The Nutrition of Isolated Cells from Vertebrates

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I HAVE BEEN asked to open this conference on "Optimal Nutrition" with a discussion of the subject as it applies to the fundamental physiological unit of all vertebrate (and other) life, the cell. This is one of the major problems of what we have come to call "tissue culture." Presumably I, as a tissue culturist, have been asked to speak on this subject because the nutrition of any organism is, after all, basically the sum total of the nutrition of its parts. Although we must never lose sight of the fact that what we are seeking is an eventual integration of all these parts into a well balanced whole, we must know what factors, in terms of individual cells, may tend to throw that whole out of balance.

DIFFERENTIAL NUTRITION

What is "optimal nutrition"? Optimal for what? For growth, for maintenance, for specific function? The thyroid concentrates iodine, the pancreas zinc, the liver copper and iron, the stomach lining cobalt. If we isolate the cells from these four types of epithelium should we expect that the optimal nutrition for all four will be the same? I hardly think so. The different layers of the adrenal are thought to manufacture each a different series of steroid compounds. Do these represent different synthetic capacities (hence different nutritional requirements), or do they, on the other hand, represent differences in the nutrition available to these cells, due to differential screening of the supply? The pituitary was once looked upon as a complex gland, but it has more recently become clear that removal of the pituitary does not eliminate all of the pituitary hormone effects, but only distributes those effects. The suspicion has arisen that the "gland" may not be the original source of all these hormones but rather a storage organ where they can be stock-piled for release in more massive quantities. If the former view is valid, then the different parts of the pituitary will have different "optimal nutrition" requirements. If the latter is true we must look to some stem tissue elsewhere as the really critical point.

These are not idle questions. One of the most dramatic demonstrations of their pertinence comes recently from Dr. Fell's laboratory in England.[1] There are in the body a great many different sorts of "epithelium." The skin is largely a protective layer and as such is thickened, keratinized. The lining of the intestine is a secretory layer as well as an absorptive one, and, as such, keratinization would interfere with its function. It is, rather, a simple columnar epithelium of glandular cells. Still a third type occurs in the lungs and nasal mucosa, where secretory function is coupled with a mechanical role in the ciliated layers. The cells originating in these three layers can all be grown in tissue culture, and if they are maintained in the usual nutrients they will all eventually take on a pattern resembling the intestinal cells. But if they are nourished with serum taken from animals, usually birds, which have been reared on a low vitamin A diet, so that they are deficient in this respect,

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the secretory function regresses and keratinization sets in. If, on the other hand, the serum is artificially fortified with excessively high vitamin A levels, there will be first a marked development of secretory function under the keratinized layers, these latter will be sloughed off, and eventually certain cells will develop active cilia, as well as accumulating mucous. The type of epithelium developed is thus sharply dependent on the amount of vitamin A available to the cells. The particular morphology of a given organ may be determined by the distribution of such substances in the body. At this level, at least, specific cell type is not internally but externally determined. The same holds true for bone. An excised femur can be caused to disintegrate into an amorphous mass of quite healthy chondroblasts without a trace of ossification, merely by increasing the level of vitamin A available to it.[2] "Optimal" nutrition can obviously be a very specific and highly localized affair!

Unfortunately, this work of Fell and Mellanby has only opened for us a tiny window into the vaults and chambers of nutritional morphogenesis. Vitamin A just happens to be one of the few substances for which we have recognized a specific formative function and which can at the same time be successfully manipulated in the simple fashion required by our present rather crude techniques. I am sure that there are many, many others. One may safely predict that the whole range of internal secretions will prove to have just such functions. I am equally certain that we need not turn to substances as complex as the hormones for examples.

WOUND HEALING

I will cite one from the woefully small number for which we have real data. It again involves epithelium. The healing of a wound involves two processes which are partly in sequence, partly concurrent. The bulk of the wound must be drawn together and filled in by fibroblast activity, by the formation of granulation tissue. And this granulation tissue must be covered by epithelium. The first starts from the depths of the wound, the second from its margins. If the growth of epithelium nearly or quite keeps pace with granulation the wound will heal with little or no scar. If epithelialization lags, the granulation tissue becomes more and more massive and irregular and difficult to cover. If covering is not complete, we have a persistent ulcer. Consequently it is of the utmost importance to stimulate rapid growth of epithelium and to hold back fibroblast activity at least to the point of balancing the two. What then distinguishes between the two types of growth? Parshley and Simms, at Columbia College of Physicians and Surgeons, set out to define these differences.[3] It develops that in a culture consisting of a mixed colony of human epithelium and fibroblasts, one of the things which will favor the development of epithelium is to increase the available phosphate concentration of the nutrient, at the same time reducing the calcium level. One can end up with pure epithelium, or pure connective tissue, or a mixed colony by adjusting the phosphate and calcium levels in the nutrient. Phosphate is chemically a lot simpler than is vitamin A! But unfortunately it is not so easy to control the local supply of phosphate to a specific area of the body, the wounded area, without also affecting other areas. The body itself has some rather effective mechanisms for controlling this general level, which mechanisms will be difficult to overcome. This study has *not* led to clinical applications. Scientifically, the way has been pointed out—but technically it is still beyond our present capacities to do anything about it!

