1978

Development of a Method for Measuring Amino Acid Transport in Cultured Cells Growing on Solid Surfaces

Jeffrey David Roth
Yale University.

Recommended Citation

DEVELOPMENT OF A METHOD FOR MEASURING AMINO ACID

TRANSPORT IN CULTURED CELLS GROWING

ON SOLID SURFACES

A Thesis

Submitted to the Yale University

School of Medicine

in Partial Fulfillment of the Requirements

for the degree of Doctor of Medicine

by

Jeffrey David Roth

1978

© Copyright by Jeffrey David Roth 1978

ALL RIGHTS RESERVED

ABSTRACT

DEVELOPMENT OF A METHOD FOR MEASURING AMINO ACID

TRANSPORT IN CULTURED CELLS GROWING

ON SOLID SURFACES

Jeffrey David Roth

Yale University, 1978

This study provides a complete set of criteria for the development of a methodology of transport measurement in cells in tissue culture. The method described is the first method available for the measurement of initial rates of uptake in cells growing on solid surfaces under physiologic conditions.

Published methods have been analyzed in detail. All of the uptake assays examined have been found inadequate for at least one of the following reasons: use of non-physiologic temperatures, use of nonphysiologic uptake incubation media, use of analogue substrates to draw con-clusions about a substrate class in general, and use of in-cubation times exceeding the period of linear transport. The use of incubation times in excess of the period of linear-ity can be traced to the use of a large cell sample size. The inadequacy of these uptake assays may be related to the

failure of most published studies to rigorously define up-take, transport, and linearity of transport measurements.

The rate and Michaelis-Menten kinetics of amino acid transport have been studied in normal and transformed mouse embryo fibroblast cells at different densities and under different conditions. Cells were plated on glass coverslips by a special method which gave uniform cell number per coverslip with standard deviations less than 5% of the mean. Uptake assays were performed in a controlled environment box in an incubation medium which matched the growth medium as closely as possible. Phenylalanine, leucine, and methionine uptake were linear for one minute and all rate determinations were made during this time at ten second intervals.

The pH of growth medium was found to vary between 7.0 and 8.0 as a function of cell density. The binding of amino acids to calf serum proteins as a function of pH in the range above was studied by an ultrafiltration method. For some of the amino acids studied, up to 20% may be bound in a nonspecific and reversible manner.

Phenylalanine and leucine uptake were studied in normal and transformed cells as a function of cell density. In general, uptake increased with increasing cell density in the transformed cells, and uptake increased at intermediate densities in the normal cells and decreased at confluence.

Preliminary experiments on the kinetics of leucine transport gave anomalous results. Analysis of the old batch of labeled leucine used in those experiments revealed that more than 90% of the tritium label was exchangeable with water, and of the nonexchangeable label, only 50% was in leucine. The use of an incubation medium with amino acids other than the one whose uptake was tested was found to interfere with kinetic determinations.

Methionine uptake was linear no longer than 75 seconds. Efflux of methionine from prelabeled cells into a buffered solution was not significant until after 40 seconds of incubation in the buffered solution. Methionine sulfoxide in fivefold excess over methionine did not inhibit methionine uptake.

The kinetics of leucine uptake by SV-3T3 were determined. K_M was 245 micromolar and Vmax was 2000 picomoles per microliter cell water per minute. The kinetics of methionine uptake by SV-3T3 cells were unchanged by starving the cells for methionine for four hours. 3T3 cells, however, have threefold lower Vmax and threefold lower K_M for methionine uptake.

Subconfluent 3T3 cells have a two component system for phenylalanine uptake, a high capacity, low affinity component and a low capacity, high affinity component. Confluent 3T3 cells have only one component with high affinity

and intermediate capacity. SV-3T3 cells starved for phenylalanine also have a two component system, whereas control SV-3T3 cells have only one component with high capacity and low affinity. The tentative conclusion is drawn that the transport of some amino acids may be regulated by the presence of a low affinity high capacity system under conditions of rapid proliferation, which is inactivated under conditions of starvation or differentiation.

Dedicated to my wife, Amy, for her patience, support, and
encouragement throughout the realization of this project.

ACKNOWLEDGMENTS

I gratefully wish to thank the following individuals:

My advisor and mentor, Dr. Arnold I. Meisler, for his special attention and concern for my development in the process of carrying out this research;

My thesis sponsor, Dr. Curtis Patton, for his unselfish willingness to give his time and energy in guiding the preparation of this manuscript;

Dr. Edward Adelberg, for his help in the early stages of this project, and for his constructive advice and criticisms of this manuscript;

Dr. Katharine Muirhead, for useful discussions on methodology and statistics, and help in chromatography;

Dr. Robert Amster, for help on the amino acid binding experiments;

Ms. Jean Dysard and Ms. Karen Jensen for their technical assistance.

