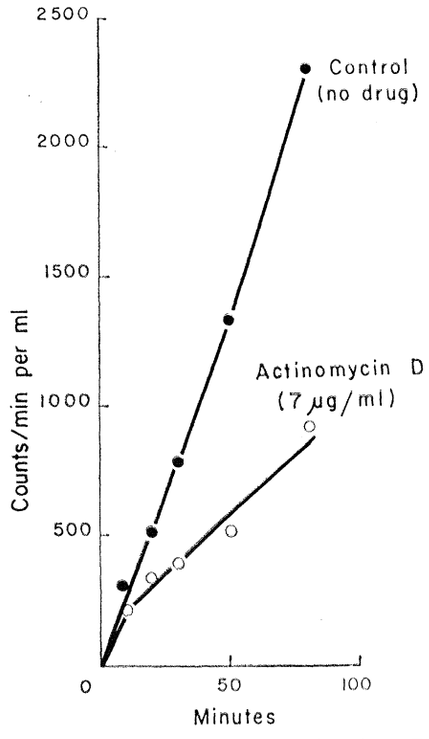

Fig. 3. Uracil-2-C^{14} incorporation into *B. subtilis* strain II 15/A in the presence and absence of actinomycin D.

ance to actinomycin in which development of resistance was assumed to result from (i) cell surface changes prohibiting entry of drug, (ii) drug binding or detoxification within cell, or (iii) enzyme inactivation of the drug. In microbial systems, the natural resistance of *Escherichia coli* to actinomycin D has been shown, by Haywood and Sinsheimer (*5*) and Mach and Tatum (*6*) who used protoplast suspensions, to be due to impermeability of intact cells to actinomycin. Our study was undertaken to establish a mechanism for the emergence of drug-resistant bacterial cell lines from sensitive parent strains.

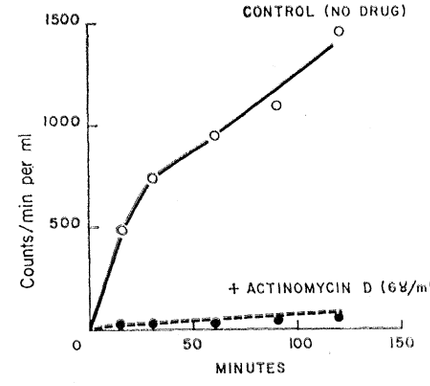

Fig. 4. Uracil-2-C^{14} incorporation into protoplasts of *B. subtilis* protoplasts II 15/A in the presence and absence of actinomycin D.

Strains of *Bacillus subtilis* (No. 6051 from the American Type Culture Collection) resistant to actinomycin D were isolated by the gradient-plate technique (*7*). Stock slants of drug-resistant isolates were maintained on heart-infusion medium that contained 100 and 1000 times as much actinomycin (10 and 100 μg per milliliter, respectively) as could be tolerated by a sensitive parent. Protoplasts were prepared with lysozyme (0.5 mg/ml) and 0.2*M* sucrose in *M*/15 phosphate buffer at *p*H 7.0. After 10 minutes incubation at room temperature, protoplasts were centrifuged, washed with the buffered sucrose solution, and suspended in Demain's synthetic basal medium (*8*) containing 1 percent glucose and 0.5*M* sucrose. Protoplast preparations were made from suspensions of intact cells adjusted to an optical density of 1.50 at 575 mμ. For studies with whole cells an optical density of 0.20 was used.

Sensitivity of either whole intact cells or protoplasts to actinomycin D was determined by measuring the incorporation of uracil-2-C^{14} into material insoluble in cold 5 percent trichloroacetic acid. Uracil-2-C^{14} (0.1 μc/ml and 10 μg/ml) was added to a suspension of protoplasts or whole cells incubated in the presence of actinomycin D (6 to 7 μg/ml) or its absence. Samples were removed at intervals and added to an equal volume of 10 percent trichloroacetic acid, filtered, and washed on Millipore filters as previously described (*9*). The radioactivity of the acid insoluble material was measured on a gas-flow counter.

The incorporation of uracil-C^{14}, a precursor of cellular RNA, into whole cells and protoplasts of the sensitive parent strain *B. subtilis* No. 6051 is completely inhibited by actinomycin D (Figs. 1 and 2) whereas only partial inhibition of uracil-C^{14} incorporation occurs with actinomycin-resistant intact cells of *B. subtilis* strain II 15 (Fig. 3). In contrast with the effect of actinomycin D on uptake of uracil-C^{14} into resistant whole cells, incorporation into protoplasts of the resistant strain of *B. subtilis* is completely inhibited (Fig. 4). The sensitivity of resistant-cell protoplasts to drug effect clearly supports the conclusion that actinomycin penetration does not occur in resistant intact whole cells.

