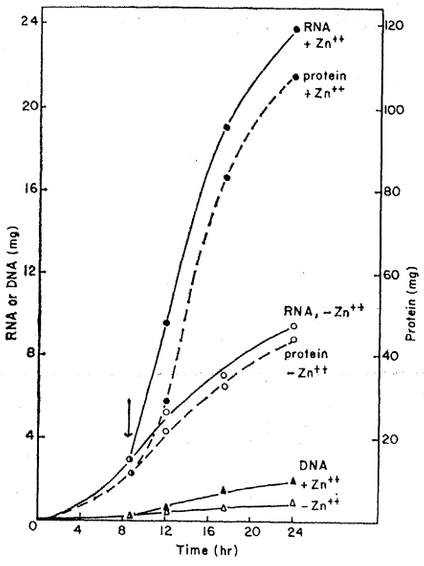

Fig. 1. Effect of zinc on rates of formation of RNA, protein, and DNA; $1.7 \times 10^{-5} M$ Zn^{++} added at time indicated by arrow.

We have determined the effect of adding 5 parts per million of $ZnSO_4$ ($1.7 \times 10^{-5} M$) to a growing culture by comparing the rates of formation of RNA, DNA, and protein in cultures with and without the addition of zinc. Cells were grown in a medium containing 9 g of glucose, 5 g of acid-hydrolyzed, salt-free, vitamin-free casein hydrolysate, 0.5 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $Fe_2(SO_4)_3$, and 0.01 g of $CaCl_2$ per liter of de-ionized distilled water. Cultures (50 ml in 250-ml flasks) were incubated on a rotary shaker (250 rev/min) at 32°C. Zinc sulfate ($ZnSO_4 \cdot 7H_2O$) was added after an initial incubation period of 8.5 hours and samples were taken at suitable intervals thereafter for analysis. Cell weight was determined by filtering on tared filter assemblies and then drying to constant weight at 70°C. Protein content was determined (*5*) after extraction of dried cells with 2*N* NaOH at 100°C for 15 minutes. Free nucleotides, RNA, and DNA were measured by the method of Ogur and Rosen (*6*). Residual glucose in culture filtrates was determined by the method of Folin and Malmros (*7*).

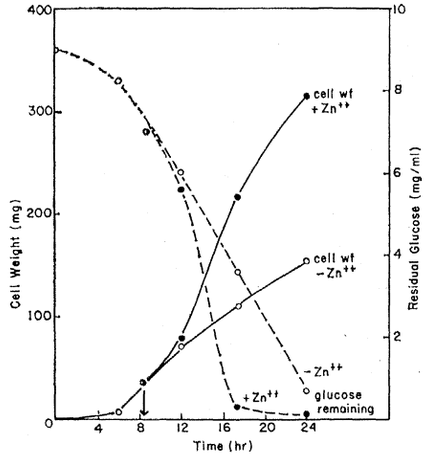

Fig. 2. Effect of zinc on growth and glucose utilization; $1.7 \times 10^{-5} M$ Zn^{++} added at time indicated by arrow.

Addition of zinc immediately stimulated RNA synthesis (Fig. 1). Protein synthesis was also greatly stimulated, but there was a lag before the rate of synthesis was maximum. The increase in RNA during the first 3.5 hours after Zn^{++} addition was 2.7 times as great in the presence of the metal as it was in its absence; in contrast, during the same period, the increase in protein was only 1.6 times as great in the presence of Zn^{++} as in its absence. In the subsequent 5.5-hour period, both RNA and protein increased at the same relative rate. The DNA levels were not increased to the same extent as were RNA and protein.

The pattern of growth and glucose utilization after stimulation by zinc was similar to that found for protein synthesis. Rates did not reach a maximum until 4 hours after the zinc was added (Fig. 2).

The early disproportionate increase in RNA occasioned by Zn^{++} is also shown in Table 1. The RNA content was greatest immediately after the Zn^{++} was added, and it decreased as other cell constituents were synthesized. Protein content was not at a maximum until 9 hours after the addition of Zn^{++}.

The mechanism by which Zn^{++} stimulates RNA synthesis is not known. The increase in free nucleotides, after addition of the metal (Table 1) may indicate a role in the synthesis of purines and pyrimidines or nucleotides, but other explanations are possible. Wacker and Vallee (*8*) found zinc associated with RNA from diverse sources and postulated that the metal may play a role in maintaining the configurational stability of the molecule. Also, it is not yet known whether zinc stimulates synthesis of all RNA's, or whether there is a specific effect on soluble, messenger, or ribosomal RNA (*9*).

