(*5*). Lymphocyte-rich cells (10^7) were added to each culture dish containing 10^6 M.R. cells and phagocytized sheep red blood cells. The plaque-forming-cell response was equivalent to that of normal spleen cells with 10^7 sheep red blood cells present throughout culture, and is indicated by the broken line in Fig. 2. These results were confirmed in subsequent experiments. A single pool of normal spleen cells was divided into various populations as listed in Table 1. A M.R. population (M.R. 1, 10^6 cells per dish; M.R. 2, 5×10^5 cells per dish; M.R. 3, 2.5×10^5 cells per dish) was incubated with 10^7 sheep red blood cells per culture dish for 30 minutes. The dishes were washed carefully, and either 10^7 normal spleen cells or 10^7 L.R. 3 cells were added to each culture dish. Both methods of culture produced a significant plaque-forming-cell response, comparable in magnitude to that produced by normal spleen cells continuously exposed to antigen. Little or no response occurred if either M.R. cells or L.R. cells alone were cultured with 10^7 sheep red blood cells; the very small response of the L.R. 1 cells can be attributed to a few macrophages remaining in this cell population. Similar results were obtained in two additional experiments of identical design presented in Table 1.

Thus, both adherent and nonadherent mouse spleen cells are essential for development of plaque-forming cells in vitro. Moreover, an amount of phagocytized antigen estimated to account for less than 5 percent of the total antigen dose is sufficient to produce a maximum in vitro immune response. That more antigen is not required for immune induction is consistent with the studies of several workers (*6*).

It appears that in the mouse spleen, production of antibody to sheep erythrocytes involves both antigen phagocytosis by macrophages and macrophage lymphocyte interaction, both processes being essential for development of lymphoid cells releasing hemolytic antibody. It has been suggested that transfer of information between two cell types involves RNA or RNA-antigen complexes (*7*). It may now be possible to determine the nature of "information transfer" between two cell types by using in vitro induction of antibody synthesis.

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5. Ten replicate hemacytometer counts were made on (i) the suspension of sheep red blood cells used to inoculate the culture dishes and (ii) the suspension of sheep red blood cells obtained by combining the recovered culture fluid and the repeated washings after the 30-minute culture interval. By this method of enumeration, as many sheep red blood cells were recovered as were inoculated. Considering the error of replicate counts to be 5 percent or less with the dilution method employed, 5×10^5 or fewer sheep red blood cells are estimated to have been phagocytized by the macrophages.
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12 October 1967

Growth of Isolated Mesophyll Cells of Arachis hypogaea in Simple Defined Medium in vitro

Abstract. *Isolated mesophyll cells from leaflets of* Arachis hypogaea *can be cultured in a simple, defined liquid nutrient medium containing minerals, with an appropriate source of ammonia, sugar, 2,4-dichlorophenoxyacetic acid, and kinetin. The significance of such a simple medium in understanding the problems of cell metabolism, growth, and morphogenesis is discussed.*

In recent years much success has been achieved in the isolation and culture of single cells of higher plants from callus cultures (*1*). In most investigations, complex growth substances such as coconut milk, yeast extract, and other substances were used as supplements to the final media, and thus the understanding of specific growth requirements of the isolated cells and of cell morphogenesis is restricted. Attempts to obtain a defined medium for the growth of such cells (*2*) showed the exogenous requirement for various metabolites, which only reveals the heterotrophic potential of the cells. The understanding of plant morphogenesis ultimately depends upon the isolation of a strain of cells that is completely autotrophic.

According to Steward *et al.* (*3*), if cells are to exhibit their full totipotency, two requirements must be met: (i) a cell must be freed from organic connections with other cells, and (ii) free cells must be nourished by a medium which is fully competent to support their rapid growth and development. It has been widely accepted that formation of embryo-like structures from single carrot cells requires the presence of coconut milk (*4*). However, Halperin (*5*) has shown that embryogenesis occurs readily in cultures of wild carrot on media containing only minerals, sucrose, vitamins, and an

Table 1. Effect of added ammonium citrate, tartrate, or nitrate on the growth and chlorophyll (Ca and Cb) formation of isolated mesophyll cells of *Arachis hypogaea* in basal medium.

