THE CULTIVATION OF RUMEN OLIGOTRICH PROTOZOA

III. WHITE CLOVER FACTORS WHICH STIMULATE THE GROWTH OF ENTODINIA

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In an attempt to cultivate entodinia in a chemically defined medium, the nutritional requirement was studied by replacing the fractions of white clover with known substances. Nutrients, including the fractions of white clover, were given to the protozoa in a particulate form to facilitate the uptake of foods.

It was found that the lipid fraction was essential and that the water-soluble fraction was stimulatory for protozoal growth in a medium containing casein, proteose-peptone, and amylopectin. The water-soluble fraction was replaceable by soluble starch, glucose, ribonucleic acid, vitamins, and minerals. The lipid fraction was further fractionated systematically, and the results showed that the essential factors were contained in the digitonin-precipitated fraction. Three sterols, *i.e.*, β -sitosterol, stigmasterol, and campesterol, were identified in this fraction. The requirement for white clover lipids by entodinia was completely satisfied by these sterols. Cholesterol was also effective, but ergosterol, lanosterol, and squalene had no effect.

Entodinia were successfully grown for more than 100 days in the medium which consisted of casein, proteose-peptone, amylopectin, soluble starch, glucose, ribonucleic acid, vitamins, minerals, and β -sitosterol.

Although rumen oligotrich protozoa have been successfully maintained *in vitro* many times, the culture medium used in each case has not been defined chemically. Attempts were made by QUINN *et al.* (1) to grow rumen protozoa in continuous culture in chemically defined media, but the protozoa were maintained for only 7 days. As reported in the previous paper (2), protozoa did not grow for prolonged periods in the medium reported by QUINN *et al.* In the previous paper, we reported that entodinia grow satisfactorily in a salt medium containing whole egg protein, Difco proteose-peptone, rice starch,

Difco yeast extract, cellulose powder, and white clover juice, although proteose-peptone, yeast extract, and cellulose powder can be omitted.

The experiments reported in this paper were conducted in an attempt to identify the essential factors present in white clover.

MATERIALS AND METHODS

Source of protozoa. The protozoa for inoculation were taken from the stock cultures which had been maintained for several months on Medium E under Condition B (2). The protozoa in these cultures were only species of *Entodinium* (principally, *E. caudatum*, *E. longinucleatum*, and *E. simplex*). The original source of the protozoa was described previously (3).

Separation of protozoa from debris and bacteria. Large fragments of the clover powder remaining in the stock cultures were removed by filtration through four layers of gauze, and small fragments and bacteria were separated by centrifugation at $400 \times g$ for 5 min. The precipitated protozoa were washed three times (suspended in 0.8% NaCl solution and centrifuged). To remove still smaller debris, these washed protozoa were incubated anaerobically for 5–6 hr in a mineral salt solution (2) with antibiotics (3) and amylopectin. Then, the upper layer with floating debris was decanted, and the protozoa at the bottom were collected by centrifugation and washed three times. These protozoa were used as an inoculum.

Basal culture media. The mineral salt solution reported previously (2) was used as the basal culture fluid, to which the following materials were added (mg/100 ml fluid): Nitrogen and energy sources were casein (Hammersten, E. Merck; 25), proteose-peptone (Difco; 25), and amylopectin (Tokyo Chemical Industry Co., Ltd.; 250). These materials are hereafter referred to as 'the basal substrate.' Supplements were vitamins and minerals (Table 1), ribonucleic acid (RNA, Daiichi Pure Chemicals Co., Ltd.; 12.5), soluble starch (25), and glucose (12.5). These supplements plus the basal substrate are hereafter referred to as 'the basal medium.'

White clover juice. Fresh leaves of white clover (*Trifolium repens*; 100 g, moisture ca. 80%) were homogenized in a Waring blender with 100 ml of water, and the homogenate was filtered through two layers of gauze. This filtrate is referred to as 'the clover juice'. The amount of the juice added was 2 ml per 100 ml medium.

