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Publication Date

1965

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THE STUDY OF METABOLIC COMPARTMENTALIZATION

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UCRL-11880
Erratum

UNIVERSITY OF CALIFORNIA

Lawrence Radiation Laboratory
Berkeley, California

AEC Contract No. W-7405-eng-48

July 12, 1965

ERRATUM

TO: All recipients of UCRL-11880

FROM: Technical Information Division

Subject: UCRL-11880, APPENDIX, Mark W. Horovitz and Grove C. Nooney,
January 1965

To supersede and replace Appendix (pages 29-36) of UCRL-11880.

OK

UNIVERSITY OF CALIFORNIA

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THE STUDY OF METABOLIC COMPARTMENTALIZATION

V. Moses and K. K. Lonberg-Holm

January 1965

UCRL 11580

The Study of Metabolic Compartmentalization

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The concept of metabolic compartmentalization is defined, and a number of possible experimental approaches for the study of this phenomenon are considered. Most of these are rejected as currently presenting insuperable technical difficulties. However, a behaviouristic approach appears promising which is capable of providing information on the nature and degree of compartmentalization but not on the intracellular localization of particular compartments.

The method is essentially to investigate the metabolic behaviour of a compound as a function of the way in which it came to exist within the cell. Thus, a particular compound might be produced from one of a number of chemical sources and in this way may conceivably be located in one of a number of different coexisting compartments. Alternatively, the compound may be taken preformed into the cell from the environment and this may facilitate its entry into yet another compartment. A varying pattern of metabolism correlated with the mode of formation or entry of the substance is then taken as an indication of compartmental separation. The method assumes that the routes of metabolism (i.e., the sequences of metabolic intermediates) are understood, so that compartmental data may be interpreted on the basis of known pathways.

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Important technical problems relating to a study of this sort are of two main types: (a) the constancy of metabolic activity of the system must be adequately controlled, and (b) the large experimental scale on which it is desirable to operate, resulting also in very many analytical manipulations, requires the development of mechanical aids to experimentation. The first class of difficulties are discussed here; the details of the mechanical devices have been considered elsewhere.

A preliminary experiment on these lines has been performed with mouse ascites tumour cells, dealing with the area of carbohydrate metabolism, in glycolysis. Some of these results are reported as being indicative of the type of data which may be obtained, and a provisional interpretation of the data has been made.

It is currently well recognized that a living cell is an exceedingly complex system containing a diverse multiplicity of structural components which are nonetheless extremely well integrated to produce a chemically and biologically efficient unit. Just how wide the range of these different subcellular units of cell anatomy is has been amply demonstrated in recent years by fractionation and electron microscope studies.

It would seem very reasonable that the large amount of subcellular structural features might be correlated with a corresponding degree of metabolic differentiation. The metabolic counterparts of the structural differentiation might be of at least two sorts: (a) certain of the cell's biochemical activities are restricted entirely to one type of subcellular structure; and (b) some biochemical activities might be found simultaneously

in different structures, which are, however, separately modulated. The intermediates in the several sequences might not be in equilibrium with one another because of such restrictive features as the relative impermeability of membranes bounding the different locations, or the organization of the enzymes of the sequences as surface structural units which do not release intermediary metabolite molecules between one catalytic step and the next.

When we look at a map of metabolic activity we notice that many metabolic sequences demonstrate branching. The extent of branching possibilities in any individual metabolic environment might, however, be much more limited than one would expect from the metabolic map, which represents the composite activity of all the individual portions of the cell. For clarity, let us consider a specific example. Glycolysis concerns a number of chemical reactions starting with glucose and producing pyruvic acid. Several metabolic roles are available to pyruvate. It may be reduced to lactate, or transaminated to alanine. In some instances it may be decarboxylated to acetaldehyde, oxidatively decarboxylated to acetate (or acetyl-coenzyme A), or carboxylated to oxalacetate. Two or more of these metabolic fates might be suffered simultaneously by pyruvate in the same organism. We can then ask the very important question: do all metabolic possibilities occur at the same time at every location within the cell and to the same extent? That is, does a molecule of pyruvic acid have the same probability of being converted to any one of its products regardless of where it is generated within the cell? If the answer is no, by reason of the differing chemical or physical environments at the various sites, then the cell can be said to be metabolically compartmentalized. More specifically, if glycolysis occurs semi-independently in a number of environments, one of which might contain lactic dehydrogenase but little or no transaminase, while

another contains transaminase but little or no lactic dehydrogenase, then the cell is glycolytically compartmentalized.

A study of metabolic compartmentalization has been made in a fairly small number of instances where it has been possible to take advantage of some special characteristic of the biological system; these have recently been reviewed (Moses, 1965). No attempts have been made, as far as we are aware, to study the degree of involvement of compartmentalization over a wide area of metabolism in one cell type, although this would be of great interest to those concerned with the interrelations of metabolism with structure. We have therefore considered ways in which metabolic compartmentalization might be tested for, and have performed some experimental studies with the approach which, in our opinion, offered the greatest promise of success.

Basically, the approaches we will discuss may be divided into two groups: those requiring cell fractionation and those involving study of the behaviour of living cells. Techniques based on fractionation may be carried out either by first fractionating the cell and investigating the metabolic activities of individual fractions, and of combinations of fractions, or by feeding the intact cell with labelled substrates and later carrying out the fractionation and investigation of the chemical nature of the labelled reaction products.

Subcellular fractionation conventionally involves a breaking of the cell followed by separation of different intracellular inclusions by differential centrifugation. It is likely that this procedure would result in unequal destruction of the metabolic potentialities of the subunits or isolated fractions. Alternatively removal of the subunits from their normal locations might release inhibitory factors, thus promoting enhancement of metabolism, and this, too, would not be expected to favour

all metabolic reactions equally. These objections to the use of fractionated subunits are the more serious because we usually cannot measure their in vivo activity and so are unable to assess the extent of alteration during fractionation. There are also the additional quantitative difficulties that (a) not all of the subcellular structures can be assigned to a particular fraction and (b) it is not possible to purify most cell components free from other contaminants. These difficulties seem insuperable, and we are led to consider other approaches.

