THE NUTRITION OF ANIMAL TISSUES CULTIVATED IN VITRO

III. Use of a Depletion Technique for Determining Specific Nutritional Requirements

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Plate 150

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The development of chemically defined media (1, 2) that will support survival and propagation of animal tissues cultivated *in vitro* has made it possible to determine the relationship of specific chemical compounds to the life processes of the cells. Studies on the amino acid metabolism of chick embryonic heart fibroblasts cultivated in synthetic medium M 150 (1, 2) have shown that certain amino acids disappear from the culture medium while others accumulate (3). Attempts to relate the disappearance or accumulation of individual amino acids to specific cell requirements, as shown by altered cell survival, proved unavailing since the standardized cultures employed (4) appeared to contain sufficient intracellular reserve nutrients to withstand prolonged cultivation in amino acid–deficient media. It became of interest, then, to investigate methods of reducing the stores of intracellular nutrients without causing irreversible cell damage. The results reported in the present communication describe such a "nutritional depletion" technique, and its use in demonstrating a coenzyme A requirement for the survival of chick embryonic heart fibroblasts. Application of this method to amino acid and other growth factor requirements of these cultures will be reported in subsequent communications.

Materials and Methods

All cultures were prepared from the heart muscle of 11-day-old chick embryos. The tissue was chopped to a fine pulp, moistened with a few drops of synthetic medium, and portions transferred to 18 × 150 mm. pyrex test tubes. Cultures prepared in this way contained from 1 to 2 mg. of tissue, wet weight, as determined by frequent weighings of replicate samples and by total protein determinations (5). The tissues were cultivated directly on the glass surface, without the use of plasma clots, in completely synthetic medium M 150 (1, 2) by the conventional roller tube technique (6). Extent of proliferation and health of individual cells were determined by frequent microscopic examinations until death of the cultures. Cumulative average survival times were calculated for the large groups of cultures in each test medium or experimental procedure. The significance of differences in survival times was calculated by the alternate t test, when necessary. Full details of these culture procedures have been reported previously (1, 2, 4, 7).

Synthetic medium M 150 (1, 2) and Hanks's balanced salt solution (8) were prepared from reagent grade chemicals. Coenzyme A was purchased from the Pabst Laboratories, Milwaukee, and fresh solutions were prepared at frequent intervals. All media and solutions were sterilized by passage through UF fritted glass filters. Amino acid metabolism of the cultures was determined by paper chromatography of the used synthetic medium, employing one-dimensional descending chromatograms with either *n*-butanol-acetic acid-water or *n*-butanol-ethanol-water as the solvent systems, followed by color development with ninhydrin. The chromatographic procedures have been described in detail in previous publications (3, 9, 10).

RESULTS

Depletion of Reserve Nutrients and Revival of the Cultures

Standardized chick embryonic heart cultures for nutrition and metabolism studies (4) are normally cultivated in synthetic medium M 150 for an initial 7-day period, before microscopic evaluation, grouping of the cultures, and replacement of the M 150 by appropriate test solutions. It appeared probable that this initial cultivation in the complete medium allowed the cells to accumulate excess reserve nutrients. Repeated washing with Hanks's salt solution, following the initial 7 days' cultivation in M 150, was found to have little or no effect upon the survival time of the cultures or upon the microscopic appearance of the cells. Attempts were then made to deplete the intracellular reserve nutrients by immediate cultivation directly in Hanks's solution. A large batch of cultures was prepared, the tissue allowed to become fixed to the glass, and 0.75 ml. of Hanks's solution added to each tube. After 3, 4, 5, and 6 days' time, groups of cultures were removed and the salt solution replaced by medium M 150. The cultures were then maintained in M 150, with twice-weekly changes of medium, until death. All the cultures treated with Hanks's solution showed a very restricted outgrowth of cells but those maintained for only 3 to 4 days in this solution revived quickly on the addition of M 150 and survived for a normal period of 42 to 44 days (1, 2). Cultures treated with Hanks's solution for 5 to 6 days failed to revive completely on the addition of M 150 and died within 25 to 30 days. The appearance of cultures depleted in Hanks's solution and their revival in medium M 150 is illustrated in Figures 1 to 4.