Since other experiments have shown that the antibiotic is not broken down in the presence of resistant cells, resistance is considered to result from a change in cell permeability.

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28 July 1964

Morphogenetic Studies with Partially Synchronized Cultures of Carrot Embryos

Abstract. *Callus tissue, derived from petiole segments of the wild carrot and containing undifferentiated meristems, can be suspended in liquid nutrient media and passed through a series of sieves to separate the meristems by size. The small meristems of fairly uniform size thus obtained will grow on defined media where their controlled differentiation into embryos can be studied.*

A simple method has been devised for studying morphogenetic problems through the use of partially synchronized cultures of embryos derived from somatic cells of the wild carrot, *Daucus carota*. Details of the embryogenesis which occurs in callus tissue derived from petioles, roots, or stems cultured on simple defined media were described elsewhere (*1*).

Segments of carrot petiole (*2*) cultured on an agar-solidified basal medium (*3*) plus adenine and 2,4-dichlorophenoxyacetic acid (2,4-D) produce callus which contains many small, approximately spherical, meristems. Such callus will multiply its fresh weight five- to tenfold during successive 30-day periods in subculture. The callus forms new meristems for a variable

number of subcultures, after which only parenchymatous cells are produced.

These meristems are preglobular and globular proembryos, since removal of 2,4-D permits their further development into mature embryos with normal morphology (Fig. 1*b*). However, such proembryos exhibit considerable morphogenetic plasticity and may develop into structures which show varying degrees of root and shoot development and may bear little resemblance to embryos. A series of such abnormal forms is shown in Fig. 1, *c–f*. These abnormal structures are commonly found along with normal embryos in callus. The nonuniform environment of a callus mass evidently allows random development along several different pathways.

To shed light on this phenomenon through the study of possible morphogenetic influences of exogeneously supplied growth regulators and metabolites, methods have been developed for routinely separating small, undeveloped meristems (Fig. 1*a*) from callus by dispersing the tissue in a liquid medium and then washing the suspension through a series of stainless steel sieves to separate the various structures by size. Meristems from petiole callus are routinely sieved after the third subculture and are pipetted onto the agar surface of a petri plate. Conditioning of the medium is reduced by using a small inoculum, a relatively large amount of nutrient medium, and a short culture period.

Morphological variability can be considerably reduced, and a near-synchronous development of hundreds of embryos approached by utilizing small meristems of narrow size range. The smallest size range obtainable with our sieves was 45 to 75 μ.

Meristems developed into embryos when cultured on the basal medium consisting of minerals, sucrose, and vitamins (*3*). In preliminary experiments, several hormones and nitrogen sources were tested for their effects on embryogenesis, particularly to determine if some improvement could be made in growth of the cotyledons which were abnormally small on the basal medium. Indoleacetic acid, kinetin, adenine, and 2,4-D were added singly to the basal medium in concentrations ranging in tenfold steps from 10^{-6} to 10^{-3} gram per liter and in several different combinations shown to promote development of globular seed embryos of another species (*4*). Indoleacetic acid and kinetin were toxic

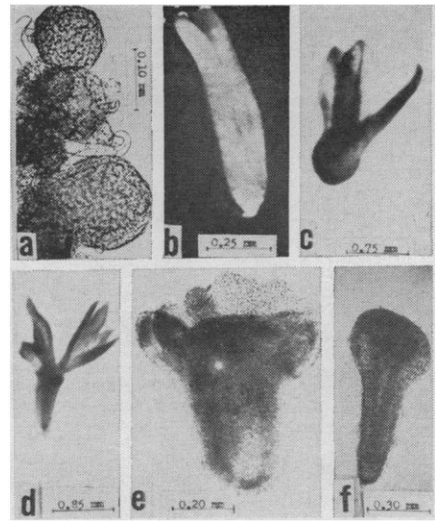

Fig. 1. *a*, Globular proembryos isolated from wild carrot callus. *b*, Adventive embryo with normal morphology. *c*, Abnormal embryo with three large cotyledons and lacking a linear hypocotyl. *d*, Abnormal embryo with expanded, dissected leaves. *e*, Abnormal embryo with ten or more leaf primordia at cotyledon node. Several primordia have been cut off to reveal full circumference of the node. *f*, Root-bearing meristem which shows no signs of a shoot apex.