It might be possible to permit the metabolic events to go on in the intact cell and then carry out the analytical procedures, including fractionation, after the period of metabolism. In this way the subunits would operate under natural conditions and the fractionation procedure would be performed only after their inactivation. An experiment of this sort might be performed by supplying the cell with a labelled substrate, preferably a volatile one, and allowing metabolism to proceed for a period under standard conditions. Samples would be removed at appropriate intervals, the reactions stopped by rapid cooling to liquid nitrogen temperature, and the water and substrate removed by lyophilization (hence the advantage of a volatile substrate). In order to prevent displacement of labelled water-soluble intermediates from their true intracellular positions, all fractionation would need to be performed in non-polar solvents. Such non-polar separations are not unknown for certain types of subcellular particles (Stocking, 1959; Heber and Willenbrink, 1964). Radiochemical analysis of each fraction would then demonstrate the locus or loci of each metabolic intermediate. Unfortunately it soon became apparent, experimentally that the conditions of criteria (a) and (b) above (that not only must the

fractions be pure, but all the cell material must be accounted for) could not be met. Attempts to separate in non-polar solvents only the chloroplasts, mitochondria and "soluble" cytoplasm from Elodea canadensis and from spinach leaves were entirely unsuccessful (V. Moses and J. Biggins, unpublished). Although mitochondria could be observed in the whole cells under the phase microscope, they are present in comparatively small amounts and could not be found at all after a non-polar fractionation. Presumably they were adsorbed, together with the "soluble" fraction, onto the much larger bulk of chloroplasts, or onto cell wall material or other debris. Following this failure of non-polar separations we abandoned all attempts to study compartmentalization by the fractionation techniques.

The next possibility considered involved the use of cellular radioautography. The idea behind this method was to investigate simultaneously during metabolism the nature of the compounds containing radioactive atoms and the position of radioactive material inside the cell as observed by direct radioautography. The object of the experiment would be to relate different stages in the biochemical reaction schemes with the appearance of radioactivity in different parts of the cell.

The experimental arrangement would be to feed a volatile radioactive substrate to a cell suspension and at suitable intervals of time remove samples for radiochemical analysis of the intermediates, and for radioautography. Samples for the latter purpose might be sprayed directly onto deeply chilled microscope slides and the cells lyophilized there. It would then be necessary to place the tissue in contact with a photographic emulsion without the intervention of a liquid so as to prevent any displacement of labelled compounds inside the cell. Later, the location of radioactivity in the preparation determined by radioautography

would be correlated with structural features observed directly under the microscope.

A main disadvantage with this approach is the difficulty of using it with ^{14}C -labelled substrates. The comparatively high energy of the β -particle emitted from this nuclide would result in an area of film blackening from each focus of radioactivity too large to permit exact definition at the site of origin of the radioactive emissions. Studies of this sort would be much easier to perform with ^3H -labelled substrates from the location point of view, but then difficulties arise from exchange reactions between ^3H released from the substrate by metabolic processes and exchangeable hydrogen atoms in other substances (Moses & Calvin, 1959).

Because of its disadvantages this method, like the fractionation techniques, was not pursued further.

The behaviouristic approach, while not providing any information of the actual physical locations of different reservoirs, permits a study to be made of the number, extent and interrelations of different compartments. This method depends on investigating the metabolic behaviour of a number of different metabolites as a function of their biochemical route of formation or of their mode of entry into the cell. Let us consider a hypothetical reaction sequence (Fig. 1), starting with substance A and proceeding via several intermediates to E. Let E represent a metabolic branch point with possible further metabolism to F, G or H. In the first instance we might consider the consequences of there existing in the cell only one type of compartmental location for this reaction sequence. In other words, all replicates of the sequence are equivalent to one another, and will all behave in the same way because the chemical and physical environment of each

individual sequence is identical. It follows, then, in consideration of the metabolism of compound B in this sequence that the final products are the same if B is made in the pathway from A, or if B enters the pathway directly from outside. Either way, the same final proportion of the products F, G and H should be formed (Fig. 1). However, it could be argued that supplying A as a substrate for the pathway is not the same as supplying B, and this might cause a rearrangement in the yields of the products. This objection can be overcome if we set up experimentally two parallel reactions, identical in every chemical respect and differing only in that in one reaction A is labelled and B unlabelled, while in the other A is not labelled and B is radioactive. Thus, by investigating the distribution of label in F, G and H in the two cases, one may compare the fate of B when it is derived from A and when it enters the sequence from some external source.

If there are two compartmental situations in which the reaction occurs we may have a situation like that illustrated in Fig. 2. Here we suppose that although qualitatively the same products (F, G and H) are produced in each sequence, there are quantitative differences in the proportions of the products in the two compartments. However, it may be the case that both A and B enter each compartment with equal ease, as shown in Fig. 2, and both contribute in a like manner to the products. We will then fail to observe the presence of the two compartments, not because they do not exist, but because we have chosen the wrong pair of test substances. In this example it might be necessary to compare A and C or A and D in order to distinguish between the two compartments.

Granted that two compartments do coexist it seems likely that A and B would not enter each compartment with equal facility. Then, as in Fig. 3, external A might enter the upper pathway rather than the lower one,

while B might be predominantly incorporated into the lower sequence. If it were further true that the proportions of products F, G and H were not identical in the two compartments, we would observe that the proportion of F, G and H made from B which was in turn derived from A (upper pathway of Fig. 3) was different from the proportion of the products produced from B originating in some way other than from A (lower pathway of Fig. 3). This may be restated in the following form: the metabolic behaviour of B depends on its route of formation or mode of entry; therefore there must be non-equilibrating reservoirs of B subject to different metabolic fates, i.e. B may exist in more than one metabolic compartment. Fig. 4 illustrates an extreme case of the situation shown in Fig. 3. Here it is supposed that the upper pathway, in which B arises from A, produces F and H, but no G. The lower sequence, in which B enters from some source other than A, produces G and H, but no F. In this case the behavioural difference between upper compartment B and lower compartment B is unequivocal.