Extraction of lipids from eggs, soybeans, and oat leaves. Content of a fresh egg, fresh oat leaves, and dried soybeans (finely ground) were extracted twice with ca. 20 volumes each of chloroform-methanol (1:1) at room temperature for 24 hr. The extracts were separated from the residue by filtration through a filter paper, and the filtrate was shaken with one-half volume of water. The chloroform layer was collected and evaporated to dryness to measure the weight. The crude lipids, after being adsorbed to amylopectin particles (see below), were used for the experiments. In each case, lipids

Table 1. Composition of vitamins and minerals for the culture of entodinia.

<i>Vitamins^a</i>			
Ascorbic acid	375	Pyridoxine-HCl	10
α -Tocopherol	10	Riboflavin	15
Biotin	50	Thiamine	10
Calciferol ^b	2	DL-Thioctic acid	25
Calcium pantothenate	50	Vitamin A ^b	25
Choline chloride	250	<i>Minerals^a</i>	
Cyanocobalamin	0.5	(NH ₄) ₆ MoO ₂₄ ·H ₂ O	25
Folic acid	5	CoCl ₂ ·6H ₂ O	2.5
Inositol	250	CuSO ₄ ·5H ₂ O	2.5
Menadione	250	FeSO ₄ ·7H ₂ O	250
Niacin	100	I ₂	0.25
<i>p</i> -Aminobenzoic acid	250	MnSO ₄ ·4H ₂ O	250
		ZnSO ₄ ·7H ₂ O	2.5

^a μ g/100 ml of medium.^b I.U./100 ml of medium.

equal to 5 mg were added per 100 ml medium.

Fractionation of white clover. In the first step, lipids were extracted from fresh leaves of white clover (100 g) with 500 ml of chloroform-methanol (1 : 1) by homogenizing in a Waring blender for 20 min. The homogenate was filtered through a filter paper to separate the extract. The residue was extracted three times with 300 ml each of the same solvent at room temperature for 24 hr, separating the extract from the residue by filtration. The extracts were combined and shaken with 800 ml of water to remove water-soluble substances. The chloroform layer was then collected and thoroughly dehydrated over K₂CO₃. This fraction is referred to as 'the total lipid fraction.' The aqueous layer remaining after removal of the chloroform layer was concentrated *in vacuo* with a rotary evaporator to *ca.* 1/100 of its volume. This concentrate was combined with the residue from the above filtration, and this mixture was extracted three times with 300 ml each of water at 5–10° for 24 hr. The extracts ('the water-soluble fraction') were separated from the residue ('the residual fraction') by filtration.

In the second step, the total lipid fraction, after the removal of chloroform, was dissolved in 500 ml of acetone. The mixture was centrifuged at 500×*g* for 5 min and the supernatant was used as 'the acetone-soluble fraction.' The precipitate, after being washed twice with acetone, was extracted with chloroform-methanol (2 : 1), and the extracts were used as 'the acetone-insoluble fraction.'

In the third step, the acetone-soluble fraction was saponified with KOH in 100 ml of ethanol-xylene (2 : 1) under reflux (*ca.* 95°) for 3 hr. After saponification, 300 ml of water was added and the unsaponified substance

('the unsaponifiable fraction') was extracted three times with 300 ml each of petroleum ether. After the aqueous layer was acidified with HCl, the saponified substance ('the saponifiable fraction') was extracted three times with 300 ml each of petroleum ether.

The unsaponifiable fraction was further fractionated as follows: This fraction was dissolved in 100 ml of 90% ethanol, and 20 ml of the ethanolic solution of digitonin (0.5%, w/v) was added to this solution. After being heated at 70° for 20 min, the mixture was kept overnight at room temperature and then centrifuged at $1,000\times g$ for 5 min. The precipitate was washed successively with 90% ethanol, acetone, and hexane. These digitonides were dissociated with 100 ml of pyridine by heating at 90° for 30 min, and extracted twice with 200 ml each of ether. This extract is referred to as 'the digitonin-precipitated fraction.' The supernatant fluid from the above centrifugation was treated with 5 ml of the digitonin solution and centrifuged again. This supernatant is referred to as 'the digitonin-supernatant fraction.'

