Differences in grazing or browsing habits of the species in this study are regarded as having little effect upon the sample values because the short physical half-life of I^{131} allows only recently contaminated plant surfaces to contribute to the concentration of the nuclide in thyroids (*6*).

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References and Notes

1. Thyroids of Maryland deer contributed by Dr. V. Flyger of the University of Maryland, those of Alaska caribou by P. Lent and O. Lønø of the University of Alaska working under the direction of Dr. W. O. Pruitt, Jr., and those of Colorado deer by D. E. Medin and A. E. Anderson, Colorado Department of Game and Fish. The assistance is gratefully acknowledged.
2. C. Blincoe and V. R. Bohman, *Science* **137**, 690 (1962).
3. M. Eisenbud *et al.*, *Science* **136**, 370 (1962).
4. L. B. Lockhart, Jr., in *U.S. At. Energy Comm. Publ. HASL-117*, (1961), p. 163.
5. K. Boddy, *Nature* **192**, 443 (1961).
6. Work performed under contract AT(45-1)-1350 between the Atomic Energy Commission and the General Electric Company.

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Albumin Replacement by Fatty Acids in Clonal Growth of Mammalian Cells

Abstract. *Clonal growth of Chinese hamster strain CHD-3 cells in a synthetic nutrient mixture supplemented with 10 micrograms of purified fetuin per milliliter exhibits an apparent requirement for serum albumin. The albumin can be replaced by linoleic acid, which occurs as a tightly bound component of most albumin preparations. Linolenic acid and corn oil can also replace albumin, while oleic acid and esters of linoleic and linolenic acids cannot.*

Synthetic nutrient mixture F10, supplemented with purified serum albumin and fetuin, will support the clonal growth of Chinese hamster strain CHD-3 cells (*1*). Minor modifications of the plating technique and the composition of the nutrient mixture permit reduction of the protein concentrations required for clonal growth to 10 μg of fetuin and 20 μg of albumin per milliliter. Under these conditions, serum albumin appeared to be essential for clonal growth.

However, the serum albumin can be replaced by an unsaturated fatty acid which normally occurs tightly bound to it. To show this, the nutrient mixture F10 (*1*) was modified by increasing the calcium chloride concentration to $1.5 \times 10^{-3}M$ and the magnesium sulfate concentration to $1.0 \times 10^{-3}M$. These concentrations were the best in titrations at the reduced protein concentrations, and represent substantial improvements over the calcium and magnesium concentrations in F10.

In the revised plating technique, the modified nutrient mixture is placed in the petri dishes, and the protein supplements are added to it with the cells. Modified nutrient, with 500 μg of fetuin (*2*) and 1000 μg of albumin per milliliter (*3*), is used to dilute the cell suspension obtained by trypsinization of a stock culture. The first stage of the serial dilution is made as quickly as possible after the cells are released from the surface of the culture bottle. A hemocytometer count is then made, and this is followed by additonal dilutions to yield a final concentration of 2000 cells per milliliter. One-tenth of a milliliter of the final dilution is then added to 5 ml of the modified nutrient in each petri dish, resulting in a suspension of 200 cells per dish, and a final protein concentration of 10 μg of fetuin and 20 μg of albumin per milliliter.

Plastic tissue-culture petri dishes (Falcon No. 3002) were used. Carefully cleaned glass dishes may also be used, but at low protein concentrations they tend to yield less consistent results. Except for these modifications, all procedures were as previously described (*1*).

In albumin replacement tests, the albumin is omitted from the diluent, and albumin replacements are added to the nutrients in the petri dishes. Fatty acids are dissolved in absolute ethanol at $1.0 \times 10^{-3}M$ and added directly to the modified nutrient mixture in the petri dishes or else are first serially diluted with the modified mixture. Care is taken never to add a total of more than 1 percent ethanol to the final medium.

Serum albumin binds fatty acids so tightly that even crystalline preparations retain them unless specially treated (*4*). In a recent gas-liquid chromatographic analysis of the fatty acids bound to a preparation of commercial albumin similar to that used in this laboratory for cell growth, Saifer and Goldman reported the presence of 43 different fatty acid peaks (*5*). One of the major peaks,

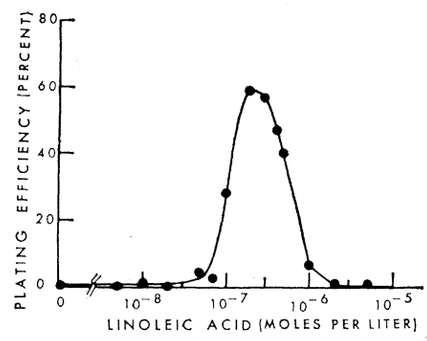

Fig. 1. Response of strain CHD-3 to linoleic acid in the absence of serum albumin. Basal medium. Modified F10 plus 10 μg of fetuin per milliliter.

comprising 20 percent of the total bound fatty acids, was linoleic acid, an essential nutrient for at least some mammals (*6*).