One of the essential requirements for the successful performance of such a study is that the cells must be in a steady metabolic state which does not alter when the labelled substrates are added but remains constant throughout the whole course of the experiment. The difficulties arising as a consequence of this requirement will be discussed later.

When one embarks on studies of this sort one cannot predict which will be the most appropriate substance to demonstrate existing compartmentalization. Nor, in the dynamic system of the living cell exhibiting complex biochemical reaction kinetics, is it easy to forecast which would be the most suitable time to observe to the best advantage such compartmental

separations which may be present. One is therefore forced to design experiments in which many substrates are investigated simultaneously, and many samples for analysis, encompassing a long period of time, are taken from each reaction vessel.

Most cells, particularly free-living protista, discriminate to a greater or lesser extent against the entry of substances into the cell from the external medium. This has to be borne in mind when designing compartmentalization experiments. In a comprehensive experiment along these lines which we report briefly below we employed Ehrlich ascites tumour cells from mice on the grounds that cells living in the more or less friendly environment of a host body would be less discriminating than those found in the much more hostile environment of the free-living forms. It has subsequently been found, however, that free-living forms, at least as represented by the bacterium Escherichia coli, are sufficiently permeable to permit the entry of a considerable number of metabolic intermediates (V. Moses, unpublished). Since the preparation of a homogeneous suspension of E.coli is much simpler than one of tumour cells, and much less damage is likely to result from manipulation, we would favour the use of such simple organisms where possible.

If one is to study the effects of several substrates simultaneously in order to cover a wide area of metabolism, it is necessary to set up a number of reaction vessels equal to the number of substrates to be tested. Each reaction vessel contains all the substrates and is in every chemical way identical with all the others. The only difference between the different vessels is that a different one of the substrates is labelled in each case. It was desired that samples be taken at times ranging from

a few seconds until about 45 minutes. This made it unsatisfactory to perform each of, say, six incubations sequentially because then the last incubation would not be finished until three hours after the first had started, at which time/cell population would be appreciably altered from the initial state. For this reason the experimental vessels were incubated simultaneously.

Briefly, then, the experimental arrangement in the experiment we have performed was as follows: It was decided to study the area of glycolysis and pentose phosphate cycle metabolism in ascites tumour cells. Six substrates were employed, in six parallel reaction vessels: glucose, fructose, glucose-6-phosphate, 6-phosphogluconic acid, 3-phosphoglyceric acid and L-lactic acid. When employed as radioactive substrates these were all uniformly labelled. The six reaction vessels formed part of a sampling machine. A mixture of the six substrates was simultaneously added to each of the reaction vessels by a mechanical syringe injection assembly. A sample of each reaction mixture was taken simultaneously at desired intervals and each sample was automatically mixed with sufficient ethanol to stop metabolism and kill the cells. The sampling device is described elsewhere (Moses & Lonberg-Holm, 1964).

Fifteen samples were taken from each reaction vessel at intervals ranging from 3 seconds to 45 minutes after addition of the substrates. Analysis of the ethanolic suspensions of dead cells was carried out by two-dimensional paper chromatography (reviewed by Moses, 1960, and modified by Crowley, Moses & Ullrich, 1963). Measurement of the radioactivity in each of the compounds formed in each sample was carried out by first locating the positions of the radioactive areas on each chromato-

gram by radioautography and then excising the spots and counting them in a semi-automatic instrument designed for this purpose (Moses & Lonberg-Holm, 1963). From a knowledge of the specific radioactivity of each substrate compound and the number of carbon atoms in the product molecules it was possible to calculate the number of μ moles of each product produced from each substrate for a standard quantity of cell at each time interval analyzed. Compartmental differences were then sought by comparing the ratios between the yields of pairs of product substances starting from each of the different labelled substrates.

It is not intended to present a full discussion of the results in this paper, but mention of a few of them might serve to illustrate the sort of data which may be obtained. Of the six substrates used, the metabolism of 6-phosphogluconic acid was so slow that it will be excluded from this discussion. It was observed that all the substrates (except lactate) were predominantly metabolized via glycolysis to pyruvate with some evidence for pentose phosphate cycle activity. Pyruvate was metabolized to lactate, alanine and further into the tricarboxylic acid cycle. Thus, in the hypothetical scheme of Eig. 1, we may equate A with glucose, B with glucose-6-phosphate, C, with fructose-6-phosphate (a product also of the free fructose used as one of the substrates), D with 3-phosphoglyceric acid, E with pyruvate, F with lactate, G with alanine and H with the tricarboxylic acid cycle.

In the experiment glucose was utilized about 300 times faster than fructose; both were metabolized mostly via glycolysis. Yet there was more citric acid produced from fructose than from glucose during the first 2 minutes of the experiment (Table 1), suggesting that glucose

carbon and fructose carbon are metabolized separately. This view is further substantiated if we compare the ratios of lactate to citrate (Table 2), citrate to malate (Table 3), citrate to glutamate (Table 4), and lactate to alanine (Table 5), all of which indicate non-equilibration of glucose and fructose carbon at the end of glycolysis and in the citric acid cycle. However, a comparison of fructose monophosphate and uridine-diphosphoglucose (Table 6) suggests that late in the experiment (from 15 minutes) glucose and fructose carbon might mix. That fructose carbon does not mix with the other substrates can also be seen by the ratios of products produced compared with ratios from the other substrates: glucose-6-phosphate (Tables 2,3 and 6); 3-phosphoglycerate (Tables 2,5, and 7); and L-lactate (Tables 3 and 4).