WARNER S. WEGENER
ANTONIO H. ROMANO

Department of Biological Sciences and
Graduate Division of Microbiology,
University of Cincinnati,
Cincinnati 21, Ohio

References and Notes

1. J. W. Foster and S. A. Waksman, *J. Bacteriol.* **37**, 599 (1939).
2. A. Nason, N. O. Kaplan, S. P. Colowick, *J. Biol. Chem.* **188**, 397 (1951).
3. D. M. Webley, R. B. Duff, G. Anderson, *J. Gen. Microbiol.* **29**, 179 (1962).
4. W. E. C. Wacker, *Biochemistry* **1**, 859 (1962).
5. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
6. M. Ogur and G. Rosen, *Arch. Biochem.* **25**, 262 (1950).
7. O. Folin and H. Malmros, *J. Biol. Chem.* **83**, 115 (1929).
8. W. E. C. Wacker and B. L. Vallee, *ibid.* **234**, 3257 (1959).
9. Supported by grant AI-04739 from the National Institutes of Health.

16 September 1963

Bromine Analysis in 5-Bromouracil-Labeled DNA by X-ray Fluorescence

Abstract. *The degree of replacement of thymine by 5-bromouracil in the deoxyribonucleic acid (DNA) of the thymine-deficient bacterium* Escherichia coli B *15 T⁻ has been determined by a nondispersive x-ray fluorescence method. A measure of the fraction by weight of bromine in the purified DNA is required for determining the degree of thymine replacement. This fraction is experimentally determined on 35-μg samples of DNA by x-ray spectroanalysis.*

A number of investigators have observed the changes in radiosensitivity of deoxyribonucleic acid (DNA) resulting from incorporating halogenated pyrimidines and purines into nucleic acids. The change in radiosensitivity of bacteria (*1*), viruses (*2*), and cell cultures (*3*) as a result of replacing the thymine of DNA by halogenated pyrimidines has been studied.

In this type of study it is essential to establish the degree of replacement of thymidine by its analog. From a review of the literature the extent of replacement has usually been determined by paper chromatography, density gradient studies, or radioactivity measurements of incorporated Br^{82}-bromouracil. Recent work in our laboratory has required a knowledge of the degree to which thymine is replaced by 5-bromouracil in the DNA of the thymine-deficient mutant *Escherichia coli B* 15 T⁻. In order to measure the degree of replacement we have developed a relatively simple technique that depends upon the determination of the content (percent by weight) of bromine in the isolated and purified DNA by nondispersive x-ray emission analysis.

It can be shown, for purified DNA which contains the four bases adenine (A), cytosine (C), guanine (G), and thymine (T), that the fractional replacement of thymine by bromouracil is given by the expression

$$F = \frac{f_{Br}}{f_T} = \frac{f_A M_A + f_T M_T + f_G M_G + f_C M_C}{f_T \left(M_T - M_{Br} + \frac{A_{Br}}{P_{Br}} \right)}$$

where M_A, M_T, M_G, M_C, and M_{Br} are the molecular weights, respectively, of the deoxyribomonophosphates of A, T, G, C, and 5-bromouracil; f_A, f_T, f_G, and f_C are the corresponding fractions in which these bases are found in the unsubstituted DNA of the organism; f_{Br} is the fraction of bases in the labeled DNA which is 5-bromouracil; A_{Br} is the atomic weight of bromine; and P_{Br} is the experimentally determined fraction by weight of bromine in DNA. The f values for *E. coli B* are well established and the molecular weights can be calculated. In calculating the molecular weights of the monophosphates it is assumed that the nucleotides are sodium salts, since they are prepared in saline solution at physiological *p*H. Thus, to ascertain the degree of replacement requires only an experimental determination of the fractional weight of bromine in DNA.

To determine this fraction we have employed the nondispersive x-ray technique (*4*), wherein inner-shell ionizations of the atoms of the sample are caused by x-rays and the energy distribution of the resultant fluorescence radiation is then determined by means of pulse-height analysis of the voltage pulses produced by a proportional counter. With this technique bromine in small amounts of DNA (10 to 35 μg) may be analyzed in short times (1-minute integration) with a high degree of precision and accuracy.