Linoleic acid (Calbiochem, "A grade") was tested as a replacement for serum albumin and was effective in promoting clonal growth of strain CHD-3 within a narrow range of concentration (Fig. 1). The optimum concentration is approximately $2.5 \times 10^{-7}M$, with essentially no growth occurring below $1 \times 10^{-7}M$ or above $1 \times 10^{-6}M$. Near the optimum concentration of linoleic acid, well-formed colonies are obtained which are almost as large as those obtained with 20 μg of serum albumin per milliliter. At 10 days, the colonies grown with $2.0 \times 10^{-7}M$ linoleic acid averaged 440 cells per colony, compared to 640 cells per colony with 20 μg/ml of albumin.

Linolenic acid, which will replace

Table 1. Replacement of serum albumin for clonal growth of Chinese hamster strain CHD-3 cells. Results are expressed as percentages of the plating efficiency obtained with 20 μg of albumin per milliliter. (The average plating efficiency obtained with albumin is 70 percent.) The esters and the corresponding free acids are at the same molar concentrations for direct comparisons. All other additives are at optimum concentrations.

Additive	Amount (μg/ml)	Relative plating efficiency
None	——	0.2
Albumin*	20	100
Linoleic acid†	0.07	83
Ethyl linoleate‡	0.08	1
Linolenic acid‡	0.03	58
Methyl linolenate‡	0.03	0
Oleic acid†	0.08	4
Corn oil§	1.0	85

* Normal human serum albumin, "Albumisol" Merck, Sharpe and Dohme. † Calbiochem "A grade". ‡ Nutritional Biochemicals "Highly purified" grade. § "Mazola" Best Foods Division, Corn Products Co.

linoleic acid in promoting the growth of rats, but not in preventing dermatitis (*6*), also supports clonal growth in the absence of serum albumin (Table 1). The usable concentration range is again very limited, and the overall results are not quite as good as with linoleic acid. Highly purified oleic acid shows only a trace of activity. Ethyl linoleate and methyl linolenate are far less effective than the free acids.

An edible corn oil, claimed to be high in polyunsaturated fats, also supports satisfactory clonal growth of strain CHD-3 in the absence of serum albumin. Once again, the useful concentration range is very narrow. No data are available on the percentage of free fatty acids in this preparation.

Erratic clonal growth of strain CHD-3 in the absence of added albumin or fatty acids is obtained at higher concentrations of fetuin (*1*). However, at a fetuin concentration of 10 μg/ml little or no growth is obtained without added albumin or albumin-replacing fatty acids (Table 1). The growth at higher fetuin concentrations is thought to represent a small contamination of fatty acids or albumin with bound fatty acids in the fetuin preparations tested.

Neuman and Tytell have described a fatty acid requirement for growth of massive populations of mammalian cells in a serum free medium (*7*). They also found the range of useful concentrations to be narrow, and best growth occurred at concentrations somewhat higher than those which support clonal growth of strain CHD-3. This may have resulted from the lessening of toxic effects by large populations of cells (*8*), and from their use of esters, which in our experience are less effective than the free acids. They also found oleate to be active, although it was required in larger amounts than the other fatty acid esters. This could represent trace impurities of more active fatty acids in the oleate, or less efficient utilization of oleic acid, which cannot be detected in the clonal growth system because of greater sensitivity to the toxicity of oleic acid, or a difference in the fatty acid metabolism of the cell strains used.

The conclusion that linoleic acid will replace serum albumin is based entirely on 10-day clonal growth experiments with Chinese hamster strain CHD-3 cells (*9*).

