1,000,000 parts. Ethylene oxide diluted 1 : 100 was also tested, but the results were inconclusive.

After the method of Hsu (*11*), mitotic indices were taken as a criterion of damage to the HeLa cells, caused by ethylene oxide. There was no significant difference in the percentages of mitotic phases between HeLa cells grown in a control medium, and HeLa cells grown in a medium which had been treated with 0.4 percent or 0.5 percent ethylene oxide.

Since sterilization with ethylene oxide is a function of concentration, exposure time, and temperature, it is difficult to specify a concentration of ethylene oxide which is 100 percent effective for sterilization. In our laboratory, under environmental conditions of 60 percent to 80 percent relative humidity and a temperature of 37°C, and for an exposure time of at least 24 hours, 1 percent by volume of ethylene oxide killed large inoculums of *Aerobacter aerogenes, Escherichia coli,* and *Bacillus megaterium* bacteriophage, which were added to the tissue culture media, as well as a yeast-like organism isolated previously from a contaminated stock bottle of HeLa cells; but sterilization of large amounts of heavily contaminated materials, or residual microbial toxins injurious to cells, was not attempted.

It is recommended that tissue culture media should be sterilized with ethylene oxide 24 hours before the media are to be used, although practical applications may depend on what cell lines are to be cultured. From the results obtained in this laboratory, it would seem entirely feasible to sterilize test chemicals or any chemical agents added to tissue culture media which cannot be sterilized by heat or which are ordinarily filtered, especially if these substances are generally used in very small quantities.

Table 1. The effects on a tissue culture medium of ethylene oxide, added 24 hours prior to inoculation of Chang liver cells or HeLa cells.

Ethylene oxide (concn.)	Plating efficiency (%)	
(μl/5ml of medium)	HeLa cells	Chang liver cells
0	47.2*	24.3*
25	63.1	85.9
50	56.3	95.8
75	23.3	74.2
100	22.0	

*These figures represent the absolute plating efficiency which was calculated from the original inoculum of cells per plate. All other relative plating efficiencies shown were calculated from the number of colonies that developed on the experimental plates, compared with the number of colonies that developed on the controls.

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Culture of Small Barley Embryos on Defined Media

Abstract. *Barley embryos, measuring 90 μ in length, grew and became differentiated on a phosphate-enriched White's medium which was fortified with glutamine and alanine as major sources of organic nitrogen, and lesser amounts of five other amino acids. The pH of the medium was a critical factor in differentiation. On this medium, excised barley embryos developed more rapidly than embryos remaining in vivo, or embryos grown on media containing coconut milk.*

In order to study many aspects of the growth and development of living embryos of vascular plants, it is necessary to excise them from surrounding parental tissues and culture them on a suitable medium. A number of investigators have successfully cultured larger, differentiated plant embryos, but cultures of small, undifferentiated or slightly differentiated embryos have rarely succeeded, and much remains to be discovered about their nutritional requirements.

In studies of embryogenesis in barley, difficulties have been experienced in the culture of embryos smaller than 0.5 mm (*1, 2*). The best results have been obtained (*3*) with media containing coconut milk, but reproducible results are difficult to obtain. Therefore, we attempted to devise a satisfactory medium of entirely known, or defined, composition.

The analysis of the organic constituents of coconut milk reported by Tulecke *et al.* (*4*) served as a starting point. No attempt was made to duplicate coconut milk in its known entirety; rather, those constituents found previously to be active in embryo cultures were used. For example, the reports of Sanders and Burkholder (*5*), that combinations of amino acids promoted the growth of small *Datura* embryos, and of Rijven (*6*) that glutamine and alanine were especially effective in promoting growth of *Capsella* embryos, suggested that these substances should be used in trial media. Other substances, such as sorbitol, inositol, indoleacetic acid, gibberellic acid, various purines and pyrimidines, and certain vitamins also were tested. Some of these were ineffective; others produced peculiar morphogenetic effects and were not included in our final medium. In addition, we studied the effects of dif-

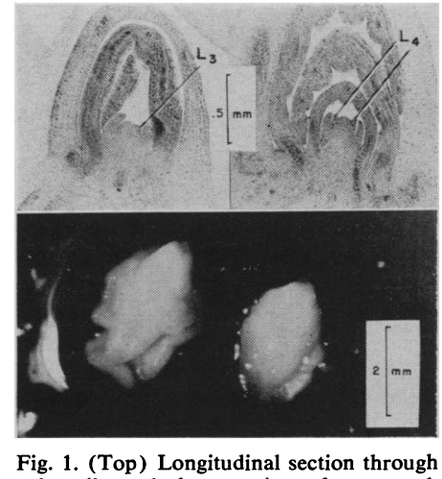

Fig. 1. (Top) Longitudinal section through coleoptile and shoot region of a normal, full-term barley embryo (left), and an embryo which was initially 0.3 mm in length, after it was cultured for 10 days on the medium defined in Table 1 (right). Number of leaf primordia in each indicated by arrows and symbols. (Bottom) Embryo cultured for 10 days on the defined medium (left) is compared with normal, full-term embryo (right).

Table 1. Composition of the medium used for culturing immature barley embryos (based on White's medium). The amounts of sucrose and agar added were, respectively, 90.0 and 9.0 g/l.