These procedures are summarized in Fig. 1. Each fraction derived from 5 g of fresh clover leaves was added to each 100 ml medium.

Preparation of particulate foods. The foods for the protozoa were prepared as follows: Nutrients (except minerals), including the juice and fractions of white clover, were dissolved in water or organic solvents, and were adsorbed to amylopectin particles (*ca.* 5 μ in diameter) by evaporating the solvents with a rotary evaporator. Then, these particles were coated with a synthetic polymer, polyvinylacetal diethylaminoacetate (Sankyo Co., Ltd; this polymer is soluble in water of below pH 5.8, and in various organic solvents), *i.e.*, the coating material (0.5 g) dissolved in hexane (100 ml) was mixed with the particles prepared as above (10 g), and then the solvent was evaporated by using a rotary evaporator. To prevent the aggregation of particles, Tween 80 (0.1 g) and Silicone oil (0.5 g) were added.

Culture conditions. Protozoa were cultured in 200-ml flasks containing 200 ml of the medium. The procedures in the initial stage were the same as those described previously (2). Culture maintenance was as follows: The particulate foods described above were given every day, and every 2 or 4 days cultures were diluted, *i.e.*, one-half volume of the culture was removed and replaced by an equal volume of a mineral salt solution (2). The minerals listed in Table 1, when supplemented, were added to this mineral salt solution. Experiments were carried out in duplicate, and the average counts of protozoa were adopted. The counting method was described previously (2, 3).

Source of sterols used for cultures. In the experiments to examine the effect of sterols on protozoal growth, the following commercial preparations were used: Stigmasterol, β -sitosterol, cholesterol, ergosterol, and lanosterol were purchased from Tokyo Chemical Industry Co., Ltd. As this preparation of β -sitosterol was found to be mixed with campesterol (β -sitosterol : campesterol = 60 : 40) by gas-liquid chromatography, another preparation (95 : 5,

Fig. 1. Fractionation of white clover.

Applied Science Laboratories Inc.) was used in subsequent experiments. Campesterol (99% pure) was also purchased from the Applied Science Laboratories Inc.

Thin-layer chromatography. Thin-layer chromatography was conducted by the method of KIRIBUCHI *et al.* (4). Glass plates (5×20 cm) were coated

with Silica Gel G (prepared according to Stahl, E. Merck) and heated at 110° for 30 min. The plates were developed with either chloroform-methanol-acetic acid-water (90 : 8 : 1 : 1) or benzene-ethyl acetate (5 : 1), and spots were detected by spraying 50% H₂SO₄ and heating at 110° for 10 min.

Gas-liquid chromatography. Sterols were analyzed by gas-liquid chromatography on a 1.5-m glass column of 4 mm in diameter, packed with Chromosorb W coated with 1.5% OV-17 (80–100 mesh). The instrument used was Shimadzu gas chromatograph GC-4ATF, equipped with a hydrogen-flame ionization detector. The column was maintained at 270°, and the pressure of carrier gas (nitrogen) was 1.0 kg/cm². Individual sterols were identified by comparing the relative retention time of the sample with that of the authentic sterols which had been identified in the laboratory of Chemistry Division, The Institute of Applied Microbiology, The University of Tokyo.

Mass spectrometry. Mass spectra were measured with a Hitachi RMU-6L mass spectrometer connected with a gas chromatograph (Hitachi K-53), under the ionizing voltage of 70 eV.

RESULTS

Attempts to facilitate the ingestion of food by entodinia

It has been described in the previous paper (2) that entodinia readily ingest particulate substances and fail to grow on soluble substances alone. Preliminary experiments showed that the essential factors in white clover juice were associated with the particles in it, since the supernatant fluid of the juice obtained by centrifugation (18,000×*g*, 20 min) had little effect on protozoal growth, and the precipitate was as effective as the juice. When the extracts of white clover were added in solution, the stimulatory effect was diminished. Therefore, it seemed necessary to supply the protozoa with foods in the particulate form.

