Selenium is an essential trace nutrient for growth of WI-38 diploid human fibroblasts

(human nutrition/trace element/clonal growth/fetal lung cells/Chinese hamster cells)

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ABSTRACT The trace element selenium is essential for clonal growth of diploid fibroblasts from human fetal lung (WI-38) in media containing small amounts of serum protein. Maximum growth stimulation is obtained when 30 nM neutralized selenious acid is added to a synthetic medium containing 1.5 mg/ml of dialyzed fetal bovine serum protein (equivalent to a 3% serum concentration). Serum appears to be a source of selenium in most culture media, since higher concentrations of serum protein or whole serum mask the selenium requirement of WI-38 cells. Selenium is also required by a Chinese hamster cell line that can be grown in a protein-free synthetic culture medium.

The essential role of selenium (Se) in animal nutrition is well established. A recent review on the biology of Se states that Se-responsive diseases have been demonstrated in over 40 species (1). These include liver necrosis in rats (2) and swine (3, 4), exudative diathesis in chickens (5), and white muscle disease in lambs (6) and calves (7, 8).

Advances have recently been made in the elucidation of the biochemical roles of Se (9). In ovine tissue, Se is incorporated into and required for formation of a 10,000 molecular weight hemoprotein of muscle and heart (10, 11). Se has been positively identified as an integral part of the enzyme glutathione peroxidase isolated from ovine and bovine erythrocytes (12, 13).

Despite the extensive evidence that Se is an essential nutrient for many different animal species, there is a lack of direct evidence for a requirement for Se in human nutrition (14). In this paper, we present evidence that Se is required for clonal growth of WI-38 diploid human fibroblasts. We believe this to be the most direct evidence that has yet been obtained that Se may also be required by humans as an essential nutrient. Evidence is also presented to show that an established line of Chinese hamster cells requires Se for clonal growth in a protein-free synthetic medium.

MATERIALS AND METHODS

Chemicals. The source of Se in all experiments was "spec-pure" grade selenious acid from Johnson Matthey Chemicals, Ltd., London, England, and was neutralized with 4 M NaOH. For convenience of presentation, neutralized selenious acid is referred to simply as " H_2SeO_3 " throughout this paper. All other chemicals used in preparation of media were from Sigma Chemical Co., except the inorganic salts, which were from Fisher Scientific.

Defined Media and Serum. All clonal growth experiments which used WI-38 cells were carried out in medium MCDB 103 (Table 1) with the omission or addition of Se as indicated in the

text. The medium used is one of a series of media presently being developed specifically for diploid human cells (ref. 25, and in preparation).

All growth experiments which used Chinese hamster cells were carried out in medium F12 (15) containing 0.1 mM cysteine instead of the indicated 0.2 mM.

Fetal bovine serum protein (FBSP) was prepared routinely as follows: 1 liter of fetal bovine serum (FBS) (Flow Laboratories, Inglewood, Calif.) was made 4.9 mM in EDTA by addition of 12.5 ml of a 0.4 M stock, previously adjusted to pH 8.0 with 4.0 M NaOH. The solution was stirred for 15 min and dialyzed against 40 liters of distilled water (4°) for 3 days with changes of the dialysis medium every 24 hr. The dialyzed protein solution was centrifuged to remove insoluble material, then frozen and lyophilized to dryness. The dry powder was stored at -20° until used. Small amounts of dry powder were dissolved and, thereby, reconstituted as needed at a concentration of 50 mg/ml in a solution containing 0.01 M Tris-HCl (pH 7.3) and 0.15 M NaCl. The resulting solution was equivalent in protein concentration to whole serum and was used as a substitute for serum as indicated in the text. It was sterilized by passage through Millipore type GS (0.22 μm) filter membranes.