when used singly at a concentration of 1.0 mg/liter, and permitted only abnormal organ formation from a few large meristems (in the 150- to 250-μ size range) which already showed signs of axial development. With 2,4-D at 1.0 mg/liter considerable growth of undifferentiated meristems occurred and many small spheres became macroscopically visible on the agar surface. With kinetin or indoleacetic acid at a concentration of 0.1 mg/liter, meristems developed rapidly into linear embryos, whereas with 0.1 mg of 2,4-D per liter, the growth of relatively undifferentiated proembryos continued. Thus there may be no concentration of indoleacetic acid or kinetin that will promote continued meristematic activity without concomitant organ formation, while 2,4-D will do so over a tenfold change in concentration. At a concentration of 0.01 mg of 2,4-D per liter, embryos developed. No single hormone or combination of hormones markedly improved cotyledon development. Embryos treated with adenine were similar over the entire range of concentrations used; in their excessive red pigmentation they resembled kinetin-treated embryos, but they showed none of the primary-root inhibition exhibited by embryos grown with high concentrations of kinetin.

In experiments on the effect of different nitrogen sources, kinetin (10^{-6} g/liter) and 2,4-D (5×10^{-7} g/liter) were used, and nitrogen was supplied at 20 m*M*/liter. It was found that inorganic nitrogen of the basal medium (nitrate and ammonium salts), or the basal medium plus 10 percent coconut milk, or a modified basal medium (inorganic nitrogen salts left out) plus glutamine, all gave similar results (Fig. 2*A*). Asparagine or a mixture of amino acids patterned after the composition of coconut milk (*5*) were somewhat inhibitory. Autoclaved and filter-steri-

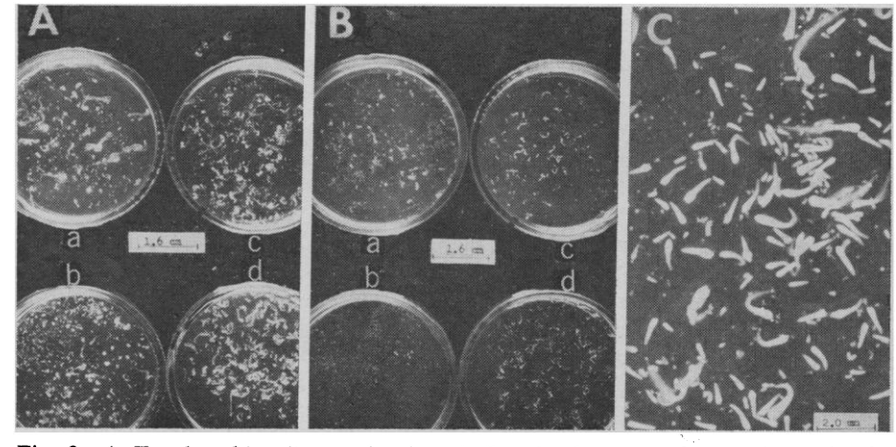

Fig. 2. *A*, Ten-day-old cultures, showing similar development of proembryos in the 150- to 250-μ size range, on undefined media (*a* and *b*) consisting of the basal medium plus 10 percent coconut milk (*a*, sterilized by filtering; *b*, autoclaved) and on defined media (*c* and *d*) in which nitrogen was supplied as the nitrate and ammonium salts in the basal medium (*c*), or as glutamine in place of the nitrate and ammonium salts (*d*). *B*, Seven-day-old cultures illustrating the rapid development of proembryos in the 45- to 75-μ size range on the same media as in *A*, except that autoclaved coconut milk was inhibitory. *C*, An enlargement of the embryos treated with glutamine (*B*, plate *d*).

lized coconut milk gave similar results with proembryos in the 150- to 250-μ size range, but autoclaved coconut milk inhibited differentiation of proembryos in the 45- to 75-μ size range (Fig. 2*B*, plate *b*), causing them to grow as peculiar flattened masses on the agar. An enlarged view of embryos on the glutamine plate (Fig. 2*B*, plate *d*) is shown in Fig. 2*C*.

Microtome sections of preglobular proembryos reveal considerable histological disorganization and often no evidence of polarity. Small meristems apparently overcome this disorganization to develop normal axial polarity. In large irregularly shaped meristems, or in tissue masses where small meristems are partially fused, the polarity which is initially established seems to be radial rather than axial. Thus embryos developing from large meristems, or from dense clusters of small meristems, do so by bud-like outgrowth in such a manner that most of the tissue from the original meristem or cluster is left as a suspensor-like remnant. Embryos from small single meristems may lack this suspensor-like component.

The techniques described here allow experimental control only of the postglobular stages of embryogenesis. Modifying these techniques may permit some degree of experimental control on defined media over the ontogenetic sequence starting with single cells.

