creased rate until the adrenals and liver have been denervated. It has been shown that adrenalin-in-beeswax increases the susceptibility of normal dogs to histamine provoked ulcer(9). The adrenalin output normally stimulated by histamine administration(10) might then, in the animals with postganglionic denervation and consequent increased sensitivity to adrenalin, be a factor in explaining their increased histamine ulcer susceptibility. This increased histamine ulcer sensitivity is abolished by operations which markedly reduce the output of adrenalin.

None of this work suggests that any of the operations described afford protection against histamine provoked ulcers. Animals given histamine for longer periods up to 45 days developed ulcers just as control animals do.

Conclusion. The potentiating effect of postganglionic sympathectomy, by a celiac ganglionectomy, on histamine induced ulcer in dogs is abolished by a bilateral thoracolumbar sympathectomy or adrenal medullectomy.

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Nutrition of Animal Cells in Tissue Culture. II. Use of Tweens in Synthetic Feeding Mixtures.*† (17797)

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A previous communication(1) from this laboratory reported the development of a synthetic feeding mixture, No. 199, that maintained chick embryo mesenchyme cells in a living, healthy state for an average period of 4 to 5 weeks, and kept certain cultures alive for over 70 days. During the course of this work, it was considered desirable to supply the cells with a source of water-soluble fatty acid and to devise a means of dissolving certain fat-soluble compounds in a vehicle other than alcohol. Consequently, an extensive investigation was made of the Tween series of surface active agents,‡ which have been widely used in bacteriological media(2). The results of this study are contained in the present report.

Materials and method. All cultures were prepared from the leg muscle of 11-day-old chick embryos; and the cells were grown di-

TABLE I.
Main Distinguishing Features of Basal Synthetic Feeding Mixtures Employed.

Mixture No.	Composition
24B	Amino acids and vitamins as in Mixt. 199(1), but stock solutions of Vit. A and D in 95% ethyl alcohol. Total alcohol in mixt., 0.1%.
199	Amino acids, vitamins, purines, pyrimidines, intermediary metabolites, and other growth factors. Stock solutions of Vit. A and D and cholesterol dissolved in Tween 80 with min. alcohol. Final conc.: .002% Tween and .01% ethyl alcohol.
281	Same as 199, but stock solutions of Vit. A and D and cholesterol prepared in 95% ethyl alcohol (without Tween). Total alcohol, 0.3%.
313	Same as 199, but with ethyl alcohol brought to the level (0.3%) used in Mixt. 281.

2. Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, v83, 409.

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‡ Produced by the Atlas Powder Company, Wilmington, Del.

TABLE II.
Average Survival Time in Days of Cultures Tested in Synthetic Mixture 281 Containing 0.3% Ethyl Alcohol and Various Levels of Tweens and Oleic Acid. (Compare Table III).*†

Substance tested	Final conc. (%)				
	.5	.05	.005	.0005	.00005
Tween 20	2.0	4.5	27.5	27.6	26.6
" 40	2.5	5.3	13.1	15.3	17.5
" 60	2.8	4.0	16.8	11.5	16.0
" 80	2.8	4.5	15.5	14.5	17.0
" 85	2.0	5.8	27.3	16.6	22.0
Oleic ac.	2.0	5.2	21.5	17.0	32.0

* Each value represents average survival time of at least 6 cultures.
† Average life of control cultures in Mixture 281 was 26.2 days, and in Mixture 199 was 33.3 days.

rectly on the surface of the glass of standard pyrex test tubes by the roller-tube technic(3). The preparation of synthetic Mixture 199, and its basic stock solutions, has already been described(1). It should be mentioned that Mixture 199 contains 0.01% ethyl alcohol. This minimal amount of alcohol was found necessary in order to dissolve vitamin A, vitamin D and cholesterol before bringing them into final solution in the Tweens. In preliminary experiments the effect of the Tweens was studied in a simple synthetic mixture (24B). The majority of the experiments, however, were carried out either in Mixture 281 or in Mixture 313, which are modifications of Mixture 199(1). The main distinguishing features of these various mixtures are indicated in Table I. It should be emphasized that all solutions tested were made up in modified Tyrode's solution(4).