Figure 2 shows the effect of particulate foods on entodinal growth. The protozoa receiving the particles to which nutrients were adsorbed grew better than those receiving nutrients in the soluble form. The coated particles supported growth better than did the non-coated ones. When the coated particles were used, limited growth of bacteria was observed by microscopic examination. Accordingly, the amount of bacterial cells taken up by the protozoa was considered to be comparatively small. Under these conditions the stable growth of protozoa was obtained. In all the experiments described below, coated particulate foods were used.

White clover factors stimulating the growth of entodinia

The first experiment was conducted to examine whether the essential factors could be extracted with either chloroform-methanol (1 : 1) or water. In the presence of the basal substrate, combining the total lipid and the water-soluble fractions resulted in good growth, but the residual fraction had no

Fig. 2. Growth of entodinia on particulate foods.

The foods containing the basal substrate and white clover juice were given in the form of; ■, solution (except amylopectin); ▲, non-coated particles; ●, coated particles. The dotted lines indicate the dilution of cultures. Details are given in the text.

effect (Fig. 3A). To determine which of the former two fractions is required by entodinia, a second experiment was made. As shown in Fig. 3B, the total lipid fraction allowed protozoal growth, but the water-soluble fraction did not. The best growth, however, was obtained when both the fractions were added, suggesting that the total lipid fraction is essential and the water-soluble fraction is stimulatory under the conditions of this experiment.

The water-soluble fraction of white clover

The purpose of the subsequent experiments was to replace the water-soluble fraction with known substances. First of all, the fraction was replaced with the vitamins and minerals listed in Table 1. As shown in Fig. 4A, in the presence of the basal substrate and the total lipid fraction, the vitamins and minerals did not sufficiently permit growth, although slight stimulation was observed. Subsequently, RNA was added in lieu of the water-soluble fraction (in addition to the basal substrate, the total lipid fraction, the vitamins, and the minerals). The results (Fig. 4B) indicate that the water-soluble fraction can be omitted when RNA is added. However, growth was slightly better in the medium containing the water-soluble fraction. As seen in Fig. 5, the lower-molecular carbohydrates had some effect, though slight, on entodinal growth (A), but no additional stimulation was obtained by the addition of sugars (B). These results seem to suggest that readily fermentable

Fig. 3. Effect of fractions of white clover on entodinal growth (I).

(A) The media contained the basal substrate and fractions of white clover; $\circ$, total lipid, water-soluble, and residual fractions; $\blacktriangle$, total lipid and water-soluble fractions; $\triangle$, residual fraction; $\bullet$, clover juice; $\blacksquare$, none. (B) The media contained the basal substrate and fractions of white clover; $\circ$, total lipid and water-soluble fractions; $\bullet$, total lipid fraction; $\square$, water-soluble fraction; $\blacksquare$, none.

carbohydrates slightly promote growth, but that no carbohydrate has any special function in entodinal metabolism. The growth with these carbohydrates was almost comparable to that observed with the water-soluble fraction. Thus, the water-soluble fraction was replaceable by known compounds.

To test whether the vitamins and minerals were necessary for growth, entodinia were cultured in the medium of the basal substrate, the total lipid fraction, RNA, soluble starch, plus glucose, with and without vitamins and minerals (Fig. 6). Omission of the vitamins resulted in the death of the protozoa, indicating that at least one of the vitamins was indispensable. Without the minerals, on the other hand, the protozoa continued growing for 20 days, although the growth rate gradually decreased during the latter period of the experiment. The best growth was obtained when both vitamins and minerals were added.

Lipids from origins other than white clover

In order to ascertain whether the essential factors exist in lipids from origins other than white clover, the effects of lipids from eggs (as an animal origin), soybeans (as a seed origin), and oat leaves (as a grass origin) were studied by using the basal medium.

Fig. 4. Effect of vitamins, minerals, and RNA on entodinal growth.

(A) The media contained the basal substrate, total lipid fraction, and the following substances; ▲, water-soluble fraction; ●, vitamins plus minerals; ■, none. (B) The media contained the basal substrate, total lipid fraction, vitamins, minerals, and the following substances; ▲, water-soluble fraction; ●, RNA (12.5 mg/100 ml medium); ■, none.

