concentrations is able to inhibit growth. Myristic acid, for example, could be derived from acetate and is known to effect a marked inhibition of *L. casei* reversible by either lactobacillic acid or unsaturated fatty acids at relatively low concentrations(9). The effects of sodium and lithium according to this hypothesis would be to relieve the inhibition by impairing synthesis of the toxic product. It is entirely possible, on the other hand, that either sodium or preferably lithium activates an enzyme system concerned with essential fatty acid biosynthesis and that potassium inhibits the system by competition with sodium and lithium. The inhibitory effects of acetate in such a system might be due to competition of acetate with fatty acid intermediates.

Superficially the synergistic inhibition of *L. casei* 7469 by potassium and acetate appears to be closely related to the genetic block in *L. casei* strains 280-16A and 280-16B, since growth of the latter strains is promoted by either lithium or sodium(3) and by a variety of DL-α-hydroxy fatty acids(2). Further investigations are being undertaken to define this possible relationship more exactly.

Summary. Growth of *L. casei* 7469 in a low-sodium, fatty acid-free medium is inhibited synergistically by potassium and acetate. Neither potassium nor acetate is significantly inhibitory when tested separately. The inhibition is readily reversed by either lithium or sodium and by a wide variety of fatty acids, the most effective of which are lactobacillic, oleic, linoleic, and linolenic acids.

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Action of Diphtheria Toxin on Cells Cultivated *in vitro*. (23335)

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Tissue culture technics that have recently proved useful in quantitative virology can also be profitably applied to the study of some toxins. This note is a preliminary report of experiments on the action of diphtheria toxin on mammalian cells cultivated *in vitro*(1).†

Methods and materials. Most of the experiments were done with monolayer cultures of cells in glass tubes. Cultures of rabbit kidney cells in tubes were prepared by the technic of Rappaport(2) and cultivated in Earle's saline containing 0.5% lactalbumen hydrolysate (Nutritional Biochemical) and 3% bovine serum. HeLa cells (strain obtained from Tuskegee Institute); 2 kinds of monkey kidney cells, a normal strain and several altered derivatives, from Dr. Raymond C. Parker (3); human epithelial carcinoma cells, HEP #2 from Dr. Alice Moore were grown in Earle's saline containing 0.5% lactalbumen hydrolysate, 0.1% yeast extract, and 20% horse serum. Additions of diphtheria toxin‡

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TABLE I. Titration of Diphtheria Toxin in Tissue Culture.

Cells	Material inoc.	Cytopathic endpoint
		L_f
Rabbit kidney	Crude toxin	$1 \times 10^{-4} - 1 \times 10^{-5}$
Monkey "	" "	*Idem*
HeLa	" "	1×10^{-3}
HEP #2	" "	"
HeLa	Purified toxin	3×10^{-3}
Altered monkey kidney (6 strains)	Crude toxin	> 1*
Rabbit kidney	Toxoid	>20*
	Boiled toxin	> 1*
HeLa	Toxin and antitoxin	> 0.1*

* No cellular changes were observed.

were made and the cultures were observed daily for cytologic changes. Titrations of toxin were carried out by adding 0.1 ml of serial ten-fold dilutions into the culture tubes containing 1 ml of medium. The cytopathic endpoint is taken as the highest dilution of toxin causing marked and progressive necrosis and detachment of the cells. Five or 6 tubes were used for each dilution.

Results. Rabbit kidney, HeLa, HEP, and normal monkey kidney cells were all susceptible to diphtheria toxin. Altered monkey kidney cells were not. With high concentrations of toxin, lysis of the sensitive cells occurred within 18 hours of addition of the toxin. With lower toxin concentrations more time elapsed before any change in the cells could be observed. Since no further changes could be observed beyond the fourth or fifth day, final readings were made on the fifth day. The results of several titrations are shown in Table I. The least amount of toxin that could be detected on rabbit kidney cells is about 10^{-4}-10^{-5} L_f units.§ Thus the method of assay as used here will not detect levels of toxin as low as detected in the rabbit intradermal inoculation test (5×10^{-6}-10^{-6} L_f) (4).

§ An L_f unit of toxin is defined as the volume in ml of toxin that gives most rapid flocculation with one standard unit of antitoxin. For our crude toxin, 1 $L_f \cong$ 15 minimum lethal doses for the guinea pig and the endpoint in the rabbit intradermal test is approximately 0.1 ml of a $1:5 \times 10^6$ dilution *i.e.* (2×10^{-6} L_f).