Since the experimental arrangement has some unique features which have not been discussed in detail (*4*), a description of some important aspects of the instrumentation follows. The essential arrangement, as applied to the analysis of bromine, is shown in Fig. 1. The elements of the sample are excited by x-rays emanating from a molybdenum target (Machlett AEG 50S tube operated at 50 kv). The gas in the proportional counter at 1 atm is 90 percent argon and 10 percent methane. The cathode material of the counter is nearly pure magnesium. Selection of cathode material is important because of the possibility that incident photons will excite the elements of the cathode, which would produce interfering lines near that of bromine. The distance from the window of the x-ray tube to the sample and from the sample to the counter window is approximately 4.3 cm. The samples are supported by a single layer of nylon between 900 and 1300 Å in thickness. The nylon support is prepared by floating a 10-percent solution of nylon in isobutyl alcohol on water and lifting the film off with a titanium frame.

To prepare the sample, purified DNA is dissolved in distilled water (1 mg/ml), and a 35-μl fraction is placed on the nylon support with a micropipette. The sample is then dried in a desiccator for about 1 hour and is then subjected to a pressure of 10^{-3} mm-Hg for 1 hour. The dried samples average about 3 mm in diameter and have an average thickness of 5 μ (calculated).

The sample is excited in a specimen chamber evacuated to a pressure of about 35 μ-Hg to reduce x-ray scattering from air. For the arrangement shown in Fig. 1, at atmospheric pressure the intensity of the radiation, in the region of the Mo-Kα line, scattered from air into the counter, is 50 times that from a 35-μg sample of DNA.

In spite of the small weight of the sample, precision of the order reported is achieved by means of a ratio method of obtaining and using the data. The counter pulses are amplified and simultaneously fed into two single-channel pulse-height analyzers. One analyzer, the "element channel," is set to count photons whose energy lies in the vicinity of the energy of the Kα line of bromine. The other, the "scattered-line channel," simultaneously counts photons in the vicinity of the energy of a scattered line in the exciting radiation, MoKα in the case where a molybdenum target x-ray tube is used.

The intensity of scattered MoKα radiation is very nearly proportional to the mass of the sample, since the sample consists of a relatively small percentage of a heavy element in a light matrix and the matrix elements scatter x-rays of these wave lengths with approximately the same intensity per unit mass. For thin samples, the photon counts in the element channel are also directly proportional to the mass.

Hence, for the samples under study the ratio of photon counts in the element channel to photon counts in the scattered-line channel is independent of the mass of the sample. After appropriate correction for background because of scattering from the specimen chamber and nylon film, all the data are expressed as ratios of simultaneous photon counts in the two channels. This ratio is useful only if the number of photons in the scattered-line channel, as a result of air scattering, is small compared to that due to scattering from the sample. This requirement, coupled with the evidence on the magnitude of the contribution from air scattering,

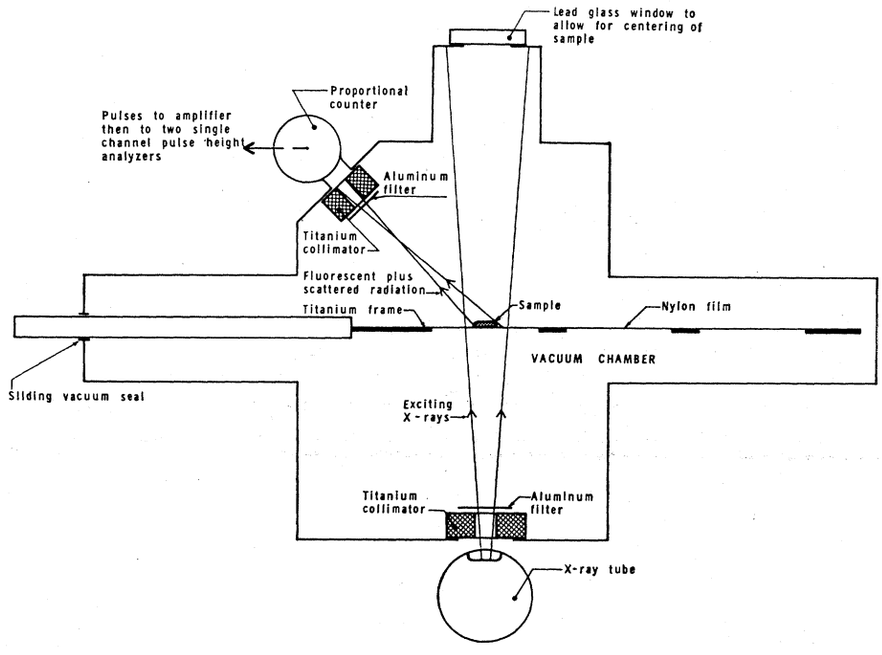

Fig. 1. Arrangement for nondispersive x-ray fluorescence analysis.

make it clear that a vacuum specimen chamber is essential to the method. By using these ratios we overcome the problem of fluctuation of x-ray tube output and make unnecessary the exact reproducibility of the placement of the samples and standards.