After cultivation in Hanks's solution for 3 days under these conditions (Fig. 1), only a scattered outgrowth of small, thin, granular, spindle-shaped cells has appeared. In contrast, a sister culture after 3 days in medium M 150 (Fig. 3) shows a confluent sheet of large healthy appearing cells. Subsequently, after 12 days' cultivation in M 150 (Fig. 2) the culture in Fig. 1 has revived completely and put forth a confluent sheet of large, healthy, spindle-shaped cells from the entire edge of the fragment. The extent of migration, size, and shape of the cells compare favorably with the appearance of the culture maintained directly in medium M 150 for 7 days (Fig. 4). From these results, it was concluded that chick embryonic heart cultures could be depleted by main-

tenance in Hanks's solution for 3 to 4 days without decreasing the subsequent survival of the cultures in medium M 150. Maintenance in salt solution for periods longer than 4 days resulted in extensive call damage, failure to revive completely in the synthetic medium, and considerably reduced survival times.

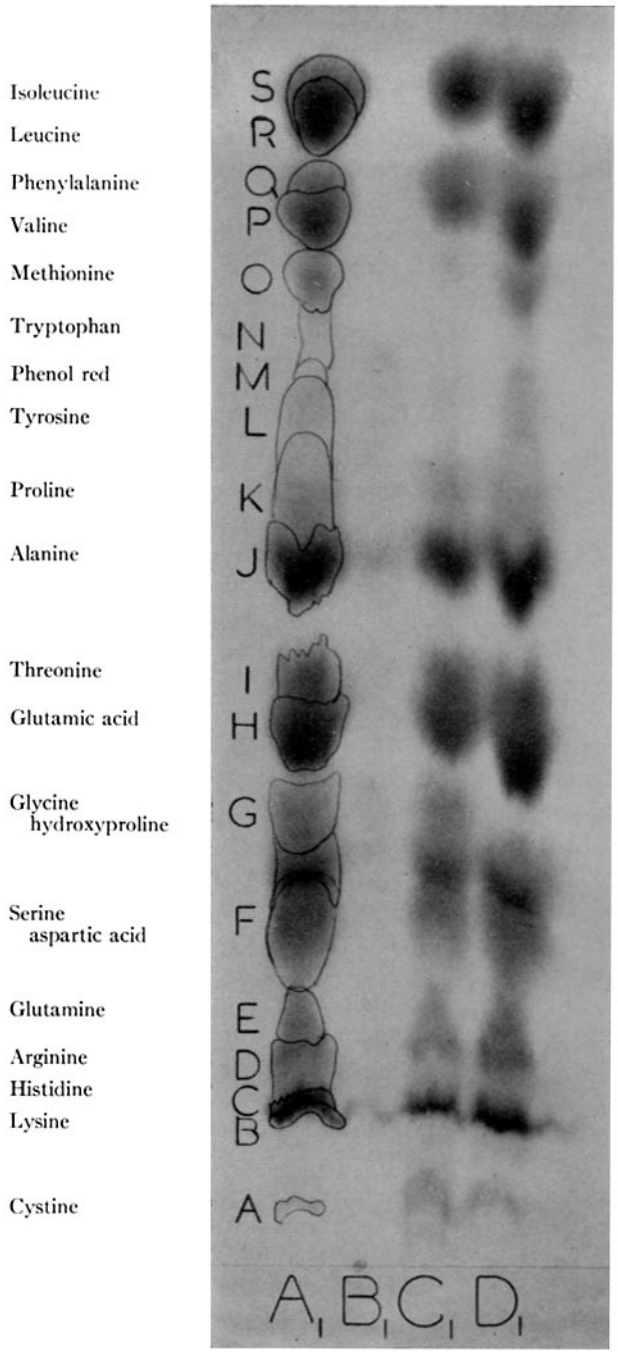
During these experiments, it was observed that rotation of the cultures during the depletion period caused loss of many of the tissue fragments. To avoid this loss, the cultures were kept stationary during the depletion period and rotation begun only after the addition of the synthetic medium. If this precaution is followed, reproducible and uniform cultures can be obtained.