TABLE II. Effect of Time of Contact on Killing of Rabbit Kidney Cells.

Amt of toxin inoc., L_f/ml	Time of contact	Cytopathic reaction
1 or .1	~1 min.	—
	1 hr	+
	2 "	+
	3 "	+
	NR	+
.01	~1 min.	—
	1 hr	—
	2 "	+
	3 "	+
	NR	+
.001	~1 min.	—
	1 hr	—
	2 "	—
	3 "	—
	NR	+

NR = Toxin not removed.
+ = Necrosis of cells. — = No necrosis of cells.

HeLa and HEP#2 cells were somewhat less sensitive than rabbit or monkey kidney cells. There was a fairly good correlation between the endpoint found for HeLa cells with crude diphtheria toxin or with a highly purified preparation.‖

That the reaction is specific is shown by the facts that a) toxoid, at a concentration 2 x 10^4 times greater than toxin, had no effect on rabbit kidney or HeLa cells; b) boiled toxin is not cytopathic; c) the action of the toxin is neutralized approximately to titer with specific antiserum; d) highly purified toxin is approximately equivalent to crude toxin (on the basis of L_f units) in cytopathic endpoint.

An experiment was performed to determine how long toxin must remain in contact with cells before its cytopathic action is no longer neutralizable by antitoxin. Cultures were inoculated with various dilutions of toxin which were allowed to remain in contact with the cells for varying lengths of time. The medium was then removed, the cell layers washed with fresh medium, and fresh medium containing antitoxin in large excess was put into the tubes. The results in Table II show that if 1 L_f unit of toxin were inoculated and allowed to remain for one hour the antitoxin could no longer suppress the cytopathic

‖ The highly purified toxin was kindly supplied by Dr. M. Cohn.

TABLE III. Toxin Neutralization Test in Rabbit Kidney Cells.

Ratio of antitoxin : toxin	Toxin titer, L_f/ml	Cytopathic reaction
11.0	10^{-1}	—
4.5	10^{-2}	—
2.3	"	—
1.4	"	—
1.0	"	+
.5	10^{-1}	+

changes. The same was true with cultures inoculated with 0.1 L_f of toxin, the only difference being that cytopathic changes took longer to appear. For toxin at 10^{-3} L_f per ml a 3 hour contact caused no effects not overcome by the antitoxin. In separate experiments, toxin was found to be completely stable at 37°C in the medium without antitoxin for at least 48 hours. These experiments indicate that toxin can within one hour either become fixed to the cells and inaccessible to antibody or initiate the metabolic events leading to cell damage.

Toxin neutralization test can be readily done in tissue culture as the results of Table III show. In these experiments varying amounts of antitoxin were added to constant amounts of crude toxin and the mixtures were incubated at room temperature for 30 minutes before adding to the cultures for assay. As in the titrations described above, the period of observation was 5 days. These results indicate that toxin is neutralized when the ratio of antitoxin to toxin is slightly in excess of 1, roughly the same relationship as found in the skin test.

An assay of the MLD for 250 g guinea pigs of the crude toxin gave a value of approximately 15 MLD per L_f.

Discussion. These experiments, preliminary to a study of the mechanism of action of diphtheria toxin and to the use of toxin as a selective agent for cell variants, serve to illustrate the relative ease and accuracy of using tissue culture in immunological research. Tissue culture may provide a system less complicated than the intact animal for elucidating the mechanism of diphtherial toxicity. In regard to the use of toxin resistance as a genetic marker, it should be noted that a group of strains—altered monkey kidney lines—can be distinguished from the parent line by resistance to high levels of diphtheria toxin. The resistance of these strains is not due to extracellular destruction of the toxin. This was ascertained by removing medium containing toxin that had been over a layer of altered monkey cells for as long as 8 days and assaying on rabbit kidney cells. The toxin titer was undiminished and was reversible by antitoxin.

Summary. Several types of mammalian cells which can be cultivated *in vitro* are susceptible to low concentration of diphtheria toxin. These cell lines may, therefore, be used as an assay system for diphtheria toxin and for antitoxin. There are altered cell lines, derived from sensitive ones which have lost their sensitivity to even very high levels of toxin. Diphtheria toxin resistance is then a possible genetic marker for study of cells in tissue culture.

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