Cells and Cultures. *Human Cells.* Diploid human fibroblasts (strain WI-38) from fetal lung tissue (16) at passage 14 were obtained from Dr. L. Hayflick (Stanford University School of Medicine, Stanford, Calif.). The cells were grown in mass culture to passage 16 in Eagle's basal medium with 10% (vol/vol) FBS, transferred and grown to passages 17 through 22 in medium F12 (15) with 10% (vol/vol) FBS. One milliliter aliquots of about 10^6 cells, passage 17 through 22, were frozen in liquid nitrogen after addition of 10% (vol/vol) dimethylsulfoxide to the medium. The frozen aliquots were used to initiate routine stock cultures for use in clonal growth experiments described below. One milliliter aliquots were thawed as needed and inoculated into Corning no. 25100 plastic tissue culture flasks (25 cm^2) containing 4 ml of medium MCDB 102 (ref. 25, Table 1) and 5 mg/ml of FBSP prepared as described above. Cultures were grown to a confluent monolayer (2-3 days) at 37° in a humidified 5% CO_2 atmosphere.

Cells were harvested from the monolayer by mild trypsin treatment as follows: the growth medium was removed and the monolayer washed with two 3 ml aliquots of 0.05% (wt/vol) trypsin (pH 8.0) in a solution of 120 mM NaCl, 5 mM KCl, 5.5 mM glucose, and 26 mM NaHCO_3 (Grand Island Biological Co., Santa Clara, Calif.). The monolayer was exposed to a third 3 ml aliquot of trypsin solution preheated to 37° and monitored visually until the majority of cells were rounded. The trypsin solution was carefully removed and 8 ml of medium containing 5 mg/ml of FBSP was added. The flask was shaken gently to remove cells from the culture surface, and the resulting cell suspension was pipetted up and down several times to break up tissue clumps. The suspension was then split 1:2 for inoculation

Abbreviations: FBSP, fetal bovine serum protein; FBS, fetal bovine serum; MCDB 101, 102, 103, etc., a series of media being developed for human diploid cells at the Department of Molecular, Cellular and Developmental Biology, University of Colorado; CHO, Chinese hamster ovary.

Table 1. Composition of media MCDB 103, MCDB 102, and F12^{a,b}

MCDB 103		Vitamins	
Inorganic salts		<i>d</i> -Biotin	3.0×10^{-8}
CaCl ₂ ·2H ₂ O	3.0×10^{-4}	Folic acid	3.0×10^{-6}
CuSO ₄ ·5H ₂ O	1.0×10^{-8}	DL- α -Lipoic acid	
FeSO ₄ ·7H ₂ O	3.0×10^{-6}	(DL-6,8-thioctic acid)	1.0×10^{-6}
H ₂ SeO ₃	3.0×10^{-8}	Niacinamide	3.0×10^{-7}
KCl	3.0×10^{-3}	D-Pantothenic acid	
MgCl ₂ ·6H ₂ O	6.0×10^{-4}	(hemi-calcium salt) ^c	1.0×10^{-6}
NaCl	1.3×10^{-1}	Pyridoxine·HCl	3.0×10^{-7}
NaHCO ₃	1.4×10^{-2}	Riboflavin	1.0×10^{-7}
Na ₂ HPO ₄ ·7H ₂ O	1.0×10^{-3}	Thiamine·HCl	1.0×10^{-6}
ZnSO ₄ ·7H ₂ O	5.0×10^{-7}	Vitamin B ₁₂	1.0×10^{-6}
Amino acids		Other organic compounds	
L-Alanine	1.0×10^{-4}	Choline chloride	1.0×10^{-4}
L-Arginine·HCl	1.0×10^{-3}	D-Glucose	1.0×10^{-2}
L-Asparagine·H ₂ O	1.0×10^{-4}	Hypoxanthine	3.0×10^{-5}
L-Aspartic acid	1.0×10^{-4}	<i>i</i> -Inositol (meso-inositol)	1.0×10^{-4}
L-Cysteine·HCl·H ₂ O	9.0×10^{-5}	Linoleic acid	3.0×10^{-7}
L-Glutamic acid	1.0×10^{-4}	Putrescine·2HCl	1.0×10^{-6}
L-Glutamine	2.5×10^{-3}	Sodium pyruvate	1.0×10^{-3}
Glycine	1.0×10^{-4}	Thymidine	3.0×10^{-6}
L-Histidine·HCl·H ₂ O	1.0×10^{-4}	Buffers and indicators	
L-Isoleucine	3.0×10^{-5}	HEPES [4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid]	2.8×10^{-2}
L-Leucine	1.0×10^{-4}	Phenol Red	3.3×10^{-6}
L-Lysine·HCl	2.0×10^{-4}	Medium MCDB 102	
L-Methionine	3.0×10^{-5}	Same as MCDB 103 except	
L-Phenylalanine	3.0×10^{-5}	H ₂ SeO ₃	0
L-Proline	3.0×10^{-4}	ZnSO ₄ ·7H ₂ O	3.0×10^{-6}
L-Serine	1.0×10^{-4}	Medium F12	
L-Threonine	1.0×10^{-4}	Same as MCDB 103 except	
L-Tryptophan	1.0×10^{-5}	Cysteine	2.0×10^{-4}
L-Tyrosine	3.0×10^{-5}	Glutamine	1.0×10^{-3}
L-Valine	1.0×10^{-4}	HEPES	0
		H ₂ SeO ₃	0
		ZnSO ₄ ·7H ₂ O	3.0×10^{-6}