Ammonium salts added (mg)	Final fresh wt. (mg)	Growth value	Chlorophyll content (μg/g fresh wt.)	
			Ca	Cb
		Citrate		
None	183.3	305.5	109.7	49.1
400	382.1	636.8		
800	189.7	316.1	53.3	24.7
1600	Insignificant growth, few pale green spheres formed			
		Tartrate		
400	342.2	570.3	36.6	20.9
800	335.3	558.8	60.2	26.2
1600	210.2	350.3	43.6	30.9
		Nitrate		
400	120.0	200.0	115.2	74.8
800	224.0	373.3	117.5	64.2
1600	51.2	85.3	105.9	57.1

Table 2. Effect of added ammonium citrate, tartrate, or nitrate on the growth and chlorophyll formation of isolated mesophyll cells of *Arachis hypogaea* in P-medium.

Ammonium salts added (mg)	Final fresh wt. (mg)	Growth value	Chlorophyll content (μg/g fresh wt.)	
			Ca	Cb
		Citrate		
400	64.0	106.6	123.2	
800	124.3	207.1	25.7	8.7
1600	131.7	219.5	29.9	7.6
		Tartrate		
400	68.6	114.3	63.4	27.6
800	133.0	221.6	68.4	33.8
1600	235.2	392.0	61.3	30.4
		Nitrate		
400				
800				
1600	91.3	152.1	34.9	23.0

auxin. He further showed that, as judged by ultrastructural studies, embryos are not formed from single cells, and he contended that if there are special "embryonic nutrients" involved they must be produced by the cells themselves. Thus, the present state of research does not really add much to the knowledge of cell morphogenesis. However, it is only through a systematic study of isolated individual cells that an understanding of the problems of cell morphogenesis, differentiation, and other biochemical and physiological aspects of growth may be achieved. For an effective approach to understanding these problems, the proper material must be chosen. Such material should have two prerequisites: (i) the starting cell should be normal, unlike the cells isolated from callus cultures, which come from an artificial environment exhibiting various biochemical, physiological and cytological modifications, and (ii) the requirements of the isolated cells should be understood in terms of the simplest compounds supporting their growth. In addition, the requirements called for by Steward *et al.* (*3*) should be met.

