terfere with glucose uptake or its utilization by these cells.

Summary. It was shown that *in vivo* grown tubercle bacilli are readily permeable to glucose and oxidize it to an even greater extent than those grown *in vitro*. It was therefore concluded that the apparent lack of respiratory response to glucose by *in vivo* grown tubercle bacilli was not due to either permeability factor or to inability of these cells to oxidize exogenous glucose. Possible causes for the apparent lack of respiratory response of $LH_{37}Rv$ cells to glucose are discussed.

1. Segal, W., Bloch, H., *J. Bact.*, 1956, v72, 132.
2. Bekierkunst, A., Artman, M., *Nature*, 1959, v184, 458.
3. Artman, M., Bekierkunst, A., *Am. Rev. Resp. Dis.*, 1960, in press.
4. Bekierkunst, A., Artman, M., PROC. SOC. EXP. BIOL. AND MED., 1960, v105, 605.

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Cultivation of Two Strains of Killer *Paramecium aurelia* in Axenic Medium.* (26194)

ANTHONY T. SOLDO (Introduced by P. L. Perlman)
Biological Research Division, Schering Corp., Bloomfield, N. J.

Certain strains of *Paramecium* contain particles, kappa, in their cytoplasm which are essentially independent genetic units capable of undergoing mutation and self-reproduction. Kappa-bearing paramecia (killers) are capable of killing other particle-free paramecia (sensitives) and are resistant to the action of a toxic agent which they produce(1).

As pointed out by Stock, *et al.*(2), destruction of kappa by chemical means without injury to the host indicates a degree of selectivity on the part of the agent tested. Of over 70 purines and pyrimidines tested by these workers, only 2-6 diaminopurine was found to reduce the kappa content of killers; certain antibiotics have also been reported to have this effect(3). The foregoing experiments were carried out with bacterized cultures. Hence, the role played by these bacteria cannot be adequately evaluated and the need for axenic (bacteria-free) cultures is emphasized.

Reexamination of the kappa system in axenic medium for its possible application to screening potential anti-cancer agents forms the basis of a future investigation. As a preliminary step this paper reports successful development of an axenic medium for 2 killer stocks of *Paramecium aurelia*.

Materials and methods. 1) *Stock cultures.* Killer stocks 51 and 299 of *Paramecium aurelia* and a corresponding sensitive strain of stock 51 were used. Stock 299, a recently discovered killer, contains particles somewhat larger than kappa, called lambda(4). Cultures were obtained from Dr. T. M. Sonneborn of Indiana University, and maintained at 27°C in a 0.15% infusion of Cerophyl (Cerophyl Labs, Kansas City, Mo.) previously inoculated (1-2 days) with *Aerobacter aerogenes;* transfers were made at intervals of 48 hours. In this manner the organisms were maintained at approximately one fission per day and kappa content ranged from 200 to 500 particles per cell. Mass cultures were prepared by doubling the volume with Cerophyl infusion until approximately 1 to 2 l of culture were obtained. Population densities approximated 500-1000 animals per ml. 2) *Sterilization of killer paramecia.* After a number of attempts at sterilization using antibiotics in combination with several washing procedures, the following method gave satisfactory results. Mass cultures of the ciliates (usually 1 or 2 l) were twice filtered through cotton to remove accumulated debris, concen-

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trated to a small volume by filtration through Whatman No. 1 paper, and placed in an apparatus designed by Claff(5) for sterilization of certain protozoa. The technic is essentially a washing procedure which takes advantage of the fact that paramecia are negatively geotropic. To enable the ciliates to digest bacteria present in vacuoles, a modification was made which allowed a stationary period of 5 hours. Neomycin (50 μg/ml) was added to the wash fluid. As a test for sterility, individual paramecia obtained by this method were plated on nutrient agar. 3) *Aceto-orcein stain for kappa.* Available technics for visualization of kappa (Feulgen and Geimsa Stains) were time-consuming. A simpler and more rapid method was developed using aceto-orcein.

The dye was prepared by refluxing 1 g of orcein (Matheson, Coleman, and Bell) in 100 ml 50% acetic-acid, cooling to room temperature and filtering through Whatman No. 1 paper. One or more animals were added to a small drop of the dye on a glass slide by means of a micropipette. A cover slip was placed over the drop so that the organisms could be observed under oil immersion. Kappa and the nuclear apparatus stained dark red against a light background. 4) *Method of assay of test culture media.* The technics for handling paramecia were essentially those described by Sonneborn(6). Individual animals were picked up in micropipettes (bore size = 150 to 200 μ) equipped with a rubber bulb on one end, and placed in a volume of test culture fluid. In practice, humidifying chambers were prepared from sterile Petri dishes (25 mm x 150 mm dia) containing three, 3-spot glass depression slides by addition of approximately 20 ml of sterile water to the bottom plate. A volume of 0.3 ml of test culture medium was added to each depression and inoculated with single sterile paramecia; plates were incubated at 27°C. Routinely, animals were removed aseptically with a sterile micropipette, stained with aceto-orcein and examined for the presence of kappa.