^a All concentrations are in moles/liter.^b For convenience the components of the media are listed in alphabetical order; this order does not reflect the sequence or grouping of components when preparing the media. (For detailed instructions for preparation of medium F12, from stock solutions, see Appendix A of ref. 21.) The same procedures are applicable for MCDB 103 except for the following: (1) the final concentrations of glutamine (stock 6a), cysteine (stock 7), and zinc sulfate (stock 11) are altered as indicated; (2) the H₂SeO₃ is incorporated into stock 11 with the zinc sulfate at 1000-times the final concentration; (3) the HEPES is dissolved in distilled water and the pH adjusted to near 7.0 with 4 M NaOH. All stock solutions except 4, 7, 11, and 12 (see below) and all solid reagents except NaHCO₃ are added. The volume is adjusted to the final figure minus the volume of the missing stocks, and the pH is adjusted to 7.3. The sodium bicarbonate is then added, resulting in a medium that is slightly alkaline in air, but at the correct pH when equilibrated in the cell culture incubator. Stock 4 (calcium, magnesium and iron), stock 7 (cysteine), stock 11 (selenium and zinc), and stock 12 (linoleic acid) are not added until immediately before use.^c The molar concentration for calcium pantothenate is given in terms of pantothenate ion. A "formula weight" of 238, based on one pantothenate ion plus one half of a calcium ion, is used to calculate the amount of calcium pantothenate (0.238 mg/liter) added to the media. The formula weight is one half of the conventional "molecular weight," which is based on two pantothenate ions plus one calcium ion.

into fresh stock culture flasks or further diluted as described below for clonal growth experiments. All experiments were done with cells at passage numbers below 30.

Chinese Hamster Ovary (CHO) Cells. The CHO cells used were derived from the Los Alamos strain of Chinese hamster ovary cells, which has a well characterized subdiploid karyotype with 21 chromosomes (17). The culture was obtained from Dr. D. M. Prescott (University of Colorado, Boulder, Colo.) who in turn had obtained it from Dr. D. F. Petersen (Los Alamos National Laboratory, Los Alamos, N.M.). The Los Alamos culture was obtained in 1962 from the laboratory of Dr. T. T. Puck (University of Colorado Medical Center, Denver, Colo.). The culture is proline dependent and is very closely related to the proline-requiring cells from CHO that were used in the development of media F7, F10, and F12 (15, 18, 19). Clonal growth experiments with CHO cells in protein-free medium

were done as previously described (15) except that the trypsin was removed before the cells were detached. The cells were then suspended in protein-free medium and shaken gently as described above for WI-38 cells.