Tweens 20 (monolaurate), 40 (monopalmitate), 60 (monostearate), 80 (monooleate) and 85 (trioleate) were prepared as 5.0% stock solutions in glass-distilled water and were further diluted to appropriate levels in glass-distilled water. Oleic acid (redistilled) was dissolved in 95% ethyl alcohol to a concentration of 5.0% and was subsequently diluted in water immediately before use. All solutions were sterilized by passage through UF fritted glass filters (Corning). Full details of the culture assay procedures were described previously(1). Under the conditions of the experiments, only differences in average survival time greater than several days were considered significant.

Experimental. Preliminary studies were carried out in which Tweens 20, 40, 60, 80 and 85, at final concentrations ranging from 0.5% to 0.00005%, were tested in a relatively simple synthetic mixture (No.24B, Table I). These initial experiments indicated a definite toxicity at 0.5% and 0.05%, whereas concentrations of 0.005% or less were not inhibitory. Accordingly, a low concentration (0.002%) of Tween 80 was incorporated in all subsequent mixtures(1). With the development of Mixture 199, which was more complete than Mixture 24B, it became possible to study the action of the Tweens under more favorable conditions, and the preliminary experiments based on Mixture 24B were repeated with Mixture 281. The results are shown in Table II.

As in the earlier experiments, it was found that Tween concentrations of 0.5% and 0.05% were decidedly toxic for the cells (Fig. 1 and 2) whereas lower levels were not. It was observed that in low concentrations of Tween 20 the cells remained alive for much longer periods than in other members of the Tween series. This longer survival was not confirmed, however, in later experiments. Free oleic acid was also tested at several levels in order to determine whether the toxic effect of high concentrations of Tweens 80 and 85 could be attributed to their content of unesterified fatty acid(5). Because only slight toxicity was observed with concentrations of oleic

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4. Earle, W. R., *J. Nat. Cancer Inst.*, 1943, v4, 165.
5. Davis, B. D., *Arch. Biochem.*, 1947, v15, 351.

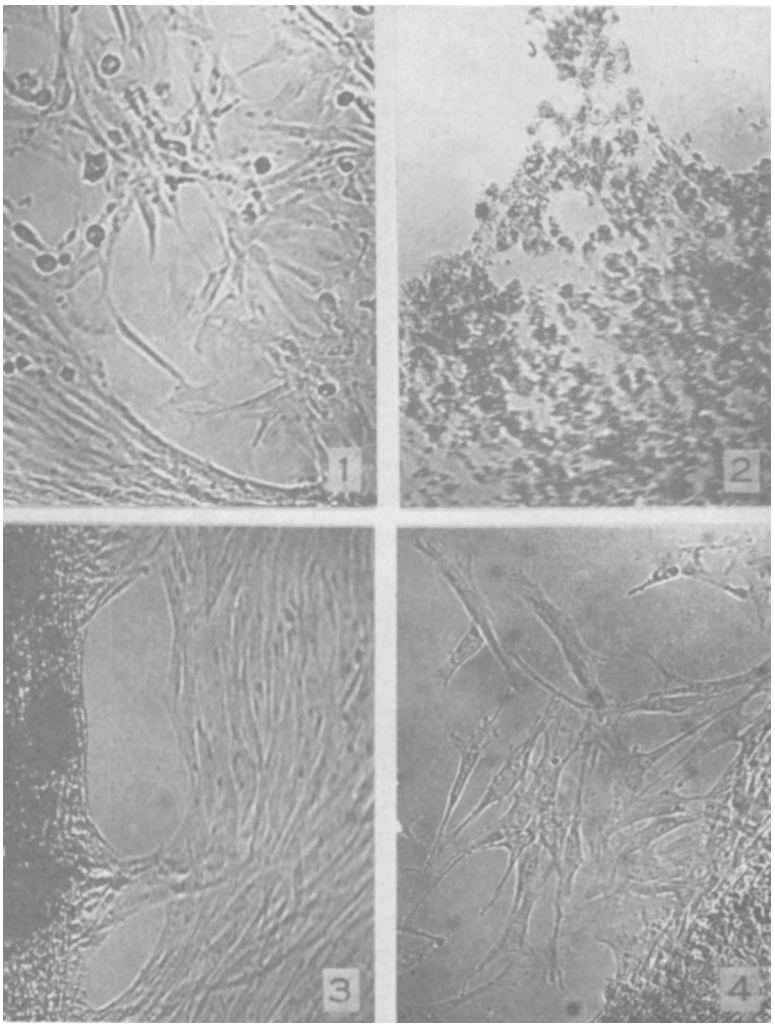

FIG. 1. Living cells from a healthy roller-tube culture of chick embryo mesenchyme tissue after 3 days in synthetic mixture No. 281 (See Table I). × 120.