More surprisingly, glucose-6-phosphate fed from the medium does not behave in the same way as glucose-6-phosphate derived from external glucose. Thus, the product ratios obtained from these two substrates demonstrate compartmental separation (Tables 3,5, and 6, and to a lesser extent Tables 2 and 7). Glucose-6-phosphate metabolism is also distinct from that of 3-phosphoglycerate (Tables 5 and 7) and L-lactate (Table 3). Phosphoglyceric acid metabolism is separate from the metabolism of both glucose (Tables 2, 5, and 7) and lactate. The only coincidence of compartmentalization suggested by this experiment is between glucose and lactate (Tables 3 and 4). This means that the relatively enormous quantities of lactate produced from glucose equilibrate with lactate in the medium, while the much smaller amounts formed from glucose-6-phosphate, fructose and phosphoglyceric acid equilibrate neither with each other

nor with lactate in the medium. A summary of these conclusions, together with some others based on evidence not presented here, is shown in Fig. 5.

The greatest technical difficulty in the type of experiment discussed here is the maintenance of a steady state of metabolism. More specifically, the problem is of two sorts: actually obtaining the biological material in a state of steady metabolism (and keeping it there), and not changing this steady state when the substrates are added. It is in any event not easy to define an overall steady metabolic state in a slow-growing cell derived from an animal or plant. One is much more confident of doing so with a micro-organism which can be shown to maintain a constant rate of doubling, for instance, for several generations. Even more certainty is provided by the use of a chemostat or similar device, but such methods are really only applicable to unicellular micro-organisms together, perhaps, with some simple colonial or filamentous forms. This is a problem which must be individually resolved in each case.

A problem which was not resolved in the experiment reported in this paper involves the requirement that the addition of the substrates must also not alter the steady state. If the addition of substrates should result in the "setting off" of a pattern of sequential metabolic transient states then the results of the experiment might reflect the shifting pool sizes and sequential uptake of differing substrates. The substrates, unlabelled, must therefore also be present before the experiment is started, and the addition of the mixed substrates, including the labelled one, must not cause significant alteration of their concentrations. Nor must the degree of utilization of any of the substrates during the period of the experiment be great enough to effect a serious concentration change.

The problem presented by these requirements is that the vast dilution of the specific activity of the labelled substrate lowers the number of counts later available in the analysis and hence the accuracy of the radioactivity measurement to an unusable level. This is particularly true when the analysis is made by two-dimensional paper chromatography where there is a very low limit for the total quantity of cell suspension* spotted onto one chromatogram; other methods of analysis are not sufficiently convenient to be used in large scale experiments.

In the experiment briefly reported in this communication we did not in fact take adequate precautions to maintain a steady metabolic state. In order to maximize the uptake of substrates the cells were first suspended in substrate-free buffer, and only at the start of the experimental incubation period were the substrates added. This should have caused transients in the concentrations of the glycolytic intermediates (Lonberg-Holm, 1959, 1962) rendering some of the data equivocal. For this reason we have presented these results only as being indicative of the sort of data which might be obtained and the type of conclusions which can be drawn from them.

The last of the essential criteria to be met in these experiments is that the cell population must be homogeneous. If it is not, one cannot know if two compartments coexist in one cell type or are present in two different cells. Obviously, it is much easier to obtain a homogeneous

* It would be prohibitively difficult at this stage to construct a machine which would not only sample the cell suspension and kill the cells but also would first separate the cells from the medium rapidly enough so as not to distort the intracellular metabolic pattern.

population with a pure bacterial culture than with a cell type extracted from a higher organism, and this represents one of the strongest technical arguments in favour of performing these experiments with micro-organisms.

It is apparent that the behavioural information which may be obtained by these simultaneous multi-substrate studies gives no clue to the structural nature of intracellular localization, but with the knowledge of the existence of a particular compartmental organization a more intelligent search can be made for structural correlations.

This work was sponsored in part by the U.S. Atomic Energy Commission. The authors wish to acknowledge the invaluable technical assistance of Miss Julia J. Chang.

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Captions for Figures

- Fig. 1 A non-compartmentalized situation in which added A or B are both metabolized in the same series of reservoirs. B behaves similarly whether derived from A or from outside the pathway.
- Fig. 2 A compartmentalized situation in which added A and B behave similarly in both series of reservoirs. These cannot then be distinguished by the use of A and B.
- Fig. 3 A compartmentalized system in which added A does not enter the same compartment as added B. It may be possible to distinguish these if they differ during subsequent metabolism as B derived from A does behave differently from B added externally.
- Fig. 4 The extreme case of the compartmentalization shown in Fig. 3. The compartment entered by A forms F; that entered by B forms G. It is therefore easy to distinguish them.
- Fig. 5 A summary of the compartmentalization tentatively proposed for ascites tumour cells.

TABLE 1

^{14}C in citrate ($\mu\text{moles/ml}$ of wet-packed cells). The reaction mixture contained the following in 2.025 ml of sodium phosphate-Locke's solution: 0.172 ml of wet-packed cells; glucose, 0.300 μmoles ; fructose, 0.288 μmoles ; glucose-6-phosphate, 0.337 μmoles ; 6-phosphogluconate, 0.159 μmoles ; 3-phosphoglycerate, 0.063 μmoles ; L-lactate, 0.718 μmoles . Incubation was at 30.5° in air.