Measurement of Cell Growth. Cell growth was measured by the highly sensitive clonal growth technique (20, 21). Cell suspensions, prepared as described above from monolayer stock cultures, were serially diluted with medium to a concentration of 1000–1500 cells per ml. Corning no. 25010 plastic tissue culture dishes (60 × 15 mm) each contained 5.0 ml of experimental medium (modified as indicated in the text). They were equilibrated with the incubation conditions and inoculated with 0.10 ml (100–150 cells) of the cell suspension. The mixtures were incubated in a 37°, 5% CO₂-humidified atmosphere for 14 days for human cells, and 10 days for hamster cells. At the end of the incubation period, the medium was removed and the colonies

FIG. 1. The effect of H_2SeO_3 on clonal growth of human diploid fibroblasts (WI-38). (A) one hundred fifty WI-38 cells (passage 25) grown 14 days in medium MCDB 103 minus H_2SeO_3 containing 1.5 mg/ml of FBSP. (B) Same as (A), but with 50 nM H_2SeO_3 added to the medium. The average colony size in photocell units in (B) is 5.0. This represents about 1000 cells or 10 doublings from a single plated cell.

were fixed with a 10% (vol/vol) formalin solution for 5 min, rinsed with water, and stained with 0.1% (wt/vol) crystal violet for 5 min. Cell growth was quantitated by a photometric measurement of the size of colonies as previously described (19). Five typical colonies were measured from each stained plate and averaged. The photometric measurement is proportional to both the area and the density of the colony.

RESULTS

Se and Growth of Human Diploid Fetal Lung Fibroblasts. The effect of Se added as selenite on colony formation from single WI-38 cells is shown in Figs. 1 and 2. With 1.5 mg/ml of FBSP in the medium, good colony formation is dependent on added Se, although some limited growth is seen in its absence (Fig. 1A). The cells begin to show a growth response above the background at nM concentrations of added Se. Optimum growth occurs at 30 nM Se. Higher levels of added Se become toxic and growth is completely inhibited at 10 μM .

It has not been possible to completely eliminate the background growth that is observed in the absence of added Se. Even with the smallest amount of serum protein that will support reasonable clonal growth (0.5 mg/ml), some of the cells attach and undergo a few cell divisions in the absence of added Se.

Whole serum or larger amounts of serum protein mask the requirement for Se (Table 2). When the medium is supplemented with 5% (vol/vol) FBS, no effect of added Se on growth can be detected. Similarly, 5 mg/ml of FBSP, treated and di-

FIG. 2. The growth response of WI-38 fibroblasts to H_2SeO_3 . The indicated amounts of H_2SeO_3 were added to clonal growth assay plates containing 5.0 ml of medium MCDB 103 minus H_2SeO_3 , 1.5 mg/ml of FBSP, and 100 WI-38 cells. Dishes were incubated, the colonies stained, and colony size determined as described in *Materials and Methods*. For each concentration of Se, the averaged colony size for three dishes and the range of variation among the dishes are shown.

alyzed as described in *Materials and Methods*, can completely replace the effect of added Se.

Se and Growth of Chinese Hamster Ovary Cells in Protein Free Synthetic Media. Fig. 3 illustrates the effect of Se on clonal growth of CHO cells in a protein free medium. Under these conditions, growth is absolutely dependent on added Se. The complete growth response of CHO cells to added Se is shown in Fig. 4. Growth is essentially optimum at 0.1 nM added Se. Detectable growth occurs in the presence of as little as 1 pM Se. The cells show a wide tolerance to added Se until growth is completely inhibited at 30 μM . This is in contrast to the response of the WI-38 cells (Fig. 2) which exhibit little growth response below 1 nM added Se and already begin to show some signs of toxicity at 0.1 μM .

DISCUSSION

The highly sensitive clonal growth assay has been used to demonstrate that Se at a concentration of 30 nM is an essential trace nutrient for growth of a human diploid cell in culture. The large multiplication factor involved in the formation of a visible colony from a single cell, plus the large volume of medium that surrounds each cell in the original inoculum, effectively eliminate the effects of any residual Se carried over with the cells from previous culture media.

Table 2. Effect of serum or serum protein concentration on response of WI-38 cells to selenium^a

Supplement	Colony size		Ratio $\left(\frac{50 \text{ nM Se}}{\text{no Se}}\right)$
	Minus Se	Plus 50 nM Se	
Whole fetal bovine serum (FBS)			
3.0% (vol/vol)	5.0	9.5	1.9
5.0% (vol/vol)	10.0	9.8	0.98
Fetal bovine serum protein (FBSP)			
1.5 mg/ml	1.0	7.3	7.3
2.5 mg/ml	6.8	10.2	1.5
5.0 mg/ml	9.5	9.5	1.0

^a Clonal growth experiments were performed as described in *Materials and Methods*. Medium MCDB 103 was prepared without H_2SeO_3 . Whole fetal bovine serum, fetal bovine serum protein, and Se as H_2SeO_3 were added as indicated. Colony size was measured photometrically as described in *Materials and Methods*.