As Hall (*5*) has shown, the fraction by weight of bromine can be obtained from the expression

$$P_{Br} = \frac{r_m - r_t}{r_s - r_t} C_{Br},$$

where P_{Br} and C_{Br} are, respectively, the fractions by weight of bromine in the sample and in a known mixture, and r_s, r_t, and r_m are the ratios obtained, respectively, with sample, pure matrix, and known mixture.

In Figs. 2 and 3, the correction for changes in mass and placement of samples, which is automatically accomplished with the ratio method, is artificially introduced by adjusting the x-ray tube current to give the same peak height at the MoKα position in each spectrum in order to show the effectiveness of the method directly and graphically.

The poor resolution in the nondispersive x-ray method limits its application to samples where interferences from extraneous lines are minimum. The absence of interfering lines in the spectra of standards made of known amounts of bromine in simulated DNA (Fig. 2) and the spectra of samples of DNA isolated from *E. coli* and salmon sperm (Fig. 3) indicate that the resolution should be adequate for the detection of bromine in DNA isolated from *E. coli* and other biological sources. Chlorine, which is always present in biological preparations, does not interfere because its Kα line lies very near that of phosphorus, sufficiently removed to be of no concern. Based on the known approximate concentrations of the elements in normal biological preparations, there is no reason to expect interferences from other elements which would disturb a bromine analysis at the bromouracil replacement concentrations of interest (0.1 to 100 percent).

Figure 4 shows that the intensity of the bromine line is linear up to a bromine content of 5 percent, as would be expected for thin samples. A 5.4-percent bromine concentration is equivalent to 100 percent replacement of thymine by 5-bromouracil in the DNA of *E. coli B*. The calibration curve (Fig. 4) obtained from standards of known amounts of bromine in simulated DNA is based on data corrected on the basis of ratios obtained after the subtraction of background.

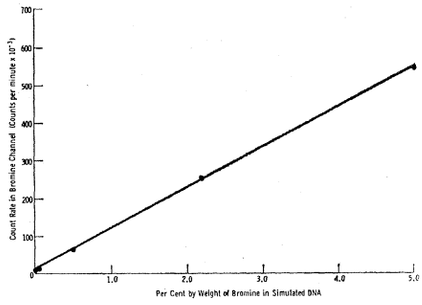

Fig. 4. Calibration curve for bromine in DNA.

The simulated DNA in this study is composed of equal molarity of deoxycytidine-5′-monophosphate and deoxyadenosine-5′-monophosphate, ammonium hydrate. Product analysis of the nuceotides supplied by the manufacturer states the purity of the monophosphates to be 100 percent, based upon nitrogen analyses, implying that the heavy metal content is below a detectable amount.

Sucrose can be used in place of simulated DNA in developing standards. The scattering, per unit mass of sucrose, or other organic samples composed entirely of carbon, oxygen, nitrogen, and hydrogen, would be very nearly equal to that of the simulated DNA since their atomic composition, as far as x-ray scattering is concerned, is very similar. There is no discernible difference between spectra taken with a pure sucrose sample and spectrum 1 shown in Fig. 2.

The limit of detectability determined from the calibration curve is about 10^{-9} g of bromine in a 35-μg sample of DNA for a 1-minute integration time. Loss of detectability is arbitrarily assigned to a signal whose magnitude is less than four times the magnitude of the counting error at background. The background count, counts in the element channel resulting from the presence of a sample with no bromine, is approximately 9000 count/min (Fig. 4). An error of the instrument, due to inadequate subtraction of that portion of the signal caused by scattering from the nylon film, slightly increases

