paper chromatography and differential solubility of the 2,4-dinitrophenyl hydrazones. α-ketoglutaric acid accumulates at the expense of pyruvic acid in the absence of added acetate. In the presence of acetate or of higher fatty acids pyruvic acid

and α-ketoglutaric acid accumulate in about equal amounts. A mixture of succinate and acetate does not replace protogen. A function other than the oxidation of pyruvic and α-ketoglutaric acids in the metabolism of *Tetrahymena* is postulated.

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Received April 28, 1952. P.S.E.B.M., 1952, v80.

Effect of Hyaluronidase on Edema Following Experimental Cold Injury.* (19605)

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The formation of edema following experimental frostbite is accompanied by rising interstitial fluid pressure and distortion of tissues which may cause obstruction of capillaries(1,3,5). The resulting stasis with limited exchange of metabolites would suggest that the accumulating edema is a contributing factor in the resulting tissue loss(3,5). Several attempts therefore have been made to control the fluid loss from the blood stream into the injured tissues(2,3,8,10,11,12). In our experiments we attempted to achieve a similar objective by use of hyaluronidase. This enzyme reduces the resistance of tissues to fluid absorption and increases tissue permeability(4,7). We have investigated whether facilitation of absorption of edema fluid with corresponding decrease in swelling would influence the appearance and extent of the resulting gangrene.

Material and methods. Six adult albino rabbits weighing 2450 to 2850 g were used. All animals were anesthetized with pentobarbital sodium intraperitoneally. Both depilated hind legs were immersed for 4 minutes in a medium consisting of ethylene glycol, alcohol and water, previously cooled to a temperature of —35°C to —37°C. The exposed extremities were allowed to thaw in air at room temperature. Hyaluronidase (Schering) was injected subcutaneously into the edematous tissues of one of the injured extremities using aseptic technic. The other extremity served as a control. One hundred and fifty turbidity-reducing units (TRU) of the enzyme, dissolved in 1 cc of distilled water, were injected in 3 different places on both the medial and lateral surfaces of the extremity. Thus a total dose of 300 TRU was injected into one extremity in 24 hours. The first injection was made 3 hours after exposure to cold, the other 24 hours later. The changes in volume of both extremities representing actual alterations in edema formation were determined by measuring the amount of water displaced during immersion of each extremity to a standard

* This work was supported by a grant-in-aid from the Defence Research Board of Canada.