FIG. 3. Clonal growth of CHO cells in protein-free synthetic medium containing H_2SeO_3 . (A) One hundred cells plated for 10 days without added H_2SeO_3 in medium F12 modified as described in *Materials and Methods*. (B) Same as (A) but with 3 nM H_2SeO_3 in the medium.

The data presented in Table 2 indicate that serum is a major source of Se in most culture media and that a substantial amount of the Se remains tightly bound to serum protein during dialysis. The limited background growth of WI-38 cells obtained in the absence of added Se is almost certainly due to bound Se in the serum protein preparation. The CHO cell, which requires far less Se for growth, and which can be grown in a protein-free medium in the presence of Se, requires a proportionally smaller amount of serum protein for growth in the absence of added Se (unpublished observations).

The fact that the CHO cells do not grow in the protein-free medium without added Se indicates that the chemicals and water used in medium preparation are quite free from Se contamination. This apparently has not always been the case. At one time, cell lines closely related to CHO could be grown in medium F12 without added Se (15). However, it has not been possible to reproduce these results recently, and it now appears that the original growth was probably due to the presence of Se as a contaminant in the chemicals used in earlier experiments. Conditions necessary for clonal growth of CHO and other Chinese hamster lines (which also appear to require Se) in the absence of serum proteins will be described in detail in a separate paper.

Addition of Se to the culture medium, together with adjustment of amino acid concentrations (25), has reduced substantially the amount of serum protein needed for good clonal growth of WI-38 cells. Preliminary observations (unpublished) indicate that further reduction of the protein requirement can be achieved by supplementation of the medium with additional trace elements and by further adjustment of the concentrations of nutrients already in the medium. Elimination of the need for protein(s) that carry Se has improved the sensitivity of the growth assay for the remaining undefined substances in serum which promote growth.

WI-38 cells require more Se for growth than CHO cells, and are also more sensitive to toxic excesses of Se. Although this effect could be due to the presence of FBSP in the medium for the human cells, unpublished experiments with other nutrients suggest that a more generalized pattern exists. The aneuploid CHO line tends to be both more efficient in the utilization of trace amounts of nutrients and more resistant to the toxic effects of nutrient excesses than the "normal" diploid WI-38 culture.

A variety of established cell lines have been grown in monolayer or suspension culture in protein-free "defined" media without added Se (22, 23). Such growth probably reflects a combination of contamination of the media that were used with

FIG. 4. The growth response to H_2SeO_3 of CHO cells in protein-free synthetic medium. The clonal growth assays were carried out as described in Fig. 3. Each point shown is the average of four separate petri dishes with the variation among dishes indicated.

traces of Se plus highly efficient utilization of such contaminants. The widespread requirement for Se in animal nutrition (1), plus the specific requirement for Se by cultured cells from two diverse species that has been demonstrated here, make it appear quite likely that all cultured vertebrate cells will ultimately be shown to require Se for growth. However, such a conclusion must be regarded as conjectural until carefully controlled experiments are run with many more types of cells in media that are known to be free from Se contamination.

The specific biochemical function of Se for the promotion of cellular growth in culture remains unknown. However, Se is known to be an integral part of the enzyme glutathione peroxidase, which plays a key role in the removal of metabolically generated peroxides from cells (1, 12, 13). Two observations from other laboratories are consistent with the possibility that Se might be involved in the protection of cultured cells from oxidative damage.