Fig. 5. Effect of carbohydrates on entodinal growth.

The media contained the basal substrate, total lipid fraction, vitamins, minerals, RNA, and various carbohydrates. (A) ▲, Water-soluble fraction; ●, soluble starch (25 mg/100 ml medium); ○, glucose (12.5 mg/100 ml medium); ■, none. (B) ▲, Water-soluble fraction; ●, glucose+maltose+cellobiose+sucrose+fructose+xylose+ribose (10:4:4:4:4:4:1, 50 mg/100 ml medium); ○, soluble starch+glucose (1:1, 50 mg/100 ml medium); ■, none.

Fig. 6. Effect of vitamins and minerals on entodinal growth.

The media contained the basal substrate, total lipid fraction, RNA, soluble starch, glucose, and the following substances; ●, vitamins plus minerals; ▲, vitamins; ○, minerals; ■, none.

The results in Fig. 7A indicate that egg lipids had some effect but they were slightly less effective than white clover lipids. As seen in Fig. 7B, however, the growth-stimulating effect was comparable among the lipids of soybeans, oat leaves, and white clover. This suggests that the essential factors exist widely in animal and plant lives, and that the lipids from plant origins are a little more effective than those from animal origins.

The total lipid fraction of white clover

In attempts to find the essential factors, the total lipid fraction from white clover was further fractionated and the effect of each fraction was studied. Figure 8A shows the results obtained when the acetone-soluble and -insoluble fractions were added. On the basal medium containing the former fraction, entodinia grew satisfactorily. The acetone-insoluble fraction did not permit growth, and combination of the two fractions showed no more effect than did the acetone-soluble fraction alone. Therefore, the essential factors were included only in the acetone-soluble fraction. Similarly, the results shown in Fig. 8B indicate that the growth-promoting factors are contained in the unsaponifiable fraction, not in the saponifiable fraction. As shown in Fig. 9A, growth was found to be stimulated by the digitonin-precipitated fraction, but not by the digitonin-supernatant fraction.

The following experiment was designed to prove that the growth factors exist exclusively in the digitonin-precipitated fraction and that other fractions have no effect on protozoal growth. As shown in Fig. 9B, no stimulatory effect was observed in the culture receiving the acetone-insoluble, the saponifiable, and the digitonin-supernatant fractions simultaneously.

Fig. 7. Effect of lipids from various origins on entodinal growth.

Entodinia were grown in the basal medium to which were added the lipids from the following origin. (A) ●, Whole egg content; ▲, white clover leaves; ■, none. (B) ●, Soybeans; ▲, oat leaves; ○, white clover leaves; ■, none.

Fig. 8. Effect of fractions of white clover on entodinal growth (II).

Entodinia were grown in the basal medium to which were added the following fractions. (A) ●, Acetone-soluble fraction; ○, acetone-insoluble fraction; ▲, acetone-soluble and -insoluble fractions; △, none. (B) ○, Saponifiable fraction; ●, unsaponifiable fraction; ▲, saponifiable and unsaponifiable fractions; △, none.

Fig. 9. Effect of fractions of white clover on entodinal growth (III).

Entodinia were grown in the basal medium to which were added the following fractions. (A) ●, Digitonin-precipitated fraction; ▲, digitonin-supernatant fraction; ○, digitonin-precipitated and -supernatant fractions; △, none. (B) ●, Digitonin-precipitated fraction; ▲, acetone-insoluble, saponifiable, and digitonin-supernatant fractions; ■, none.

Identification of the growth factors

Since the essential factors are precipitated by digitonin, it is highly possible that they are steroids having a 3-hydroxy group of the β -configuration, or are higher alcohols. The presence of steroids in white clover leaves was confirmed by the following qualitative tests: The digitonin-precipitated fraction was positive to the LIEBERMANN-BURCHARD (5, 6) and the SALKOWSKI (7) reactions, indicating that white clover contains abundant sterols. Since the fraction was negative to the ROSENHEIM (8) and the TORTELLI-JAFFÉ (9) tests, it was considered that the fraction does not contain sterols having a conjugated double bond in the B-ring, nor a double bond at the position C-8 to any detectable degree.