FIG. 2. Cells from a degenerating culture from same series after 3 days in synthetic mixture No. 281 plus 0.05% Tween 60. (Compare Fig. 1). × 120.

FIG. 3. Living cells from a healthy roller-tube culture of chick mesenchyme tissue after 32 days in synthetic mixture No. 199 (containing 0.002% Tween 80). × 120.

FIG. 4. Living cells from a healthy culture of chick embryo mesenchyme tissue after 31 days in synthetic mixture No. 199 with a higher level of Tween 80 (0.005%). × 120.

acid far in excess of those that could conceivably be present in the Tween solutions, even after storage(6), the commercial preparations were employed without further purification. The beneficial effect of 0.00005% oleic acid, shown in Table II, was not obtained in previous or subsequent experiments.

It was observed that cultures maintained in Mixture 281 plus low concentrations of Tween survived for much shorter periods than those in Mixture 281 alone or in Mixture 199 (Table II). These findings suggested that even low concentrations of Tweens appear

6. Davis, B. D., *Arch. Biochem.*, 1947., v15, 359.

TABLE III.
Average Survival Time in Days of Cultures Tested in Synthetic Feeding Mixtures Containing Tweens at 0.0005% and Ethyl Alcohol at 2 Levels.*

Compound tested	.3% alcohol	.01% alcohol
Tween 20	27.6	28.6
" 40	15.3	36.0
" 60	11.5	28.5
" 80	14.5	37.2
" 85	16.6	33.5
Controls	25.4†	33.3‡

* Each value represents average survival time of at least 12 cultures.
† Mixture 281.
‡ Mixture 199.

TABLE IV.
Effect of Various Concentrations of Tween 80 upon the Survival Time of Cultures Maintained in a Synthetic Feeding Mixture Containing 0.01% Ethyl Alcohol.*

Tween 80, %	Survival, days
0.5	2.2
0.05	7.0
0.005	31.2
0.002†	33.3
0.0005	40.5
0.00005	32.7

* Each value represents average survival time of at least 6 cultures.
† Mixture 199.

somewhat toxic when incorporated in media containing a relatively small amount of ethyl alcohol (0.3%). It was decided, therefore, to attempt to avoid injurious levels of alcohol by dissolving the alcoholic concentrates of vitamins A and D and cholesterol, at their appropriate dilutions, directly in the levels of Tween to be tested. Table III shows the survival time of cultures in the 5 Tweens at a level of 0.0005% and in 2 levels of alcohol. It is apparent from the results of these experiments that a reduction in the alcohol content of the medium eliminates the toxic effect associated with the presence of low concentrations of Tweens. It is also interesting to note that the presence of alcohol does not appear to influence the survival time of cultures maintained in media containing a low concentration of Tween 20. In this respect, Tween 20 would seem to differ from the other Tweens that were tested.

For studies on cell nutrition, the addition of an unsaturated fatty acid to the synthetic mixtures was considered preferable to the addition of a saturated one. From the Tweens available, therefore, Tween 80, a monooleate, and Tween 85, a trioleate, were selected. It was found, however, that solutions of Tween 85 rendered the mixture so turbid that it was difficult to observe the cells, and for this reason all subsequent studies were confined to Tween 80. The effect of various concentrations of this Tween, tested in Mixture 199 containing a minimal amount of alcohol (0.01%), is shown in Table IV. These results confirmed the toxic levels of Tween 80 already established and indicated that concentrations from 0.005% to 0.00005% are suitable for studies in animal cell nutrition (Fig. 3 and 4).