Inorganic				Organic			
Primary and secondary nutrients	Amount (mg/l)	Minor nutrients	Amount (mg/l)	Vitamins	Amount (mg/l)	Acids	Amount (mg/l)
$MgSO_4 \cdot 7H_2O$	730.0	$MnSO_4 \cdot 4H_2O$	3.0	Niacin	1.25	Malic acid	100.0
$Ca(NO_3)_2 \cdot 4H_2O$	290.0	$ZnSO_4 \cdot 7H_2O$	0.5	Thiamine	0.25	Glutamine	400.0
Na_2SO_4	200.0	H_3BO_3	0.5	Pyridoxine	0.25	Alanine	400.0
KNO_3	160.0	$CuSO_4 \cdot 5H_2O$	0.25	Ca pantothenate	0.25	Leucine	20.0
KCl	140.0	$Na_2MoO_4 \cdot 2H_2O$	0.25			Tyrosine	10.0
$NaH_2PO_4 \cdot H_2O$	800.0	$CoCl_2 \cdot 6H_2O$	0.25			Phenylalanine	10.0
		Fe citrate	10.0			Cysteine	10.0
						Tryptophane	10.0

ferent concentrations of sucrose and minerals, and various *p*H values.

In the medium composed of the substances listed in Table 1, excised barley embryos as small as 90 μ in length grew and differentiated. Nearly all embryos longer than 100 μ developed normally (Fig. 1); although some of the embryos measuring from 90 to 100 μ differentiated normally, many of them exhibited a "thalloid" character and grew as irregular masses of tissue without leaf or root primordia. This type of development has also been observed in barley embryos cultured on a medium containing coconut milk (*3*), although on this medium, roots and leaves did differentiate in some embryos. It seems probable that coconut milk contains other factors which are required primarily for initiation of primordia, and that this action is separate from the maintenance of embryonic growth and differentiation in vitro.

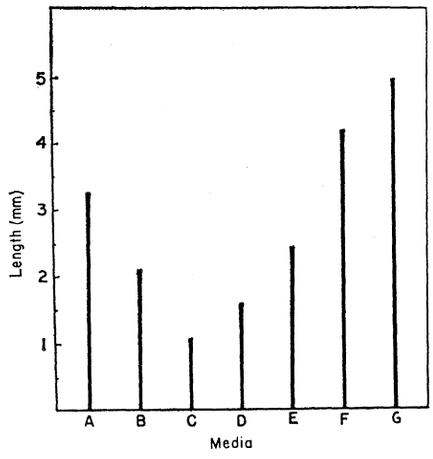

Fig. 2. Maximum length attained by barley embryos in vivo (*A*), by 0.4-mm embryos cultured for 10 days on White's medium plus 20 percent coconut milk (*B*), White's medium (*C*), White's medium plus 400 mg/l alanine (*D*), White's medium plus 400 mg/l glutamine (*E*), White's medium plus 400 ml/l each of glutamine and alanine (*F*), and on the medium listed in Table 1 (*G*).

However, it is necessary to develop a medium capable of sustaining embryonic growth before investigating such primordium-initiating substances as may be present in coconut milk.

To test the medium in Table 1, embryos ranging in length from 0.3 to 0.4 mm were cultured. Their growth was compared with the growth of similar embryos cultured on other media. The results of these experiments are presented in Fig. 2.

The normal, full-term barley embryo (Fig. 2) is about 3.2 mm long, and has three well-defined leaf primordia, one primary and four lateral roots, as well as other peculiarly embryonic structures. After being cultured for 10 days on media containing coconut milk, embryos which measured 0.4 mm when excised attained a length of 2.0 mm, and had two leaf primordia and up to five roots. Other embryonic structures usually were moderately well formed. With White's medium alone, 0.4-mm embryos grew to a length of about 1.2 mm, and had only one leaf primordium and not more than three root primordia. The addition of glutamine to White's medium, modified by increasing the phosphate level, resulted in greatly increased embryonic growth, but seemed to favor particularly the enlargement of the scutellum. Alanine, by itself, scarcely accelerated growth and differentiation, but in combination with glutamine increased both growth and differentiation. This occurred only on media in which the *p*H was adjusted to a value between 4.0 and 5.2; above 5.2, growth occurred but there was little differentiation. The optimum *p*H was about 4.9.

The addition of malic acid appeared to enhance the effect of glutamine and alanine as well as to serve as an effective buffering agent. Addition of leucine, tyrosine, phenylalanine, cysteine, and tryptophane produced additional growth. Embryos initially 0.4 mm long reached a length of 5.0 mm in 10 days and developed four leaf primordia and five roots.

The importance of maintaining low *p*H values in media used for culturing small barley embryos is supported indirectly by other investigations. Ziebur *et al.* (*1*) found that excised barley embryos smaller than 0.5 mm could not be grown on media containing casein hydrolysate, amino acids, or high concentrations of phosphate ions, although larger embryos grew well on such media. The *p*H of their media was stated to be 5.6. Cockerline (*2*) tested a variety of media in which the *p*H was adjusted to 5.7, but was unable to grow barley embryos that were smaller than 0.3 mm when excised. It was reported previously (*3*), that amino acids contributed little to the growth of small barley embryos; however, the *p*H values of the media in these earlier experiments were higher than 5.2. Undoubtedly, the failures in all these instances were due, at least in part, to the relative alkalinity of the media.

The complete medium appears to be rather specific in its action. Embryos of rye, wheat and *Capsella* grow erratically on it. Our few attempts to grow barley zygotes on this medium were unsuccessful, but it is significant that with larger embryos, growth and differentiation appeared to be normal. Furthermore, embryonic growth beyond that occurring naturally is a commonplace occurrence. One embryo, cultured for 5 weeks, attained a diameter of 14.4 mm, primarily through a tremendous overgrowth of the scutellum. The overgrowth tissue appeared to be of a thalloid nature rather than callus, for it was a convoluted, laminar type (*7*).

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