Each medium was tested with 3, 6 or 9 individual paramecia. When populations of 64 to 128 cells were reached (5-6 days), the animals were transferred to tubes containing 4 ml of test media. Surviving tube cultures were tested for sterility in the following media: thioglycollate, nutrient broth, 0.5% yeast extract plus 1% glucose, and 0.3% beef extract. If no bacterial growth was observed after 2 weeks at 27°C, the protozoan cultures were considered sterile.

Results. The ability of bacterized Cerophyl to support the growth of killer animals suggested trial of this medium supplemented with killed bacteria. Consequently, cultures of *Aerobacter aerogenes* were killed by heat, solvents and freeze-thaw procedures and added to Cerophyl medium alone and in combination with various crudes. None of the media tested was effective in supporting the growth of killers. Feeder cultures of living bacteria placed in dialysis sacs and suspended in a variety of culture media were also ineffective in cultivating the ciliates.

A medium consisting of 7 vitamins, a yeast preparation (AIF-*acetone insoluble fraction* of a hot water extract of Fleischman's yeast), proteose-peptone and stigmasterol, which supported the growth of a sensitive strain of *P. aurelia*(51) was available(7,8). This medium supported the growth of killer animals when supplemented with Edamine S (an enzymatic digest of lactalbumin-Sheffield) but killers lost their kappa after 5-8 fissions.

Constituents of the medium were tested, in various concentrations and combinations, for effects upon growth and maintenance of kappa. Increased concentrations of the yeast fraction (AIF) were toxic. Dialysis of the preparation removed some of the toxic material and made possible the testing of larger amounts. Media supplemented with dialysed AIF allowed growth of some clones in which kappa was maintained for 10 to 15 fissions before being lost. A fresh fraction, (NDF - *nondialysable fraction*(9)) prepared by dialysing a hot water extract of Fleischman's yeast against running deionized water and distilled water for 48 hours, was found to be an effective replacement for AIF in supporting the sustained growth of killers and maintenance of kappa in the cells.

Table I shows the final composition of the medium and Table II the essentiality of vari-

TABLE I. Composition of Axenic Medium* (μg/ml Unless Otherwise Specified).

Compound	Conc.	Compound	Conc.
Ca pantothenate	2.5	K_2HPO_4	250.0
Nicotinamide	2.5	KH_2PO_4	250.0
Pyridoxal HCl	2.5	Ethylenediaminetetraacetic acid (disodium salt)	5.0
Riboflavin	2.5		
Folic acid	1.25	$MgSO_4 \cdot 7H_2O$	25.0
Thiamine HCl	7.5	$Fe(NH_4)_2(SO_4) \cdot 6H_2O$	6.25
Biotin	.625 mμg		
Stigmasterol	.2	Proteose-peptone	5.0 mg
$MnCl_2 \cdot 4H_2O$	.125		
$ZnCl_2$	.015	Edamine S†	10.0 "
$CaCl_2 \cdot 2H_2O$	12.5		
$CuCl_2 \cdot 2H_2O$	1.25	NDF (yeast fraction)‡	5.0 "
$FeCl_3 \cdot 6H_2O$	.31		

* Proteose-peptone, Edamine S, salts and stigmasterol may be autoclaved together. NDF and vitamins are added aseptically. pH is 6.9.

† Enzymatic digest of lactalbumin (Sheffield Chemical).

‡ Details of preparation can be found in Miller (8).

ous components. While proteose-peptone is not required for growth of the stock 51 killers, it is necessary for maintenance of kappa. If the NDF fraction of medium No. 8 is replaced with nondialysed AIF, the cells become sensitive. Apparently, in addition to a factor (factors) present in proteose-peptone, an additional factor (factors), either absent or present in small quantities in AIF and present in NDF, is required for maintenance of kappa. Killers of stock 299 may be cultivated without loss of lambda in media containing NDF and Edamine S or proteose-peptone.

Maintenance of stocks. Killers of stock 51 are best grown at 27°C as single cell isolates in depression slide culture where they divide at a rate of slightly more than one fission per day. However, tube cultures have been successfully maintained by transferring small volumes (0.1 to 0.3 ml) to fresh medium at 3-day intervals. Kappa populations have been estimated at several thousand particles per cell; killers exhibit strong killing activity when tested against suitable sensitive strains in axenic medium.

Stock 299 has now been maintained at 27 C in tube cultures for several months. Subcultures are made every 5 days and population densities range from 13,000 to 17,000 cells per ml. Periodic examination of the ciliates reveals the presence of an estimated 500 particles per cell although considerable variation has been noted in cells taken at various stages of the growth cycle.