Amino Acid Metabolism of Depleted Cultures

Although depletion in Hanks's solution for 3 to 4 days did not decrease the survival time of the cultures, it appeared possible that alterations in metabolism might have occurred. Accordingly, the amino acid metabolism of depleted cultures was examined, employing the techniques used previously for non-depleted cultures (3). The results of a typical experiment are recorded in Text-fig. 1. Medium M 150, added to the cultures after removal of the depleting fluid, and left on the tissue for 4 days, was examined on paper chromatograms. A marked decrease of nearly every amino acid was seen (Text-fig. 1, C_1), when compared with the amino acid content of fresh, unused M 150 (Text-fig. 1, A_1). The second change of M 150 (Text-fig. 1, D_1), similarly examined after 4 days on the cells, shows the characteristic pattern of amino acid uptake and accumulation previously established for chick embryonic heart cultures (3). Increases in the glutamine, glutamic acid, alanine, and methionine region, are clearly shown, whereas in the previous change of M 150 (Text-fig. 1, C_1) these regions show decreases. Subsequent changes of M 150, which are not included on this chromatogram, agreed closely with the patterns shown by the second change. From these results, it would appear that the amino acid metabolism of the depleted cultures is atypical in the first change of M 150 following removal of the depleting fluid, but is restored to its typical pattern by the second change of M 150. Chromatographic analysis of the depleting fluid itself (Text-fig. 1, B_1) shows that a small amount of ninhydrin-positive material has been released into the Hanks's solution during the depletion process. It is of interest to note that, in previous experiments (3), no release of ninhydrin-positive materials was detected during the degeneration and death of chick embryonic heart tissues cultivated for prolonged periods in medium M 150.

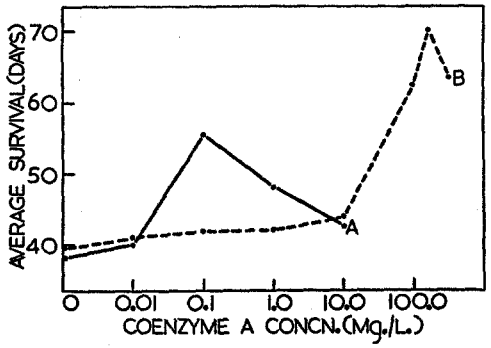
Coenzyme A Requirement of Chick Embryonic Heart Cultures

To establish the suitability of the depletion method for determining the specific nutritional requirements of tissue cultures, graded levels of coenzyme A were added to medium M 150 and the effect of these media on the survival of depleted and non-depleted chick embryonic heart cultures measured. The



TEXT-FIG. 1. Pattern of amino acid changes in medium M 150 following depletion of chick embryonic heart cultures in Hanks's solution. Chromatogram developed with butanol–acetic acid–water mixture and sprayed with ninhydrin. A_1, control, M 150; B_1, Hanks's solution after use for 3 days as depletion medium; C_1, first 4-day change of medium M 150 after removal of depletion medium; D_1, second 4-day change of medium M 150.

results of these experiments are shown in Text-fig. 2. With the normal, non-depleted cultures (Text-fig. 2, Curve A), a response to coenzyme A was obtained, with maximal effect at a concentration of 0.1 mg. per liter (0.1 μg. per ml.). When nutritionally depleted cultures, prepared as described above, were employed (Text-fig. 2, Curve B), a much greater response was obtained and the maximal effective concentration was found to be 200 mg. per liter. These results suggest that the depletion method has increased the level of coenzyme A required for maximum survival of the cultures by 2,000-fold and has also increased the magnitude of the response to this metabolite.