On thin-layer chromatography, the digitonin-precipitated fraction (Fig. 10B and C) migrated to the same R_f value as that of stigmasterol. (Under the analytical conditions employed, sterols such as β -sitosterol, stigmasterol, campesterol, cholesterol, and ergosterol showed the same R_f value.) In the case of the unsaponifiable fraction (Fig. 10A), a clear spot appeared at the position corresponding to stigmasterol, with four faint spots in other positions. The latter spots are not steroids, because they were quite different in color from the former.

The gas chromatographic analysis of the unsaponifiable fraction (Fig. 10D) gave three major peaks, which corresponded to β -sitosterol, stigmasterol, and

Fig. 10. Chromatograms of sterols in white clover.

(A, B, and C) Thin-layer chromatograms: Stigmasterol (a), unsaponifiable fraction (b), and digitonin-precipitated fraction (c) were spotted on the chromatoplates coated with Silica Gel G. Solvent systems were chloroform-methanol-acetic acid-water (90 : 8 : 1 : 1, A and B) and benzene-ethyl acetate (5 : 1, C). (D) Gas chromatogram: The unsaponifiable fraction was chromatographed on Chromosorb W coated with 1.5% OV-17, at 270°, under the nitrogen gas pressure of 1.0 kg/cm², by using a hydrogen flame ionization detector. The peaks a, b, and c correspond to campesterol, stigmasterol, and β-sitosterol, respectively.

campesterol, based on the relative retention time. The peak area corresponding to β-sitosterol was the greatest.

These three peaks were further identified by mass spectrometry. The mass spectrum of the peak corresponding to β-sitosterol was identical with that of an authentic β-sitosterol; *m/e* 414 (*M*⁺), 399 (*M*⁺-CH₃), 396 (*M*⁺-H₂O), 381 (*M*⁺-CH₃-H₂O), 273 (*M*⁺-branched chain), 255 (*M*⁺-branched chain-H₂O), 231 (D-ring fission), 213 (D-ring fission-H₂O), 145 (C-ring fission-H₂O). Similarly, the peak corresponding to stigmasterol was; *m/e* 412, 397, 394, 379, 273, 255, 231, 213, 145, and the peak corresponding to campesterol was; *m/e* 400, 385, 382, 367, 273, 255, 231, 213, 145.

Replacement of white clover lipids by known compounds

Figure 11A shows the results obtained when the mixture of β-sitosterol, campesterol, and stigmasterol was added to the basal medium instead of white clover lipids. These sterols were as effective as the digitonin-precipitated fraction.

Subsequently, the effect of fatty acids (palmitic, stearic, oleic, linoleic, linolenic, and arachidonic acids) and lecithin (a mixture of α- and β-forms, prepared from eggs) was tested, because it was speculated that the unsaturated fatty acids and lecithin in white clover, even though these compounds were

Fig. 11. Effect of sterols, fatty acids, and lecithin on entodinal growth.

Entodinia were grown in the basal medium to which were added the following materials. (A) ●, Digitonin-precipitated fraction; ▲, β -sitosterol, campesterol, and stigmasterol (3 : 2 : 5, 1.0 mg/100 ml medium); ■, none. (B) ▲, Digitonin-precipitated fraction; ○, fatty acids (palmitic, stearic, oleic, linoleic, linolenic, and arachidonic acids, each 0.5 mg/100 ml medium); ●, lecithin 0.5 mg/100 ml medium; △, fatty acids and lecithin; ■, digitonin-precipitated fraction, fatty acids, and lecithin; □, none.

included in the material, might be oxidized during the process of extraction, fractionation, and preservation. As shown in Fig. 11B, the fatty acids and lecithin had little effect on the protozoa and could not substitute for the digitonin-precipitated fraction.