Seconds	Glucose	Fructose
3	0.0	0.15
7	0.40	1.0
10	0.62	1.4
15	0.75	1.7
20	0.94	1.9
30	1.1	2.1
45	1.3	2.2
60	1.6	2.3
90	1.9	2.3
120	2.2	2.2
180	2.5	2.1
450	2.9	1.8
900	4.1	1.5
1800	5.2	0.82
2700	6.2	0.13

TABLE 2

^{14}C in lactate/ ^{14}C in citrate

Seconds	Glucose	Fructose	G-6-P	3-PGA
3	inf	0	inf	-
7	435	0	inf	-
10	472	0	inf	inf
15	685	0.39	inf	inf
20	695	0.70	inf	inf
30	855	1.2	inf	inf
45	959	2.1	inf	inf
60	941	3.1	inf	inf
90	1056	6.1	inf	inf
120	990	9.4	inf	inf
180	910	20	inf	inf
450	825	34	inf	9.9
900	543	54	inf	11
1800	367	114	inf	14
2700	283	777	inf	25

TABLE 3

^{14}C in citrate/ ^{14}C in malate

Seconds	Glucose	Fructose	G-6-P	Lactate
3	0	0.26	0	0
7	0.08	1.6	0.	0.08
10	0.11	2.0	0	0.13
15	0.10	2.3	0	0.19
20	0.12	2.4	0	0.23
30	0.12	2.5	0	0.29
45	0.17	2.5	0	0.35
60	0.24	2.5	0	0.41
90	0.32	2.3	0	0.51
120	0.42	2.1	0	0.60
180	0.54	1.9	0	0.77
450	0.83	1.4	0	1.5
900	1.4	0.97	0	1.8
1800	2.2	0.47	0	1.4
2700	3.0	0.11	--	1.2

TABLE 4

^{14}C in citrate/ ^{14}C in glutamate

Seconds	Glucose	Fructose	Lactate
3	0	inf	--
7	0.18	inf	0.21
10	0.22	inf	0.38
15	0.21	inf	0.41
20	0.22	inf	0.50
30	0.20	inf	0.60
45	0.21	inf	0.64
60	0.22	inf	0.59
90	0.21	inf	0.53
120	0.22	inf	0.44
180	0.22	inf	0.35
450	0.19	inf	0.29
900	0.21	inf	0.19
1800	0.21	inf	0.08
2700	inf	inf	0.04

TABLE 5

^{14}C in lactate/ ^{14}C in alanine

Seconds	Glucose	Fructose	G-6-P	3-PGA
3	6.6	--	inf	0
7	41	--	inf	0
10	42	--	inf	3.4
15	47	inf	inf	5.9
20	45	inf	inf	6.1
30	46	inf	inf	4.7
45	47	inf	inf	2.8
60	46	inf	inf	1.8
90	49	inf	inf	1.1
120	47	inf	inf	0.85
180	41	inf	inf	0.95
450	30	15	6.9	0.99
900	23	12	8.0	4.8
1800	16	12	13	18
2700	14	12	214	37

TABLE 6

^{14}C in fructose monophosphate / ^{14}C in uridinediphosphoglucose

Seconds	Glucose	Fructose	G-6-P
3	7.2	--	0
7	3.9	inf	0
10	2.9	inf	2.3
15	2.0	inf	2.9
20	1.5	inf	4.3
30	1.0	inf	6.9
45	0.71	9.0	2.6
60	0.62	6.9	28
90	0.54	4.7	20
120	0.50	3.7	15
180	0.46	2.4	13
450	0.43	0.81	11
900	0.51	0.55	9.9
1800	0.71	0.52	16
2700	1.1	0.56	inf

TABLE 7

¹⁴C in lactate/¹⁴C in phosphoenolpyruvate

Seconds	Glucose	Fructose	G-6-P	3-PGA
3	28	--	inf	--
7	163	--	inf	--
10	213	--	inf	inf
15	306	inf	inf	30
20	371	inf	inf	13
30	541	inf	inf	6.3
45	773	inf	inf	2.7
60	991	inf	inf	1.6
90	1655	inf	inf	0.88
120	3111	inf	inf	0.62
180	inf	inf	inf	0.68
450	inf	inf	inf	0.91
900	inf	inf	39	0.87
1800	inf	inf	19	0.86
2700	inf	inf	113	0.73