Discussion. The toxicity of lipids for animal cells in tissue culture has long been assumed. Baker and Carrel(7) found that crude lipids of serum, chicken brain, chicken liver, egg and embryo tissues exerted an inhibitory action on the growth of fibroblasts; and Mayer(8) observed a toxic effect with lipids from brain tissue. Margoliash(9) found that both adult and embryo tissue extracts, used as growth stimulants for tissue cultures, could be considerably improved by extraction with fat solvents. Carminati(10), on the other hand, observed that a synthetic distearyl lecithin had a distinct growth-promoting effect on chick heart cultures. Also, Davidson and Waymouth(11) reported that defatted chick embryo extract is less effective than whole extract in promoting growth and that cultures deteriorate more quickly in defatted plasma than in whole plasma. It would seem, then, that a detailed study of the effect of individual lipid fractions is necessary before the relationship of lipids to cell growth and survival can be established with any certainty.

The present experiments have established that all Tweens tested are definitely toxic to animal cells in tissue culture at concentrations

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8. Mayer, E., *Skand. Arch. Physiol.*, 1937, v75, 1.
9. Margoliash, E., *Science*, 1947, v105, 369.
10. Carminati, V., *Arch. exp. Zellforsch.*, 1933, v13, 661.
11. Davidson, J. N., and Waymouth, C., *Biochem. J.*, 1946, v40, 568.

of 0.5% and 0.05%. These results are at some variance with those reported for bacteria. Dubos and Davis(2), who studied the growth of tubercle bacilli in liquid media, found that Tweens 20 and 40 were inhibitory at 0.005% or higher, but that Tween 80 was not inhibitory up to 0.3%. The present experiments have shown no marked differences in the degree of toxicity of different members of the Tween series, since all Tweens tested have been consistently toxic at a concentration of 0.05%. It should be pointed out, however, that no detailed comparative studies on the relative value of the various members of the Tween series have been reported for bacteria.

The present experiments have established that oleic acid and the various Tweens are toxic to animal cells in tissue culture at approximately the same concentrations. The toxic level of oleic acid for tissue cells (0.5% to 0.05%) is somewhat higher than that reported for certain lactic acid bacteria, where levels of 0.004% and 0.001% were found to be inhibitory(12,13). It is also interesting to note that the toxic action of oleic acid for tissue cells is not prevented by the presence of cholesterol, calciferol or α-tocopherol, which are present in Mixture 199. These compounds were found(13) to reverse the inhibitory effect of oleic acid upon lactic acid bacteria.

The results reported in this communication, which are based on approximately 1200 cultures, have not revealed a beneficial effect upon cell growth or survival that can be attributed definitely to Tween alone or to oleic acid. Although these studies have not established whether lipids in general are toxic for tissue cultures, they have shown that certain esterified fatty acids can be used without harmful effects provided suitable concentrations are employed. Since it is possible that animal cells in tissue culture may require a source of fatty acids, the use of non-toxic concentrations of Tween would appear to be desirable in synthetic media studies. The ability of these surface active agents to dissolve fat-soluble vitamins and cholesterol and to hold them in solution in aqueous media can also be utilized as a convenient means of eliminating all but minute traces of alcohol from the synthetic mixtures.

It should be emphasized that the present experiments were carried out in media that were completely synthetic. Also, the use of long-continued homogeneous cell strains was not considered practicable since these are ordinarily cultivated in a plasma coagulum and must be supplied with a nutrient fluid containing chick embryo extract. It was felt that the lipases present in such complex natural media would hydrolyze any Tween added and so confuse the results. For the same reason, Tween toxicity values obtained by rat feeding experiments(14) cannot be related to the present findings.

Summary. The effect of Tweens 20, 40, 60, 80 and 85, and oleic acid, upon the survival of chick embryo mesenchyme cells maintained in a completely synthetic feeding mixture has been studied over the concentration range of 0.5% to 0.00005%. Levels of 0.5% and 0.05% were definitely toxic whereas lower concentrations were not. Relatively small amounts of ethyl alcohol (0.3%) were inhibitory when used together with low concentrations of Tweens. Reduction of the alcohol content to a minimum (0.01%) makes it possible to employ the Tweens as a source of water-soluble fatty acids and also to dissolve fat-soluble compounds in aqueous synthetic feeding mixtures.

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