The effect of several sterols on the entodinal growth was next examined. Figure 12A shows that cholesterol was effective, stigmasterol more effective, and the mixture of β -sitosterol and campesterol (60 : 40) the most effective, whereas ergosterol was ineffective. This experiment was repeated three times, with substantially the same results each time. The effect of β -sitosterol and campesterol was further examined by using more purified commercial preparations. In this experiment, the effect of lanosterol and squalene was also examined. As shown in Fig. 12B, β -sitosterol (95% pure) was more effective than campesterol (99% pure). Lanosterol and squalene were ineffective.

Thus, white clover could be satisfactorily replaced with known compounds. In the basal medium to which β -sitosterol (1.25 g/100 ml) was added, entodinia were successfully maintained for 100 days, when the experiment was stopped. The population densities during the period were $2\text{--}15 \times 10^4/\text{ml}$.

Fig. 12. Effect of various sterols on entodinal growth.

Entodinia were grown in the basal medium to which the following sterols were added (each 1.25 mg/100 ml medium). (A) ●, Mixture of β -sitosterol and campesterol (60 : 40); ▲, stigmasterol; ○, cholesterol; △, ergosterol; ■, none. (B) ○, 95% β -Sitosterol; ▲, 99% campesterol; ○, lanosterol; △, squalene, ■, none.

DISCUSSION

As far as the authors are aware, the present studies report the first successful attempts to maintain and grow rumen protozoa *in vitro* for an extended period in a chemically defined medium, although the compositions of protein and RNA are not specified. Entodinia were grown on casein, proteose-peptone, amylopectin, soluble starch, glucose, RNA, vitamins, minerals, and β -sitosterol, but it is clear that the protozoa do not require all of these ingredients. Proteose-peptone is not necessary, but it facilitates the preparation of particulate foods because of its ready solubility. The protozoa can grow without soluble starch and glucose (Fig. 5), but these easily fermentable carbohydrates seem to promote growth slightly.

That vitamins and minerals are required was shown (Fig. 6), although the signs of mineral deficiency were slow to appear. Undoubtedly some of the vitamins and minerals are essential for protozoal metabolism. Especially when bacterial activities are limited, it appears that at least some of the vitamin B group must be supplemented.

RNA slightly stimulated growth (Fig. 4B), but it is not certain whether nucleic acids are indispensable or merely stimulatory, *i.e.*, whether entodinia are unable to synthesize the constituents of nucleic acids, or whether they are able to synthesize them but with limited rapidity. Unfortunately, this cannot be confirmed unless the protozoa are grown in the absence of bacteria.

Nevertheless, protozoa must undoubtedly utilize nucleic acids. COLEMAN (10, 11) reported that *Entodinium caudatum* incorporated nucleic acid bases into its nucleic acids with limited interconversion of the bases. It is probable, in the rumen, that protozoal nucleic acids are more or less derived from those of bacteria or feeds. It is known that purines, pyrimidines, and related compounds are required by some species of *Tetrahymena*, *Paramecium*, and other ciliates (12).

This is the first evidence to show that sterols stimulate the growth of rumen protozoa, although the requirement of sterols by other ciliates such as *Paramecium* (13–15), *Tetrahymena* (16–18), *Colpodium* (19, 20), and *Stylonychia* (21) has long been known. The exact role which sterols play in protozoal metabolism is not known, but sterols may be incorporated as structural constituents of the body or they may have some hormone-like function. The responses of entodinia vary with different sterols, just as reported for free-living ciliates. Active sterols may be converted to other compounds which are metabolically active.

The effect of dietary diethylstilbestrol on a number of protozoa in the rumen has been studied (22, 23), but it seems difficult to relate those data to the sterol requirement, because the diet used in those studies contained sterols and diethylstilbestrol differs from sterols in its structure.

There was no evidence that entodinia require fatty acids and lecithin, although the requirement of fatty acid by *Paramecium* has been shown (24, 25). However, the possibility should not be excluded that in the present experiments entodinia in fact may have ingested bacterial fatty acids, though the quantity taken up was considered to be quite small. Again this cannot be confirmed until protozoa are grown in axenic cultures.

The present experiments were carried out with limited growth of bacteria, but it seems obvious that the protozoa took up a small quantity of bacterial body constituents and metabolites. To gain more precise information on the nutrition of rumen protozoa, axenic cultures are eagerly desired.

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