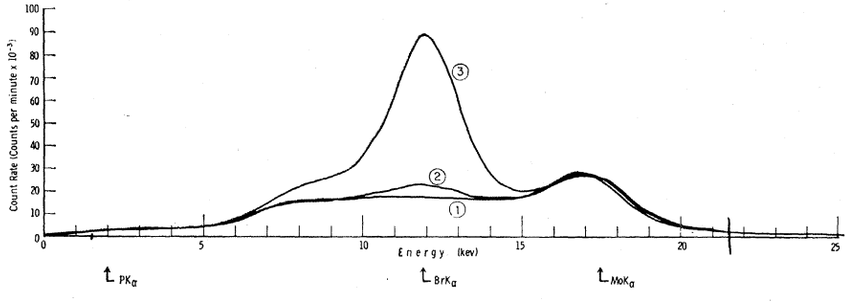

Fig. 2. X-ray nondispersive emission spectra of simulated DNA samples (35 μg) with varying, known amounts of bromine: (i) no measurable bromine, (ii) 0.05 percent bromine, and (iii) 0.5 percent bromine. The peak at 17.5 kev results from scattering of Mo-Kα radiation emitted from the molybdenum target x-ray tube. This scattered line is used in obtaining ratios as described in the text. The relatively strong P-Kα lines produced by the phosphorus in DNA are not detectable owing to attenuation in the aluminum filter employed.

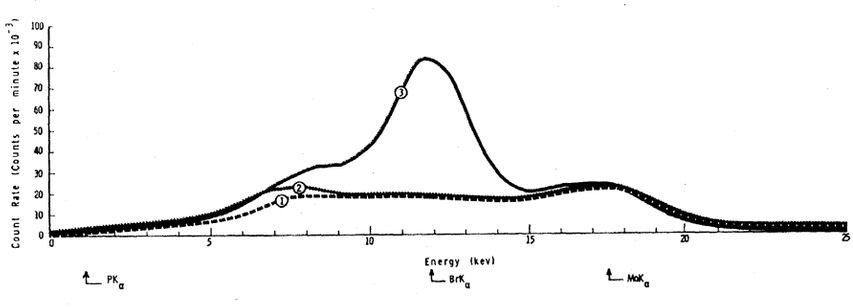

Fig. 3. X-ray nondispersive emission spectra of isolated and purified DNA from (i) salmon sperm, (ii) *E. coli B* obtained from General Biochemical, and (iii) E. *coli B* 15 T$^-$ grown in a mineral medium containing bromouracil. The fractional replacement for the last sample, as determined by measurement of bromine count, is 12 percent.

this limit of detectability. An experimental determination of precision on samples of simulated DNA, having a bromine content equivalent to about 1 percent replacement, indicates an error of approximately ± 1 percent. This error is the relative standard deviation of the determinations, for one-minute integration, of fractional bromine content of eight separate 35-μg samples taken from the same preparation of simulated DNA with added bromine.

The weight of the sample need not be accurately determined, since the fraction by weight of bromine in DNA, P_{Br}, comes directly out of the experimental determination of ratios and the ratios are independent of the mass of the sample. Assays have been carried out on samples as small as 10 μg, although with this size of sample there is a loss of precision compared to 35-μg samples.

The technique is also applicable to the analysis of iodine, and it can be used to determine the degree of substitution of iodinated as well as brominated purine and pyrimidine analogs. Because of the higher ionization energy of the K shell of the iodine atom and the higher energy of the I-Kα photon, two basic changes in the instrument are required to obtain an equivalent order of magnitude of detectability for iodine. The x-ray tube must have the capacity to operate at a voltage of at least 100 kv, and the proportional counter must be filled with a gas having a higher absorption for I-Kα than the argon has. The use of a krypton gas at 1 atm should be adequate. The Kα lines of bromine and iodine may be resolved easily since they are sufficiently separated. This indicates the feasibility of a simultaneous analysis for iodine and bromine with a single proportional counter (*6*).

L. ZEITZ
R. LEE

Division of Biophysics, Sloan-Kettering Institute for Cancer Research, New York

References and Notes

1. S. Greer, *J. Gen. Microbiol.* **22**, 618 (1960); Z. Lorkiewicz and W. Szybalski, *Biochem. Biophys. Res. Commun.* **2**, 413 (1960).
2. A. W. Kozinski and W. Szybalski, *Virology* **9**, 260 (1949); F. W. Stahl, J. M. Grasemann, L. Okun, E. Fox, C. Laird, *ibid.* **13**, 98 (1961).
3. B. Djordjevic and W. Szybalski, *J. Exptl. Med.* **112**, 509 (1960).
4. T. Hall, *Science* **134**, 449 (1961).
5. ———, in *Advances in X-ray Analysis*, W. M. Mueller, Ed. (Plenum Press, New York, 1958), p. 297.
6. We thank Dr. H. Moroson for the DNA samples used for spectra in Fig. 4.