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References and Notes

1. R. G. Ham, *Exptl. Cell Research* **29**, 515 (1963).
2. Fetuin is prepared by $(NH_4)_2SO_4$ fractionation of fetal calf serum. See R. G. Ham and T. T. Puck, in *Methods in Enzymology*, S. P. Colowick and N. O. Kaplan, Eds. (Academic, New York, 1962), vol. 5, p. 90.
3. "Albumisol," Merck, Sharpe and Dohme.
4. F. E. Kendall, *J. Biol. Chem.* **138**, 97 (1941); D. S. Goodman, *J. Am. Chem. Soc.* **80**, 3892 (1958); ———, *Science* **125**, 1296 (1957).
5. A. Saifer and L. Goldman, *J. Lipid Research* **2**, 268 (1961).
6. R. T. Holman, *J. Am. Med. Assoc.* **178**, 930 (1961).
7. R. E. Neuman and A. A. Tytell, *Proc. Soc. Exptl. Biol. Med.* **104**, 252 (1960).
8. H. W. Fisher and T. T. Puck, *Proc. Natl. Acad. Sci. U.S.* **42**, 900 (1956).
9. Contribution No. 206 from the Department of Biophysics, Florence R. Sabin Laboratories, University of Colorado Medical Center, Denver. Aided by a grant from the National Foundation, by U.S. Public Health Service training grant No. 5 T1 GM-781-05, and by grant DRG 337F from the Damon Runyon Memorial Fund for Cancer Research. I thank Dr. Theodore T. Puck for his interest in this work and for his many valuable suggestions, and Jane Treber for excellent technical assistance.

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Vitamin E Oxidation with Alkaline Ferricyanide

Abstract. *Oxidation of* dl-*α-tocopherol or its model, 6-hydroxy-2,2,5,-7,8-pentamethylchroman, with alkaline ferricyanide yields a variety of products. In addition to the known dimeric keto ether, two new products, compound A, a trimer, and compound B, an isomer of compound A, were ioslated. Ultraviolet and infrared absorption spectra and paper chromatography indicate that compound A might be identical with liver metabolite "O" which results from the addition of α-tocopherol to the diet.*

Oxidation of *dl-α*-tocopherol (*1*) with alkaline ferricyanide has been reported to yield a yellow oil with a strong ultraviolet (UV) absorption band at 235 to 236 mμ and with a weak band at 300 mμ (*2*). The results of acid cleavage excluded structure I (see Fig. 1 for structural formulae) analogous to the product (II) of the alkaline ferricyanide oxidation of methyl substituted hydroquinone monoethers. Our studies with Stuart atomic models have shown that structure I is sterically strained while structures of type II are not.

Draper, Csallany, and Shah (*3*) reported a UV absorption maximum at 295 mμ for this ferricyanide oxidation product and postulated that it was identical to liver metabolite "O" described by Alaupovic *et al.* (*4*).

Recently Nelan and Robeson (*5*) reinvestigated this oxidation and isolated a yellow oil absorbing at 300 mμ with a weaker band at 337 mμ. Chemical studies on this oil indicated that it was a keto ether and structure III was proposed. However, no mention was made of the other three possible isomers of compound III, namely compounds V, VI, and VII.

In our studies of the alkaline ferricyanide oxidation of both *dl-α*-tocopherol and its model, 6-hydroxy-2,2,5,-7,8-pentamethylchroman, we have found by the use of silica-gel thin-layer chromatography that a variety of products are produced. Since many of these products are isomeric, some being dimers and some trimers, elementary analysis is not a criterion for establishing purity. Thin-layer chromatography has proven extremely valuable for separation of these products in pure form. In addition to the keto ether (III or IV), we have isolated two new products, compounds A and B. The ratio of these various products is somewhat dependent upon the time allowed for the oxidation to take place. After 6 minutes no tocopherol remains. The yield of yellow dimeric oil (III) is 65 to 75 percent; that of compound A, a colorless wax, 2 to 3 percent; and that of compound B, 2 to 3 percent. The remainder is composed of compound IX, trace amounts of other oxidation products, and some decomposition products.

Parallel studies with the model compound, 6-hydroxy-2,2,5,7,8-pentamethylchroman, yielded crystalline products. The time required to oxidize this chroman completely was greater than in the case of *dl-α*-tocopherol. A yellow crystalline keto ether (IV) was isolated free from compound A by silica-gel chromatography. Its melting point was 77° to 79°C, its composition $C_{28}H_{36}O_4$, its molecular weight in benzene by the vapor pressure osmometer method (Mechrolab) was 433 (calculated 436).

Nelan and Robeson (*5*) reported a melting point of 126° to 127° for this compound. We have found it difficult to separate compound IV from compound A, which is higher melting, except by repeated chromatography and fractional crystallization. The infrared- and ultraviolet-absorption characteristics of compound IV agreed with those reported by Nelan and Robeson and with those of the tocopherol analog