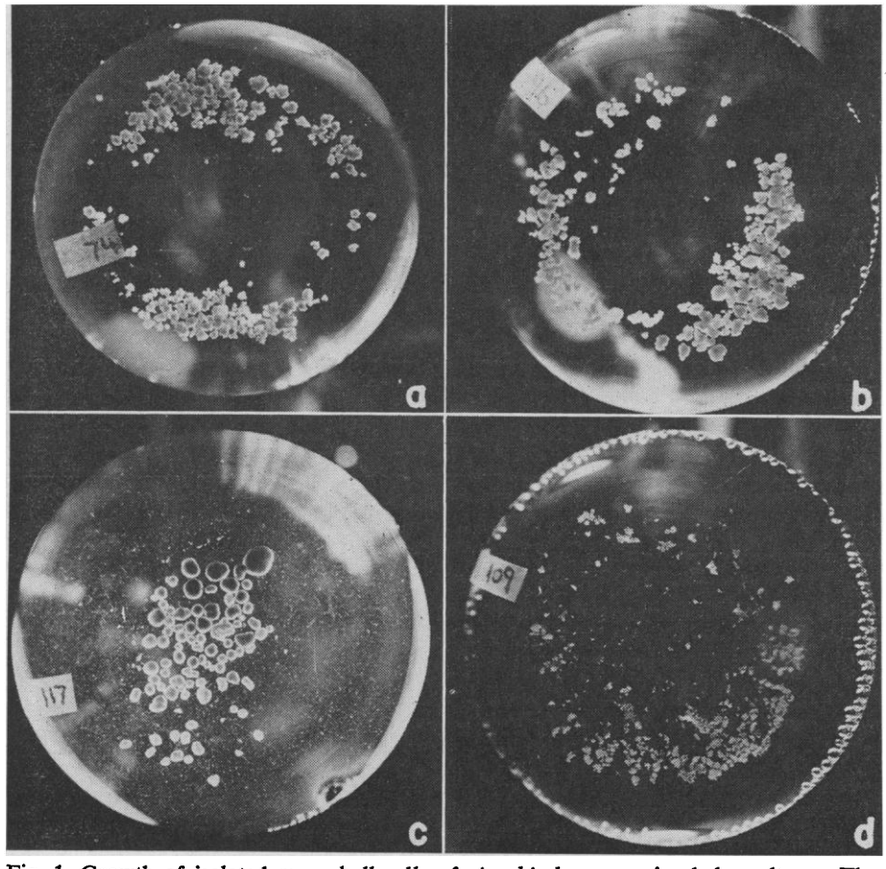

Fig. 1. Growth of isolated mesophyll cells of *Arachis hypogaea* in shake cultures. The average fresh weight of inoculum per flask was 0.6 mg. Bottom of the flask is photographed. (a) Forty-day-old culture in P-medium plus 1600 mg of ammonium citrate (× 0.82); (b) 43-day-old culture in P-medium plus 1600 mg of ammonium tartrate (× 0.87); (c) 62-day-old culture in P-medium plus 1600 mg of ammonium nitrate (in presence of sodium nitrate) (× 0.88); (d) 30-day-old culture in P-medium plus 1600 mg of ammonium nitrate (in absence of sodium nitrate) (× 0.87).

Table 3. Effect of added ammonium nitrate on the growth and chlorophyll formation of the isolated mesophyll cells of *Arachis hypogaea* in the absence of sodium nitrate from the P-medium.

Ammonium nitrate added (mg)	Final fresh wt. (mg)	Growth value	Chlorophyll content (μg/g fresh wt.)	
			Ca	Cb
400	77.7	129.5	18.8	12.3
800	206.8	344.6	27.6	20.7
1600	273.1	455.1	25.4	10.7

Haberlandt (*6*) demonstrated that one of the best sources for such cells is the mesophyll from leaves, but he was unable to cultivate these cells. The growth potential of isolated mesophyll cells from the leaflets of *Arachis hypogaea* has been conclusively demonstrated by time-lapse studies (*7, 8*). We now report on the growth of these cells in media simpler than those reported earlier.

The technique of isolating and culturing mesophyll cells is described elsewhere (*7, 8*). An average fresh weight of inoculum per Erlenmeyer flask containing 40 ml of liquid medium was 0.6 mg. Growth increments (fresh weight) were measured as averages of four to five replicates per experiment after a growth period of 30 days, the growth value being the ratio of final weight to initial weight. Mesophyll cells are primarily photosynthetic and under conditions of culture they produce a tissue which is usually green. This characteristic makes these cells suitable for studies of autotrophic growth and determination of factors responsible for the formation of chlorophyll. The chlorophyll content of tissue obtained from isolated mesophyll cells in various media was determined. The tissue was ground in a mortar, extracted with 80 percent acetone, and centrifuged; the absorption of the extract was measured spectrophotometrically, and the quantitative estimations of chlorophyll *a* (Ca) and *b* (Cb) were made by the formula given by Arnon (*9*).

The basal medium included minerals as listed by Joshi and Ball (*7*), with the following supplements (milligrams per liter): sucrose, 20,000; casein hydrolyzate free of salt and vitamins, 400; 2,4-dichlorophenoxyacetic acid, 1; kinetin, 1; thiamine hydrochloride, 0.4; myo-inositol, 100. The final medium was sterilized by filtration. Thiamine and myo-inositol are not essen-

tial for the growth of the cells, but they are routinely added to the medium. The basal medium excluding casein hydrolyzate is designated P-medium. The inorganic sources of nitrogen tested were ammonium citrate, ammonium tartrate, or ammonium nitrate.

In the medium containing only minerals, sucrose, auxin, and kinetin, there was some growth of the isolated cells (from 0.6 mg to 6 mg). In a medium with casein hydrolyzate but no sugar, growth was better (0.6 mg to 27 mg). When both casein hydrolyzate and sugar were present, there was marked increase in growth (0.6 mg to 183.3 mg). Thus, besides sugar, an organic nitrogen source (casein hydrolyzate) is apparently a primary need for the growth of these isolated cells. Trials were made with individual amino acids (at concentrations contained in 400 mg of casein hydrolyzate). Only additions of glutamic acid, lysine, and proline gave some growth response. Increased concentrations of casein hydrolyzate (800 to 1600 mg/liter) did not improve the growth; 400 mg/liter appeared to be the optimum requirement. However, if the cells were supplied with a mixture of organic (casein hydrolyzate) and inorganic (NH_4^+) sources of nitrogen, there was significant increase in the growth (Table 1).