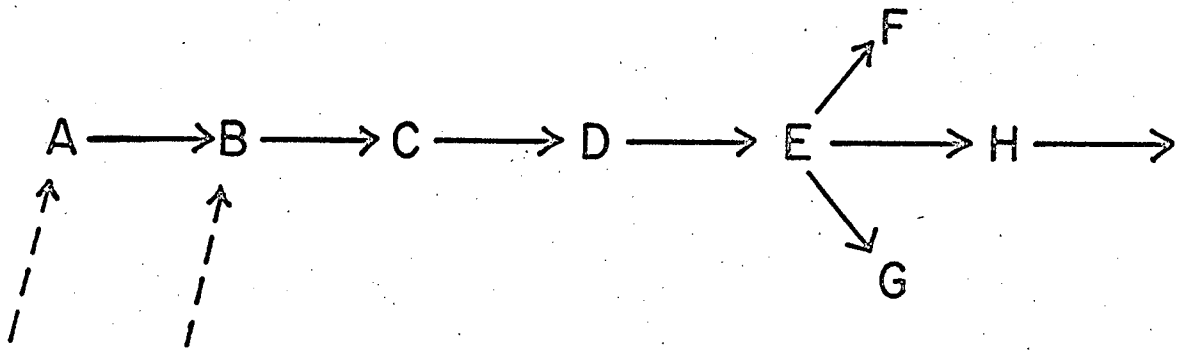


Fig. 1

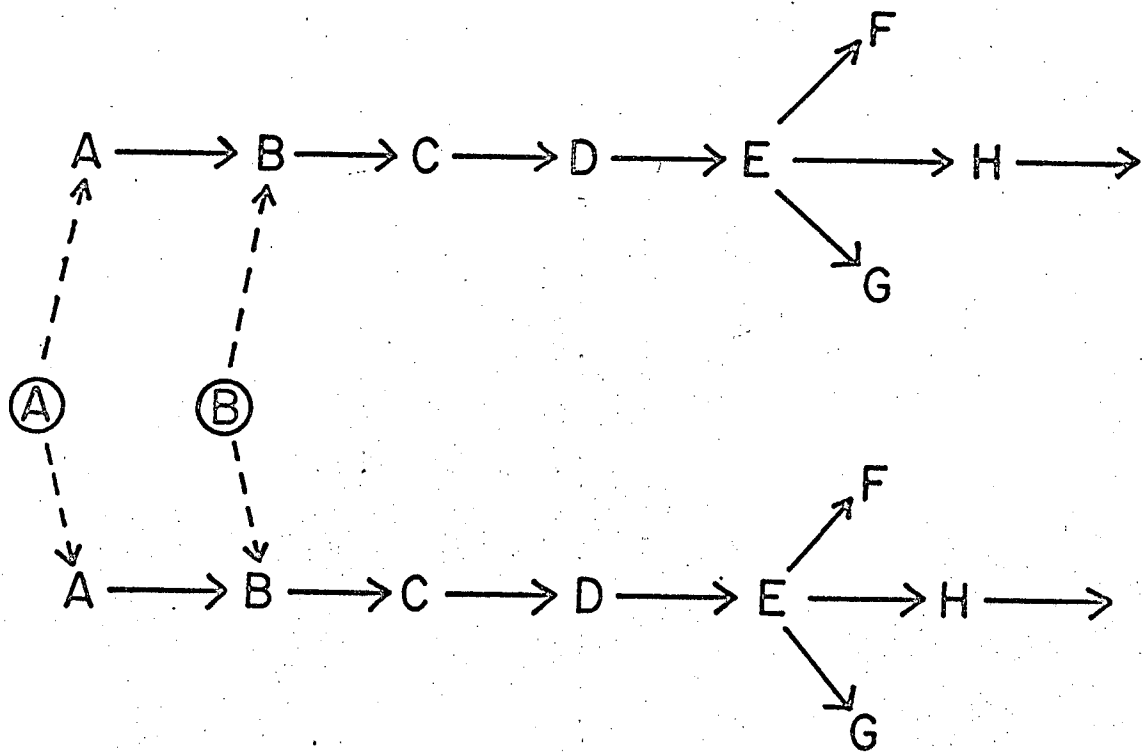


Fig. 2

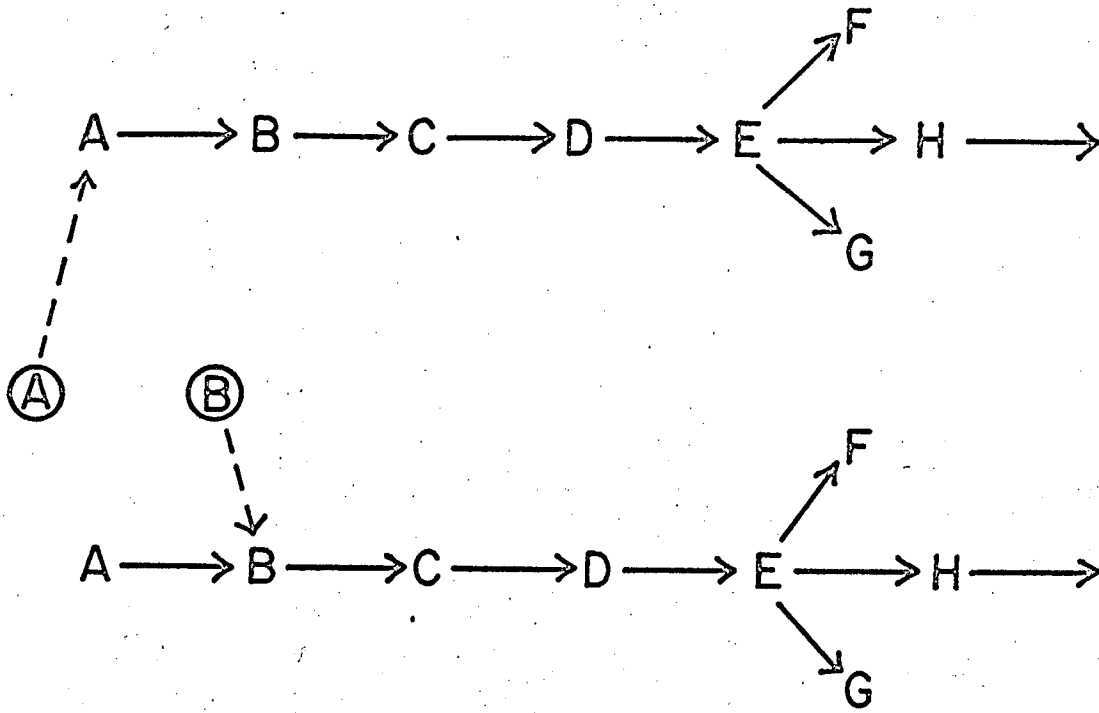


Fig. 3

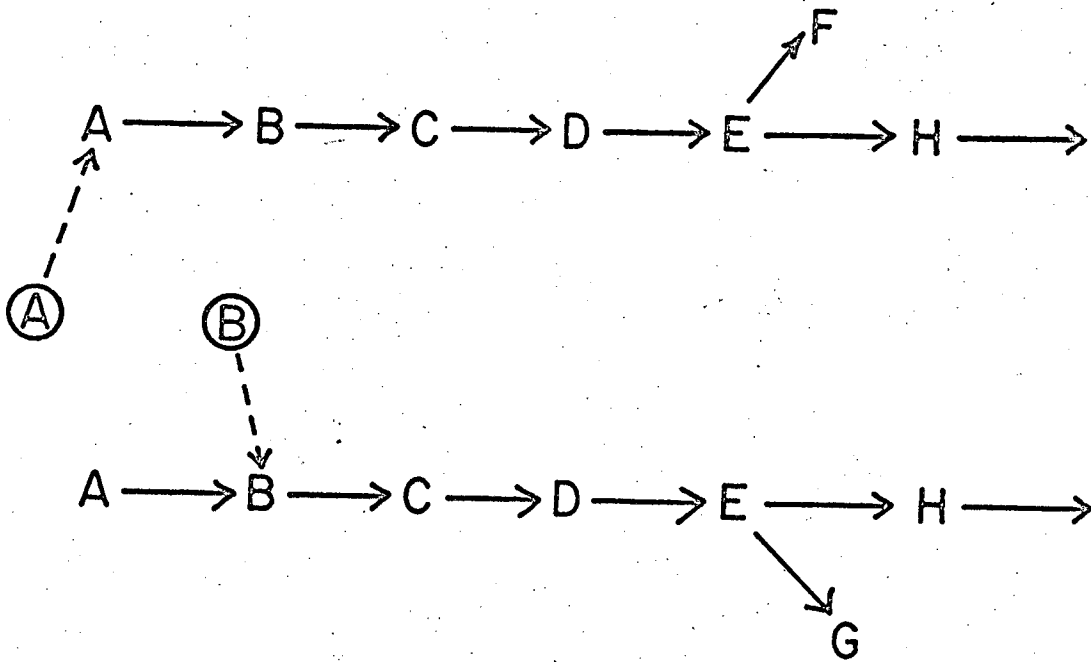
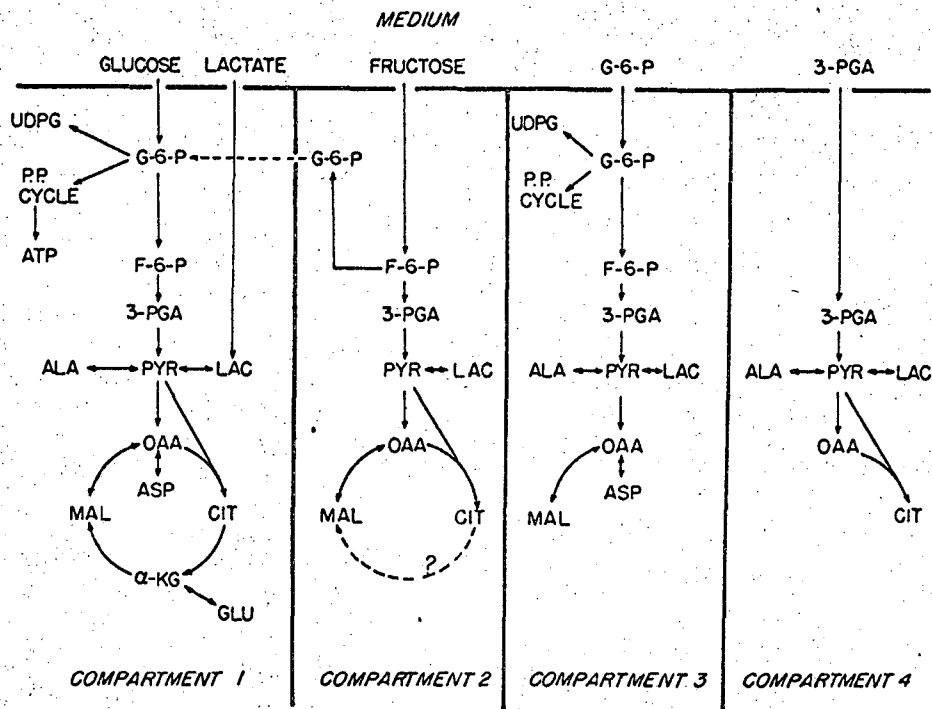


Fig. 4



MU-32472

Fig. 5



A P P E N D I X

ON INTERPRETING COMPARTMENTALIZATION EXPERIMENTS

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Introduction

In the preceding main text, Moses and Lonberg-Holm describe a methodology for experiments to determine metabolic compartmentalization. Although they show experimental results and draw conclusions from one such experiment, those authors only hint at their methods for analyzing the experimental data. It is clear, however, that those methods are predominately qualitative. This Appendix outlines a methodology for the quantitative analysis of this and similar experiments. The methods we describe are applications of common techniques in the analysis of tracer kinetics, and we review them partly to display the novel context of metabolic compartmentalization, partly to emphasize experimental and analytical difficulties in this context, and partly to suggest the failure of certain plausible qualitative considerations.

Definitions and Assumptions