TEXT-FIG. 2. Effect of coenzyme A concentration on survival of freshly explanted chick embryonic heart cultures. Curve A, non-depleted cultures; Curve B, cultures depleted for 3 days in Hanks's solution.

DISCUSSION

In many studies on cell nutrition, synthetic media have been supplemented with serum or other uncharacterized fractions of natural materials (11–14), but the extent of the contribution of these materials to cell survival and propagation has not been precisely determined. Previous studies from this Laboratory have shown that such uncharacterized substances will prolong cell life (2) and that media designed to supplement dialyzed serum and embryo extract are completely inadequate in the absence of these materials (4). In the present experiments, the degree of response to a single metabolite has been shown to be affected markedly by the nutritional condition of the cells at the beginning of the experiment. From these observations, it would appear that determination of the basic nutritional requirements of cells and tissues cultivated *in vitro* should be based on the use of completely chemically defined media and the reduction to a minimum of intracellular reserve nutrients. Unless such rigidly controlled conditions are employed, it appears difficult, if not impossible, to relate cell survival and propagation to specific components of the medium. For this reason, current studies on tissue cell nutrition and metabolism in this Laboratory are based entirely upon the use of completely synthetic media.

The present experiments have shown that freshly explanted chick embryonic tissues may be depleted of nutritional reserves by initial cultivation in a simple salt solution. Provided the depletion is not continued too long, the cultures revive completely on the addition of the synthetic medium and survive for a normal period. Paper chromatographic studies on these cultures have shown that the normal pattern of amino acid metabolism is quickly established after removal of the depleting fluid. It is apparent, then, that the depletion process has not resulted in alteration of the metabolic pattern of the cells nor in damage to the vital cell processes. It is of interest that the critical 3 to 4 day depletion period represents approximately half the survival time of this type of culture in salt solution alone (1) and that similar depletion for 6 days did not reduce the ability of chorioallantoic membrane cultures to support the propagation of influenza virus (15). This nutritional depletion technique has now been used on more than 3,000 cultures over a 15 month period and has been found to yield reproducible results.

The production of individual amino acid and vitamin deficiencies has been reported with the L strain and the HeLa strain (13, 14, 16, 17), but in these studies the media employed contained varying amounts of serum, or the residue from dialyzed serum, and these uncharacterized materials may have influenced the nature of the deficiencies obtained. In the present experiments, entirely synthetic media were employed and an over-all nutritional deficiency was produced, followed by correction of the general deficiency except for the compound under test. It was considered that production of such a complete deficiency would create sensitive conditions for the detection of nutritional factors required in low concentration. This supposition has been confirmed in extensive use of the depletion technique.

Studies on coenzyme A have shown that non-depleted cultures do require this compound, but the level required for maximum response is increased 2,000-fold, and the degree of response is also increased, by the depletion method. It should be emphasized that, in both cases, the responses were obtained in the presence of pantothenate (1) and thus represent specific requirements for the coenzyme form of this vitamin. Further studies are in progress to determine whether fragments of the coenzyme A molecule will elicit a similar response. It appears unlikely that the beneficial effect of coenzyme A can be attributed to its content of sulfhydryl groups, since the addition of high concentrations of cysteine to the synthetic medium did not increase the survival time of the cultures (unpublished observations).

The present experiments represent the first clear cut demonstration of a specific coenzyme A requirement for tissues cultivated in a completely synthetic medium. Recent studies with a mouse fibroblast cultivated in media supplemented with dialyzed serum (14) have shown a requirement for either pantothenic acid or coenzyme A. Pantothenate was found to possess approxi-

mately ten times the activity of the coenzyme form but no data were reported on the activity of either compound in the presence of the other. In earlier studies with the L strain (18), coenzyme A was found to be beneficial in the presence of both di- and triphosphopyridine nucleotides, but no effect from coenzyme A alone was observed. The nutritional depletion technique reported in this communication, by reducing to a minimum the effect of stored and carried-over growth substances, together with the use of completely synthetic media, should make it possible to determine with greater certainty the precise nutritional requirements of tissue cultures.