Discussion. In an attempt to cultivate stock 51 killers in a medium consisting of salts, lemon factor (stigmasterol), NDF and proteose-peptone, van Wagtendonk *et al.*(9) reported that the animals underwent one to 4 fissions and died. They pointed out that their results indicated that the presence of kappa in the cell modified the growth requirements of the killers. The additional requirements of the killers, reflected in the medium reported here (Edamine S, EDTA, and increased amounts of NDF), support this idea.

It is of interest to note the differences in nutritional requirements between the killer stocks. Common to both stock 51 and 299 is the NDF; replacement of NDF by AIF for stock 299 results in loss of lambda and indicates the essentiality of a factor(s) present

TABLE II. Essential Constituents of the Axenic Medium and Maintenance of Particles.

	Components of medium			Growth			Retention of particles	
Medium*	Proteose-peptone	Edamine S	NDF	51 (S)	51 (K)	299 (L)	51 (K)	299 (L)
1				0	0	0		
2	+			0	0	0		
3		+		0	0	0		
4			+	0	0	0		
5	+	+		0	0	0		
6	+		+	+	0	+	†	+
7		+	+	+	+	+	—	+
8	+	+	+	+	+	+	+	+

* Medium consists of salts, vitamins and stigmasterol.

† Kappa lost in Medium No. 7 after 5-8 fissions.

in yeast. Moreover, proteose-peptone and Edamine S, both of which are requirements for growth and maintenance of kappa of stock 51, may be substituted for each other without loss of lambda in stock 299.

The complexity of the growth requirements of killers as they differ from sensitives and the differences in these requirements between stocks serves as an indication of the intricate relationships between these remarkable particles and the cells of the host. More detailed analysis of nutritional requirements of the killers is presently being undertaken, to develop a chemically-defined medium. In this way, greater insight into the nature of the particles themselves as well as identity of the factors responsible for their maintenance may be obtained.

Summary. Two killer stocks of *Paramecium aurelia*, 51 and 299, have been cultivated axenically in a medium consisting of vitamins, a salt mixture, stigmasterol, proteose-peptone, an enzymatic digest of lactalbumin and the nondialysable fraction of a hot water extract of yeast. All components are necessary for growth of the ciliates and for maintenance of the kappa character. Differences in nutritional requirements of the two stocks have been noted. As far as is known, this is the first instance of cultivation of a kappa-bearing strain of *Paramecium* in bacteria-free medium.

Since this paper was submitted for publication, stocks 138 and 139 of *P. aurelia*, containing respectively μ and π particles, have been placed in axenic culture (medium No. 8 as given in Table II). The organisms have been maintained in tube culture for two months without loss of particles.

1. Sonneborn, T. M., *Adv. in Virus Res.*, Acad. Press, Inc., New York, 1959, v6, 229.
2. Stock, C. C., Jacobson, W. E., Williamson, M., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 874.
3. Williamson, M. Jacobson, W. E., Stock, C. C., *J. Biol. Chem.*, 1952, v197, 763.
4. Schneller, M. V., *Proc. Indiana Acad. Sci.*, 1958, v67, 302.
5. Claff, L. C., *Physiol. Zool.*, 1940, v13, 334.
6. Sonneborn, T. M., *J. Exp. Zool.*, 1950, v113, 87.
7. Tarantola, V. A., van Wagtendonk, W. J., *J. Protozool.*, 1959, v6, 189.
8. Miller, C. A., van Wagtendonk, W. J., *J. Gen. Microbiol.*, 1956, v15, 280.
9. van Wagtendonk, W. J., Conner, R. L., Miller, C. A., Rao, M. R. R., *Ann. N. Y. Acad. Sci.*, 1953, v56, 929.

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Influence of Dietary Protein Levels on Ascorbic Acid Status of the Rat.* (26195)

M. SRINIVASAN, M. V. L. RAO AND H. N. BHAGAVAN†
(Introduced by E. L. Severinghaus)
Central Food Technological Research Institute, Mysore-2, India

There appears to be no report regarding the influence of dietary protein on biosynthesis of ascorbic acid in the rat. Prompted by a chance observation that rats receiving a high protein diet excreted relatively large amounts of ascorbic acid in urine, we investigated the influence of dietary protein on the Vit. C status of the rat. In the first instance, the effect of variations in dietary protein levels was examined.

Materials and methods. Adult male albino rats (Wistar strain) with body weights in the range 180-200 g were employed in all experiments. Animals were distributed according to the randomized block design for the various test treatments. Diets fed had the following composition: Protein (Polson's lactic casein)—according to choice; fat (refined peanut oil)—10%; vitaminized starch—1%; salt mixture‡—2%; and corn starch—to 100.

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† Present address: Dept. of Animal Nutrition, Univ. of Illinois, Urbana.

‡ Hubbell, Mendel and Wakeman mixture.