TISSUE CULTURE STUDIES

I cite these few examples largely to establish a framework. While you may perhaps *hope* that I will talk about *your* problems I am sure you do not expect me to ignore my own. Each of us has his hobby horse. Mine is cell nutrition *outside* the body, in tissue culture. So I am sure you will allow me a brief canter over the fields which lie open to me.

How do we go about studying cell nutrition? Obviously by isolating a cell and then controlling its nutrition. To what extent can we do this? The classic tissue culture nutrient for connective tissue consists of 40 per cent serum

—chick or rabbit serum if you prepare your own, horse serum if you buy it, human placental cord serum if you are attached to a big hospital, plus 20 per cent embryo-extract—again chick embryos or beef embryos if you prefer, plus 40 per cent of a balanced salt solution. One can control what goes into the salt solution. That is no problem. One may exert some slight control over the serum by choosing the nutritional background of the chickens or horses from which it is derived as Fell and Mellanby did (but what control do those who use placental cord serum have over the nutritional history of *their* sources?) and one can exert little or no control over the nutritional content of the embryo-extract. Fell and Mellanby did a masterful piece of nutritional work in their vitamin A studies, but against what odds! The classic method is simply not designed for that kind of thing! Baker and Carrel, Ebeling, Fischer, and a few others have tried to analyze serum and embryo-extract. But the 40:20:40 formula still stands as standard for most work.

Ten years ago I set out to try my hand at correcting this state of affairs. Over the past quarter century there has grown up a large literature on the nutrition of protozoa, bacteria, yeasts, fruit flies, dogs—and *Homo sapiens*. It is a motley literature, but certain patterns keep recurring. A considerable number of elements are required by all and can be supplied by the same salts. The fact that snails have copper in place of iron in their hemoglobins, while vanadium is the replacement in certain tunicates, are exceptions to the general rule. The fruit fly—*Drosophila*, the protozoan—*Tetrahymena*, and the mammal—*Canis domestica* all require for maintenance the same eight amino acids plus, in each case, certain others which are more specific. All organisms require certain vitamins. One is by no means working entirely blindly when he goes to the chemist's shelf and takes down this bottle, and this, and this, to *build* a nutrient, though he obviously risks the chance of missing some crucial substance which may mask the effectiveness of all the rest. In 1946 I published one such mixture which would support contraction of chick heart muscle for about 40 days, as against 5 or 6 days in a simple balanced salt-dextrose solution.[4] In 1949 I improved this by addition of four more amino acids, doubling the survival of cells:[5] More recently, Dr. Waymouth has improved the result greatly by simply trebling the level of phosphate-bicarbonate buffer.[6] These defined nutrients did not support prolonged *growth* of tissues, but only survival and metabolic maintenance. More recently we have taken the step which every experimenter must make periodically, going back to see just what we have accomplished in terms of improving the classic approach.

The defined portion of the classic nutrient was confined to the balanced salt solution. What would happen if, for this balanced salt solution, we substituted one of our more complex defined nutrients and then tried progressively reducing the concentrations of the remaining unknowns? This we have done, using one of Earle's single-cell strains of mouse cells as test organisms.[7] I cannot generalize as to other types of cells, but it is highly probable that such generalization will eventually prove possible (Table I).

In the first place embryo-extract is *not* necessary for rapid growth of these cells. For about a year now we have maintained our stock cultures without any embryo-extract at all. They grow almost if not quite as fast as in the classic nutrient—quite fast enough for all routine purposes. In the second place, the serum level can also be drastically reduced. Routinely, we still maintain a 5 per cent level of serum in our stock cultures, as compared to the 40 per cent in the classic formulae, with 95 per cent of a defined nutrient. This *can* be still further reduced. In a 2:98 per cent nutrient, Strain L cells grow very slowly, so slowly that it takes 6 to 8 weeks to reach a level of cell density sufficient to justify subculturing, as against the 8 to 10 days which cultures in a 5:95 per cent nutrient require for the same growth. In a 1:99 per cent nutrient the same cells multiply just enough to balance cell losses, and a colony will have about the same density at the end of