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2. Callus derived from petiole segments is exceptionally prolific in the regeneration of meristems. This callus arises from parenchyma in the vascular bundles (unpublished observations of E. K. Abendroth). Excised carrot embryos have also been used to obtain cell suspensions which, on media containing coconut milk, will form adventive embryos [F. C. Steward, M. O. Mapes, A. E. Kent, R. D. Holsten, *Science* **143**, 20 (1964)].
3. The basal medium used in this study consisted of Murashige's minerals [M. Lin and J. Staba, *Lloydia* **24**, 139 (1961)], 2 percent sucrose, thiamin hydrochloride at a concentration of 5.0 mg/liter, and nicotinic acid at 5.0 mg/liter. Adenine at 2.0 mg/liter was not required for initial formation of the callus or for meristem development but was beneficial to growth of the callus after several subcultures. 2,4-Dichlorophenoxyacetic acid was used at a concentration of 0.1 mg/liter.
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6 July 1964

Placenta of the Indian Elephant, Elephas indicus

Abstract. *The placenta of the Indian elephant is incompletely annular and zonary macroscopically and occupies the equator of an ovoid chorioallantoic sac. The amnion is fused with the chorion over the zone. Microscopically, the placenta is labyrinthine and endotheliochorial with a rudimentary marginal hematoma. Both macroscopically and microscopically it resembles the placentas of the carnivores, particularly the racoon, the cat, and the dog.*

Classification of the Indian elephant placenta (*1*) is based on scattered reports, almost all published prior to 1908 (*2–5*). Some are incomplete as regards microscopic anatomy (*2*); one is based on less than a full-term gestation (*3*), and others on only portions of the placenta (*4*). In the report of an extensive survey of Eutherian mammalian placentation by Mossman in 1937, the Indian elephant placenta is described as "zonary with villous patches at each end of the chorionic sac. Zone apparently hemochorial and villous patches syndesmochorial, but this is uncertain" (*1*). Recently, two full-term intact Indian elephant placentas were obtained, examination of which indicates that placentation in the Indian elephant is not hemochorial.

On 15 September 1963 and 24 September 1963, respectively, viviparous full-term male and female elephant calves (*Elephas indicus*) were born at the Portland Zoological Gardens. Both calves were sired in captivity by the same bull, a Siamese Indian elephant (Bangkok, Thailand). The mothers were, respectively, a Siamese Indian elephant (Bangkok, Thailand) and an Asiatic Siamese elephant (Saigon, Vietnam). The gestational period for both pregnancies was 634 days plus or minus several days. Intact placentas were delivered and obtained at periods of 2 hours and 30 minutes and 3 hours and 28 minutes after calving, respectively. Weights and dimensions are given in Table 1.

For both calves, the chorionic sac was ovoid (Fig. 1*A*) and was encircled on the maternal surface by an annular equatorial placental zone (Fig. 1*B*). On cross section, the zone was dome-shaped. The lateral convexities of the maternal surface of the zone were smooth and green-brown, and bordered a central elevated spongy area which, like the bulk of the sectioned surface, was a deep red-purple (Fig. 1*C*). In the placenta of the male calf, the annulus was bilobed, consisting of one small and one large lobe separated by distances of 5 and 12 cm, while in that of the female calf the zone was incomplete over a distance of 15 cm. Small (2 to 3 cm in diameter) flattened areas of brown friable tissue were present at the poles of the chorionic sac. The remainder of the maternal surface was smooth, opaque, and pink-tan. The fetal surface was opaque with a faint purple hue. It was characterized by an intricate network of fetal vessels with complex vascular crossings. Numerous plaques averaging 2 cm in diameter were distributed along the course of these vessels (Fig. 1*D*). The amnion was filmy and delicate, and was attached to the chorion over that portion bordered by the placental ring. Over the remainder of the chorionic surface it seemed to be separated from the chorion by a well-defined space (see *1*, plate 22, Fig. D). The umbilical cords, at the fetal extremity, consisted of two arteries and one vein, each measuring 1 cm in diameter. As a result of two venous branchings, but only one arterial branching, the cord entered the chorionic surface of the placenta as four separate arteriovenous pairs, two of which supplied the margins of the villous ring and two entered the center of the ring. In the placenta of the male calf, a pedunculated purse-like yolk sac, measuring 7 by 7 by 1.5 cm, was in proximity to one of the four umbilical vessels. This structure was not identified in the female placenta.

Microscopically, the placenta is labyrinthine and consists of delicate

Table 1. The weights and dimensions of the Indian elephant placentas.

Placental weight (kg)	Diameters of placental zone (cm)	Dimensions of sectioned surface of zone (cm)	Length of umbilical cord (cm)
Male calf, weighing 68.1 kg			
11.3	50 × 42	13 × 5.5	110
Female calf, weighing 109 kg			
11.8	58 × 48	16 × 6.0	114