25 October 1963

Zoospores in *Scenedesmus obliquus*

Abstract. *In basal medium without a nitrogen source a 3-day-old culture of* Scenedesmus obliquus *produced zoospores, which were biflagellated, had a parietal chloroplast, lacked a pyrenoid, and were apparently not walled. Identical results were produced with new clones. A reconsideration of the relationships and classification of this organism is now essential.*

Scenedesmus, which might be the most widely distributed fresh-water algal genus (*1*), has been studied extensively both in nature and in the laboratory. The genus was monographed twice during the last 50 years (*2*) with the taxonomic disposition of many isolates based on prolonged observation of laboratory cultures. Reproduction is said to occur solely by the production of nonmotile spores which become arranged in a definite pattern within the parent cell before release as a colony.

Beginning with Beijerinck's work with *S. acutus*, in which only unicells were formed in basal medium (*3*), there have been many investigations dealing with pleomorphism (*4*). Stages of *Scenedesmus* resembling *Dactylococcus, Chlorella, Oocystis*, and *Ankistrodesmus* are common in culture.

When *Scenedesmus obliquus* (Fig. 1) (*5*) was grown in basal medium (*6*) from which the ammonium nitrate had been withheld, a few zoospores were observed (Fig. 2). To eliminate the possibility of a mixed culture, we established new clones. Upon subsequent starvation zoospores appeared in six clonal cultures (*7*). No motility or any evidence of foreign organisms were noted in control flasks of basal medium. Aseptic procedures were used throughout.

Growth in basal medium, from which the ferric chloride, magnesium sulfate, or the potassium phosphates had been withheld, did not stimulate motility. With ammonium nitrate starvation, zoospores were evident after 3 days in continuous fluorescent illumination (4400 lu/m^2) and motility terminated after 36 hours. Although there were not usually great numbers of zoospores, and the effects of starvation were apparent, a parietal chloroplast, two flagella of equal length, and an occasional stigma were observed. Pyrenoids were always present in vegetative cells, but were not seen in the zoospores. Apparently, they do not possess a wall; elongate, fusiform and spherical types were observed. When zoospores in a hanging drop became quiescent they assumed a spherical shape and lost their flagella, but in the absence of a nitrogen source there was no further development. Techniques for transferring zoospores to a basal medium, and at the same time retaining their viability, will have to be developed.

With the presence of flagellated cells, induced by conditions which stimulate sexuality in some species of *Chlamydomonas* (*8*), the existence of a sexual phase in *Scenedesmus* is now a possibility.

Some time ago, Fritsch (*9*) suggested that a motile stage might be found eventually in members of the family Coelastraceae, in which *Scenedesmus* is placed. Zoospores and gametes are known in the colonial forms of the related Hydrodictyaceae. Furthermore,

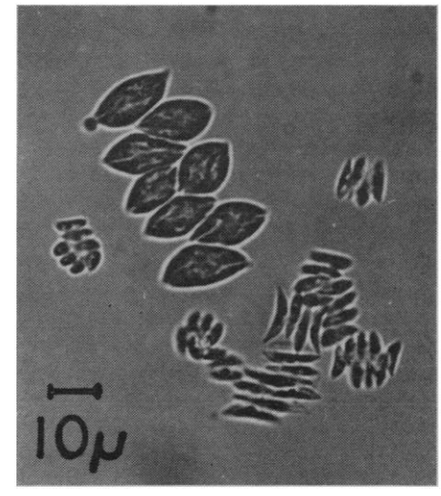

Fig. 1. *Scenedesmus obliquus* colonies.

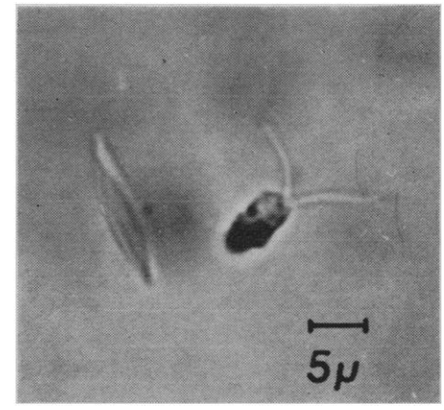

Fig. 2. A zoospore from an ammonium nitrate starved culture of *S. obliquus*. A wall from a mother cell is seen at the left. Material killed with I_2KI.