We shall continue use of the term compartment in the structural sense of Moses and Lonberg-Holm, and we shall regard the experiment they describe as consisting of several distinct parallel subexperiments or sections, each of which involves the introduction of a single labeled substance. Let us include an extracellular compartment of substrate, so that all label is introduced directly into a compartment. We suppose the metabolic system to behave identically in each section; we let $S_{i,j}$ denote the substance i in compartment j ; and we define for all sections two functions: $U_{i,j}(t)$ is the amount of unlabeled $S_{i,j}$ at time t , and the transfer rate $R_{i,j;k,l}(t)$ is the instantaneous rate at time t of direct conversion of $S_{i,j}$ into $S_{k,l}$. By direct conversion we mean conversion involving no intermediate substance of present interest. For a specific section we define $X_{i,j}(t)$ to be the amount of label on $S_{i,j}$ at time t divided by the number $A_{i,j}$ of tracer atoms in each labeled

molecule of $S_{i,j}$. Measurements in the experiment described yield values for $X_i(t) = \sum A_{i,j} X_{i,j}(t)$ for certain i , the sum extending over all compartments j . The $R_{i,j;k,\ell}(t)$ are assumed to depend on t only through their dependence on all $U_{i,j}(t)$. We further assume the label on each substance in each compartment to be homogeneously distributed there.