TABLE I

Defined Maintenance Nutrient for L-Strain Mouse Cells

The control nutrient (W11) contains the following final concentrations in milligrams per liter:

Fructose	8500	Glycine	100
NaCl	7000	Cysteine HCl	1
KCl	375	Glutathione	1
$MgSO_4$	275	Thiamine	1
$Ca(NO_3)_2 . H_2O$	210	Riboflavin	1
$NaHCO_3$	1102.5	Ca-pantothenate	1
Na_2HPO_4	115	Pyridoxine	5
KH_2PO_4	52	Nicotinic acid	5
$Fe(NO_3)_2 . 9H_2O$	1.375	*i*-Inositol	5
L-Cystine	7.5	*β*-Alanine	5
L-Glutamic acid	140	Choline HCl	10
L-Aspartic acid	60	*d*-Biotin	1
L-Leucine	156	Folic acid	1
DL-Isoleucine	104	Ascorbic acid	0.5
L-Phenylalanine	50	Vitamin A	0.1
L-Methionine	130	*β*-Carotene	0.1
L-Threonine	130	Vitamin B_{12}	0.025
DL-Valine	130	Phenol red	4
L-Lysine HCl	156	Ethanol	1
L-Arginine HCl	78		
L-Histidine HCl	26		
L-Proline	50		
L-Tryptophan	40		

8 weeks as at the beginning, although mitoses are still not uncommon. In a 0:100 per cent nutrient—that is, one in which only ingredients off the chemical shelf are used—no growth occurs and there is a gradual loss of cells, yet cultures kept in such a solution remain viable for many weeks, and will resume normal growth within a matter of hours when transferred to a 5:95 per cent nutrient. We have not quite attained our goal of being able to define *all* the requirements for cell growth, but we are certainly approaching it.

Perhaps some of you will say "But this goal *has* already been attained, in Parker's nutrient #703."[8] I should not wish to deny that. But I would remark that the formulae with which we in this Laboratory are working contain less than half the number of ingredients of #703. And many of those omitted are among the very expensive ones—the co-enzymes for example. We are aiming at simplicity as well as definition—something that can eventually be put into the hands of little laboratories as well as big ones. That is not an impossible goal. One of our most spectacular improvements came from changing the buffer concentration—something that one might have supposed was pretty well established!

OBJECTIVES

When we attain this preliminary goal, and only then, can we hope to attack on a rational and really hopeful basis, some of these critical problems: how to supply the optimal nutritional conditions to a healing wound; how to overcome resistance to skin and gland transplants; how to promote the growth of a severed nerve before the surrounding fibroblasts can penetrate the sheath and turn the nerve back upon itself, as now so often happens; how to encourage a damaged gland to resume its function without tipping it over into a state of malignancy; and, by no means least, how to encourage a none-too-perfect brain to attain the highest functions of which it is inherently capable! Nutrition can be optimal for function, or for repair, for growth or for maintenance, or for eventual regression in unwanted members. Each will have unimagined complexities, once we can strip off the irrelevant and get down to facts. When we can tell you, from laboratory studies, what is the optimal nutritional background for each type of cell in the body at each stage, and for each of its normal and abnormal functions, we can give you the information with which to rear and maintain a perfect body. Holmes' "Deacon" had those secrets. We may search hopefully for them.

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Vitamins: Haves and Have-nots

"The need for vitamins of various animals and the ingenious way in which they are supplied make a truly fascinating study. For example, we know that the hervivora make their own B group vitamins in the rumen and/or series of stomachs with which many of them are supplied. The carnivora on the other hand have no rumina and no such ability and are therefore dependent on their own prey to supply their needs. One of the most curious facts recently brought to light is the way in which the rabbit supplies its needs for vitamin B. This, it would appear, is made largely in the caecum and colon. It is passed during the night as 'night soil' and reingested by the rabbit the following morning. From the current issue of *Family Doctor* we learn that 'Guinea pigs and men and apes are the only animals that need to have vitamin C in their food. All the other animals can make their own vitamin C.' One is not surprised to learn that man needs to have almost everything given him; he seems able to synthesise almost nothing but a little vitamin B in his intestine, and that amount is clearly not enough for his needs judging by the alacrity with which, given half a chance, he develops beri-beri. Our cousins, the monkeys, one is not surprised to learn, fall into the same category. But what about the guinea pig? Why should he be unable to do what the rabbit, the ox, the horse, the dog, the cat and all the rest including even the lowly mouse can manage? What factors in his evolutionary background have brought this about? He must have gotten his C *gratis* at one stage. How and when was that?"
—*Med. Press* 235: 456, 1956.