As the higher growth values were obtained, there was a decrease in the amount of chlorophyll in the tissue produced by the isolated mesophyll cells. This fact is comparable to findings of experiments with *Euglena*. When grown at fairly high temperature for several successive generations, such cells replicate faster than the chloroplasts, giving rise to successively paler cells. The mesophyll cells exhibited a higher chlorophyll content when grown in the basal medium containing added ammonium nitrate than they did in medium with added ammonium citrate or tartrate.

To obtain some idea about the mettabolic potential of the cells, and eventually to achieve their autotrophic growth, we omitted casein hydrolyzate from the basal medium (P-medium) and replaced it by one of the ammonium salts (Table 2; Fig. 1, a–c). At concentrations of 2000 to 2400 mg/liter, growth was insignificant.

Among the nutrients of the P-medium is $NaNO_3$, and additional NH_4NO_3 in the medium increased the concentration of NO_3^- relative to that of NH_4^+. The lack of growth at a lower concentration of NH_4NO_3 in the presence of $NaNO_3$ in the P-medium could be due to the fact that the isolated mesophyll cells lack an efficient nitrate-reductase system and thus cannot reduce nitrate to ammonia. Therefore, the presence of increased NO_3^- may in some way check the availability of NH_4^+ which could be absorbed by the cells. As soon as certain balance between NH_4^+ and NO_3^- is attained in the medium, NH_4^+ becomes available to the cells. In addition, the *p*H of the medium favors the uptake of NH_4^+ over that of NO_3^-. According to Arrington and Shive (*10*), a low *p*H favors nitrate uptake, and a high *p*H favors ammonia uptake. However, we made no such experiments with isolated mesophyll cells.

In the absence of $NaNO_3$ from the P-medium, the addition of NH_4NO_3 resulted in an increased growth (Table 3). The growth increased as the amount of ammonium nitrate added to the medium was increased; maximum growth was attained at 1600 mg/liter (Fig. 1d).

Thus, the isolated mesophyll cells can synthesize their own metabolites from the mineral nutrients. Ammonia seems to be one of the crucial requirements. For these cells, the only requirements for growth (by division and enlargement) are the minerals with an appropriate ammonia source, and energy source (sugar), auxin, and kinetin. The tissue mass produced by the isolated mesophyll cells in such a medium in shake cultures, when transferred to agar medium (*11*) supplemented either with ammonium citrate or tartrate, could grow continuously if subcultured at regular intervals.

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25 October 1967

Comparative X-Ray and Neutron Diffraction Study of Bonding Effects in s-Triazine

Abstract. *X-ray and neutron-diffraction data were combined for study of deviations from spherical symmetry of the atomic charge distributions in the small organic molecule* s-*triazine. The results indicate that density has migrated from the atomic regions into the bonds and into the nitrogen lone-pair region. Refinement procedures for x-ray data, which do not take these bonding effects into account, give parameters containing small but measurable errors.*

In conventional refinement of x-ray data by least-squares techniques, all atoms are assumed to have spherical symmetry. In reality many atoms in molecules are in an asymmetric environment. Thus the centroid of the atomic charge density often does not coincide with the atomic nucleus, because of the presence of overlap density in the bond regions and lone-pair density in certain nonbonding regions (*1*). Atomic positions, as determined by x-rays, may therefore differ slightly from positions determined by neutron diffraction, which correspond to the locations of the atomic nuclei. Similarly, anisotropic x-ray temperature parameters may contain small contributions resulting from the diffuseness of the valence electron cloud due to bonding, rather than from genuine thermal motion.

I undertook to investigate these effects. The molecule of *s*-triazine was selected because (i) it is a simple molecule containing only first-row atoms, so that bonding effects are relatively important; (ii) it contains lone electron pairs on the nitrogen, aromatic C–N bonds, and C–H bonds, all of which are features of interest; (iii) as shown by Wheatley (*2*), the planar molecule oc-