SUMMARY

1. Cultivation of freshly explanted chick embryonic heart tissues in Hanks's salt solution for 3 to 4 days has been shown to create a general state of nutritional deficiency in the cultures. Provided the depletion is not prolonged beyond 4 days, the cultures subsequently revive and survive to a normal period in synthetic medium M 150.

2. Paper chromatographic studies on the culture medium have shown that the amino acid metabolism of the depleted cultures is restored to a normal pattern within a few days in medium M 150.

3. By the use of the nutritional deficiency technique, a coenzyme A requirement for this type of culture has been established.

4. The application of these findings to tissue cell nutrition and the possible hazards of using serum, or other uncharacterized additions, either to establish cultures, or as part of the experimental medium, are discussed.

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EXPLANATION OF PLATES

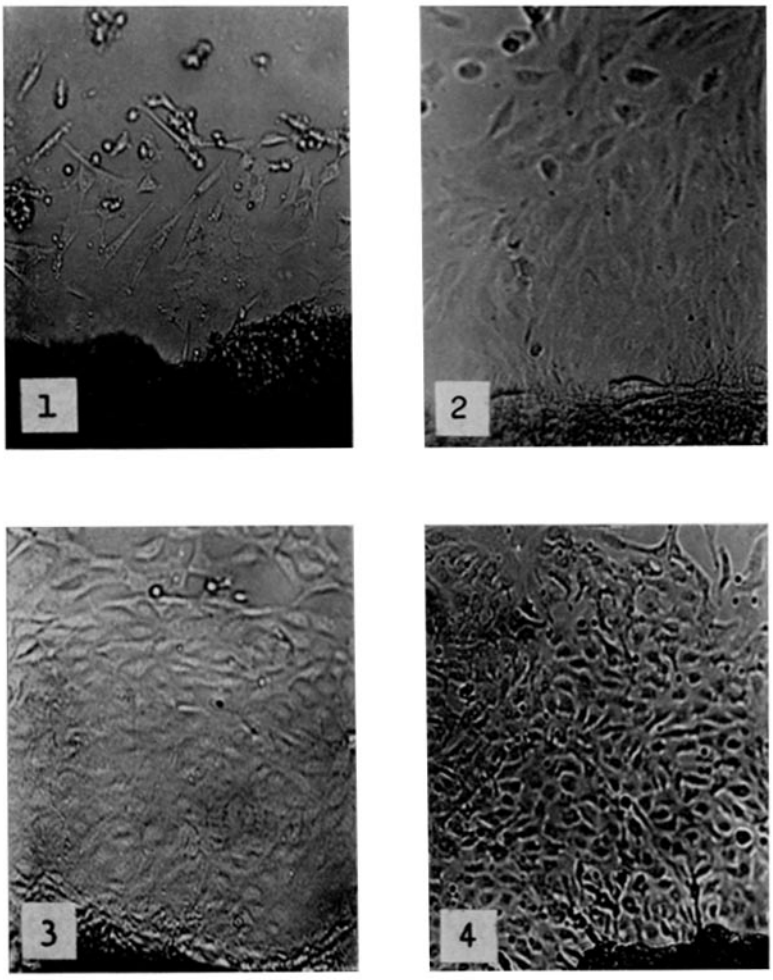
PLATE 150

FIG. 1. Freshly explanted chick embryonic heart tissue cultivated in Hanks's solution for 3 days. × 105.

FIG. 2. Culture area shown in Fig. 1 12 days after replacement of Hanks's solution by synthetic medium M 150. × 105.

FIG. 3. Freshly explanted chick embryonic heart tissue cultivated in medium M 150 for 3 days. × 105.

FIG. 4. Culture area shown in Fig. 3 after 7 days' cultivation in medium M 150. × 105.



(Morton, Pasieka, and Morgan: Nutrition of animal tissues *in vitro*. III)