Taylor *et al.* (24) studied clonal growth of WI-38 cells in medium IMEM-ZO, which qualitatively contains most of the ingredients of MCDB 103 except Se, although a number of nutrient concentrations are quantitatively different. They obtained good growth at reduced oxygen tensions, but found that 20% oxygen was quite toxic and that the toxicity varied with the lot of serum that was used. If the serum that they used was from cattle pastured in areas of the country that are relatively deficient in Se, their cells could have been marginally deficient in glutathione peroxidase and therefore particularly sensitive to oxidative damage. We have not done specific experiments on oxygen toxicity. However, plating efficiencies near 50%, with no indication of toxicity, were consistently obtained in the experiments reported here and in a previous paper (25). All such experiments were performed in incubators gassed with 95% air and 5% carbon dioxide (saturated with water vapor) at an altitude of 5400 feet (1647 m). The partial pressure of oxygen that the cells were exposed to corresponds to approximately 16–17% oxygen at sea level (cf. note added in proof).

At the other extreme, Dr. Lester Packer (personal communication) has found that WI-38 cells in a maintenance medium which contains 1% serum can be protected from toxic effects of 50% oxygen by 0.1 ppm Se (1.25 μM). Several key experiments remain to be done before we can draw definite conclusions about the role of Se. All currently available evidence appears to be compatible with protection from peroxides, but other roles have also been suggested for Se in whole animal nutrition and they cannot yet be ruled out for cultured cells.

A requirement for Se in human nutrition has not been clearly

established (1, 14). Dietary Se at concentrations around 0.1 ppm is unquestionably an essential nutrient for many types of animals. Children suffering from kwashiorkor have reduced amounts of Se in their blood and weight gain during recovery is reported to be faster with Se supplementation. However, kwashiorkor is a complex deficiency disease and it is difficult to attribute effects to single causes (cf. discussion by Leander, ref. 14).

Direct demonstration of a human nutritional requirement is difficult to achieve. The problem becomes even more difficult for a trace nutrient, which must be meticulously removed from all components of the diet without the destruction of other nutrient values, and which may have to be depleted for a long period before deficiency symptoms appear. In the past, the use of cultured cells to study human nutritional requirements has been severely limited by the necessity for the use of either large amounts of serum to obtain growth of normal cells, or of highly evolved established cell lines whose nutrient responses are significantly different than those of recently isolated normal cells. The improved medium described in this paper, which will support clonal growth of WI-38 cells when supplemented with small amounts of dialyzed serum protein, makes the use of cultured cells as a tool in studying human nutrition much more feasible. Experiments currently in progress indicate that further reductions in the serum protein requirement for growth of WI-38 cells can be achieved, and that with less protein in the medium it will be possible to demonstrate specific requirements for additional inorganic trace nutrients for growth of the cells.

The present data, which show that a cultured human cell population requires Se, plus widespread evidence that many animal species including primates require Se (1, 14), strongly support the likelihood that Se will ultimately be proven to be an essential nutrient for humans. Many other cultures of human cells need to be examined, however, before firm conclusions are drawn. The possibility that WI-38 and certain other cell types could have specialized requirements for Se that are not representative of cells in general cannot be ruled out completely by the current data.

Note Added in Proof: We have confirmed the report by Taylor *et al.* (24) that reduced oxygen tension improves clonal growth of WI-38 cells. The benefit is greatest without added Se, but improvement is also seen with optimum amounts of Se in the medium. Reduced oxygen (1% oxygen, 94% nitrogen, 5% carbon dioxide) does not fully replace the need for Se. Growth is improved by the addition of 30 nM Se at all oxygen concentrations tested (1, 5, 10, and 20%). Several other lines of human diploid fibroblast-like cells derived from amniotic fluid, fetal lung, and newborn foreskin also require selenium.

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1. Frost, D. V. & Lish, P. M. (1975) "Selenium in biology," *Annu. Rev. Pharmacol.* **15**, 259-284.
2. Schwarz, K. & Foltz, C. M. (1957) "Selenium as an integral part of factors against necrotic liver degeneration," *J. Am. Chem. Soc.* **79**, 3292-3293.
3. Eggert, R. G., Patterson, E., Akers, W. T. & Stokstad, E. L. R.