The Kinetic Equations

With the foregoing definitions and assumptions, we may write the system of ordinary differential equations (e. g., Sheppard, 1962)

$$\frac{d X_{i,j}(t)}{dt} = \sum_{k,\ell} [R_{k,\ell;i,j}(t) X_{k,\ell}(t) - R_{i,j;k,\ell}(t) X_{i,j}(t)], \quad (1)$$

where i and k range over the number of substances and j and ℓ range over the number of compartments. These equations are satisfied also by the functions $U_{i,j}(t)$ and are valid for every section. These equations account for intracellular metabolism as well as for ingestion of substrate, excretion or leakage from cells into the surrounding substrate, and leakage from one compartment into another. Ingestion and leakage are signaled by nonzero transfer rates of the form $R_{i,j;i,k}$. Initial conditions for the $X_{i,j}(t)$ are provided by the experimental mode of tracer introduction: If the introduction occurs at $t = 0$, then all $X_{i,j}(0)$ are known (in fact, only one of them is not zero). Thus the solutions of the differential equations (1) for different initial conditions yield the tracer distributions for different sections. Various compartmentalization hypotheses are reflected in equations (1) by the range of subscripts j and ℓ and in the specification of certain $R_{i,j;k,\ell}$.

In general, the transfer rates appearing in equations (1) are not known. Even for metabolic pathways which are well understood, only rough estimates of the nonzero transfer rates can be given. In order to solve equations (1) with given initial conditions, it is therefore necessary for one to determine

or approximate the transfer rates on the basis of measurements from the experiment. The difficulty of such determination and the necessity of solving equations (1) are greatly influenced by the existence of steady states.

Steady States

A steady state of the unlabeled system or of the tracer system is a situation in which is constant each $U_{i,j}(t)$ or each $X_{i,j}(t)$, respectively. If both systems are in steady states, then the $X_{i,j}$ are proportional to the $U_{i,j}$. For the left-hand sides of equations (1) are then zero, and we obtain the same system of linear, homogeneous algebraic equations for the steady-state values $X_{i,j}$ as for the steady-state values $U_{i,j}$. Thus if tracer is introduced into the separate compartments 1 and 2 in experimental sections 1 and 2, we have, writing Y for X in section 2,

$$\frac{A_{k,1} X_i}{A_{i,1} X_k} = \frac{X_{i,1}}{X_{k,1}} = \frac{U_{i,1}}{U_{k,1}} \quad (\text{Section 1})$$

$$\frac{A_{k,2} Y_i}{A_{i,2} Y_k} = \frac{Y_{i,2}}{Y_{k,2}} = \frac{U_{i,2}}{U_{k,2}}, \quad (\text{Section 2})$$

for each pair of labeled substances in the same metabolic pathway. Of course, if there is transfer, even by diffusion, from one compartment to the other, then

$$A_{k,1} X_i / A_{i,1} X_k = A_{k,2} Y_i / A_{i,2} Y_k.$$

However, inequality of those ratios clearly indicates compartmentalization. In principle, then, an experiment to determine the existence of compartmentalization with both systems in steady states requires only four measurements.

If the unlabeled system is in a steady state, then each $R_{i,j;k,l}$ is constant, and the differential equations (1) have constant coefficients. Methods are available (Berman, Shahn & Weiss, 1962; Davidon, 1959) by which to determine these constants in order that the resulting solutions $X_{i,j}(t)$ should

best approximate the experimental data in the least-squares sense. The adequacy of this approximation may then be assessed in relation to experimental error, perhaps formally with statistical tests such as the chi-squared test. Applied to two compartmentalization hypotheses, this method yields a comparison between them. The decisiveness of the comparison depends strongly on the number of measurements available relative to the numbers of substances and compartments involved. In particular, preliminary analysis indicates that the data of Moses and Lonberg-Holm are insufficient for a decisive comparison of a single-compartment hypothesis for their experiment with the four-compartment hypothesis of Fig. 5. The analysis was not completed due to the doubtfulness of the steady-state conditions maintained in the experiment, as explained in the last part of the Moses and Lonberg-Holm paper.

If neither system is in a steady state, then an excessive amount of data is required for one to determine a best-fitting solution of the differential equations. If, however, the quantities $U_{i,j}(t)$ and consequently $R_{i,j;k,l}(t)$ may be assumed to vary slowly (as verified by independent experiment), then a parameterization of the transfer rates may be attempted, with subsequent determination of the constant parameter values as suggested for the steady-state case. Naturally, the determination of more unknown constants requires more experimental data. The decisiveness of such non-steady-state analysis is weakened by its dependence on the possibly arbitrary parameterization of the transfer rates.

The determination of values for the unknown transfer rates most compatible with both the measurements and the compartmentalization hypothesis in the stated sense does not establish these values as chemically correct; nor is the best-fitting hypothesis necessarily metabolically correct. The main effect of the analysis described is to limit the range of hypotheses to be further investigated.

Qualitative Considerations

Qualitative analysis of the differential equations describing a metabolic system and its compartmentalization may also be useful. Conclusions may be reached which contradict otherwise plausible qualitative statements. For instance, consider the system of Fig. 3 under the assumption of a steady metabolic state and the hypothesis of a single compartment. The differential equations of the system show that if the ratios of activities of the substances F, G, and H are to be identical in the two sections indicated, then each ratio is a constant, and the rates of conversion or destruction of F, G, and H must be equal. (This can easily be demonstrated by equating the derivatives of a ratio in the two sections and invoking the independence of the initial conditions.) Identification of F with the lactate, G with the alanine, and H with the tricarboxylic acid cycle of the Moses and Lonberg-Holm experiment implies that F, G, and H do indeed suffer further conversion. It follows then that the ratios mentioned may be the same from section to section under the hypothesis of a single compartment only if the rates of conversion of lactate, of alanine, and of the contents of the tricarboxylic acid cycle are all equal. Since such equality is quite unlikely, the ratios are unlikely to be identical in different sections. Thus in this respect the experimental data are consistent with the hypothesis of a single compartment even for the metabolic steady state.

This work was supported by the
U. S. Atomic Energy Commission

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