- (1957) "The role of vitamin E and selenium in the nutrition of the pig," *J. Anim. Sci.* **16**, 1037.
4. Grant, C. A. & Thafvelin, B. (1958) "Selenium and hepatitis diaetetica of pigs," *Nord. Veterinaarmed.* **10**, 657-663 (in Swedish).
5. Scott, M. L., Noguchi, T. & Combs, G. F., Jr. (1974) "New evidence concerning mechanisms of action of vitamin E and selenium," *Vitam. Horm.* **32**, 429-444.
6. Muth, O. H., Oldfield, J. E., Remmert, L. F. & Schubert, J. R. (1958) "Effects of selenium and vitamin E on white muscle disease," *Science* **128**, 1090.
7. Muth, O. H., Schubert, J. R. & Oldfield, J. E. (1961) "White muscle disease (myopathy) in lambs and calves. VII. Etiology and prophylaxis," *Am. J. Vet. Res.* **22**, 466-469.
8. Nelson, F. C., Hidioglou, M. & Hamilton, H. A. (1964) "The effect of prophylactic treatment of pregnant beef cows on the incidence of nutritional muscular dystrophy. A field trial," *Can. Vet. J.* **5**, 268-273.
9. Stadtman, T. C. (1974) "Selenium biochemistry," *Science* **183**, 915-922.
10. Whanger, P. D., Pedersen, N. D. & Weswig, P. H. (1972) "Characteristics of a selenium-binding protein from lamb muscle," *Fed. Proc.* **31**, 691.
11. Whanger, P. D., Pedersen, N. D. & Weswig, P. H. (1973) "Selenium proteins in ovine tissues. II. Spectral properties of a 10,000 molecular weight selenium protein," *Biochem. Biophys. Res. Commun.* **53**, 1031-1035.
12. Oh, S. H., Ganther, H. E. & Hoekstra, W. G. (1974) "Selenium as a component of glutathione peroxidase isolated from ovine erythrocytes," *Biochemistry* **13**, 1825-1829.
13. Flohe, L., Gunzler, W. A. & Shock, H. H. (1973) "Glutathione peroxidase: a selenoenzyme," *FEBS Lett.* **32**, 132-134.
14. Levander, O. A. (1975) "Selenium and chromium in human nutrition," *J. Am. Diet. Assoc.* **66**, 338-344.
15. Ham, R. G. (1965) "Clonal growth of mammalian cells in a chemically defined, synthetic medium," *Proc. Natl. Acad. Sci. USA* **53**, 288-293.
16. Hayflick, L. (1965) "The limited *in vitro* lifetime of human diploid cell strains," *Exp. Cell Res.* **37**, 614-636.
17. Deaven, L. L. & Petersen, D. F. (1973) "The chromosomes of CHO, an aneuploid Chinese hamster cell line: G-band, C-band and autoradiographic analyses," *Chromosoma* **41**, 129-144.
18. Ham, R. G. (1962) "Clonal growth of diploid Chinese hamster cells in a synthetic medium supplemented with purified protein fractions," *Exp. Cell Res.* **28**, 489-500.
19. Ham, R. G. (1963) "An improved nutrient solution for diploid Chinese hamster and human cell lines," *Exp. Cell Res.* **29**, 515-526.
20. Puck, T. T., Marcus, P. I. & Ciecuira, S. J. (1956) "Clonal growth of mammalian cells *in vitro*. Growth characteristics of colonies from single HeLa cells with and without a 'feeder layer,'" *J. Exp. Med.* **103**, 273-284.
21. Ham, R. G. (1972) "Cloning of mammalian cells," *Methods Cell Physiol.* **5**, 37-74.
22. Higuchi, K. (1973) "Cultivation of animal cells in chemically defined media, a review," *Adv. Appl. Microbiol.* **16**, 111-136.
23. Katsuta, H. & Takaoka, T. (1973) "Cultivation of cells in protein- and lipid-free synthetic media," *Methods Cell Biol.* **6**, 1-42.
24. Taylor, W. G., Richter, A., Evans, V. J. & Sanford, K. K. (1974) "Influence of oxygen and pH on plating efficiency and colony development of WI-38 and Vero cells," *Exp. Cell Res.* **86**, 152-156.
25. Ham, R. G., Hammond, S. L. & Miller, L. L. (1976) "Critical adjustment of cysteine and glutamine concentrations for improved clonal growth of WI-38 cells," *In Vitro*, in press.