TABLE OF CONTENTS

v

LIST OF TABLES

viii

LIST OF FIGURES

INTRODUCTION

A. THEORETICAL CONSIDERATIONS

The published work on transport in cells in tissue culture has often been less than rigorous in its definition of transport, in its control of the relevant parameters in the transport assay, and in its conclusions from the data generated thus far. Rigorous criteria when established and adhered to provide the bases for a method which generates meaningful data. Reviews of the literature on cell transport seldom explicate the methods used by different workers, methodological differences among workers, or which methods are appropriate to which questions (14, 86, 94, 95, 127, 139, 147, 149, 159, 169, 182). The extent to which disagreement exists as to the appropriate approach to transport methodology is shown below. A thought experiment in transport will illustrate how definition of transport terminology, control of relevant parameters, and data handling affect the interpretation of results under ideal conditions.

1. A Thought Experiment

X is a substrate which is known to be actively transported by some cells. The concentration of X inside the cell

1

is maintained at a level ten times greater than the level outside of the cell, and X is not bound within the cell by proteins or by other means.

The transport assay may be divided into three phases: the cells as prepared for the assay, the environment where the transport takes place, and the data obtained from the cells which are passed through the transport environment. For the sake of simplicity, the thought experimenter uses one cell which is standardized with respect to cell cycle phase, duration of time in culture, and any other relevant parameters. The transport environment might be most simple from a chemical standpoint given physiologic saline at room temperature. But simplicity from a physiologic standpoint is more probably related to the cell's familiar environment. Then the most simple environment will consist of the cell growth medium, which is altered only by the addition of some means of measuring the transport of X.

X* is a substance which is indistinguishable from X by the cell, but which is detectable by experimental means; X* can be added to the growth medium at one thousandth the concentration of X without significantly altering the cell's environment. Only the transport of X* is directly measured; the transport of X is calculated from the observed uptake of X* and the specific activity of X*. At the moment at which X* is added, it is present only outside of the cell. At

some time after this moment, the cell is removed from its environment and washed. The amount of X* associated with the cell is determined, and this procedure is repeated for different time intervals.

During a short period of time, the rate of transport of X and the pool size of X in the cell are relatively constant if the cell adequately regulates its supplies free of major perturbations. The balance in pool size is determined by entry of X into the pool by transport, macromolecular degradation, and synthesis of X, and by exit from the pool by efflux, macromolecular synthesis, and degradation of X. During the initial moments of the transport assay, the number of molecules of X* in the cell rises linearly, since transport is relatively constant over this short interval, and efflux or transformation of X* within the cell is not yet significant. After some longer period of incubation in the transport environment, X* approaches non-negligible concentration in the cell, and the increase in X* is no longer linear. Moreover, the absolute maximum amount of X* that will appear in the free pool of X in the cell after an infinite incubation is determined by the cellular pool size of X and the specific activity of X*, because the cell can not distinguish between X and X* to concentrate X* above its specific activity.

The data from the experiment then assumes the shape of a curve which represents transport as a function of time.

This curve can be divided into three parts: an initial linear part, a middle complex curvilinear part, and the final part which is linear and parallel to the time axis corresponding to a net rate of transport of zero at equilibrium.

The interpretation of data from this thought experiment depends entirely on the part of the transport curve that is used. When rates are calculated from the linear portion of the curve, those rates reflect the influx of substrate that is constantly occurring during the part of the cell's growth during which measurements were made. Rates calculated from the curvilinear part of the curve, unless corrected by appropriate equations that correct for efflux of substrate from the pool, do not admit of easy interpretation. Rates calculated from the final equilibrated part of the curve reflect the fact that pools of substrates are in equilibrium over short time periods, or alternatively may be used to estimate pool size when the magnitude of the absolute accumulated label X* is used. Results are then expressed as amount of substrate accumulated per cell, per unit cell volume, or other convenient index depending on the purpose of the study. When biochemical concentrative ability is an important factor, the use of cell water as the index is preferred, whereas if fluxes in the membrane were to be considered, the use of unit surface area of the membrane as

the index might be more appropriate. Neither of these indices would bias the results obtained from different cell lines, or the same cells in different conditions.

This thought experiment has been successfully carried out in bacteria, blood cells and tumor cells in suspension, and protozoa. Corresponding work in cells in tissue culture is just beginning to approach the questions of definition, rigor of controls, and interpretation of data; this situation parallels the earlier work in bacterial transport about which Pardee stated "(Transport) is at present a highly active area; no one seems to believe any-one else's results, so that I hope that we will have some active discussion" (148).

Rigorous work in transport in mammalian cells has been done using suspension cultures (33, 35, 62, 63, 64), but these studies by their construction leave unanswered all of the problems unique to cells that only grow attached to solid surfaces. A glance at the bibliography of this study indicates that these problems have received much attention. The controversies arising from the multitude of answers to these problems lead one to examine the premises underlying the supposed answers.

2. Definitions

Definitions play a major role in the confusions and controversies surrounding work in biological transport. The

most frequent error is to equate "transport" with "uptake."
Christensen defines transport as "the process by which a
solute is transferred from one phase to another being in
the same initial and final states in the two phases" and
further defines "active" as "that transport which is able
to operate against a concentration gradient." (35). This
operation against a concentration gradient necessitates
the use of energy. A common logical error is the assumption
that the converse of this latter statement is also true; that
is the use of energy implies the operation against a concen-
tration gradient.

Christensen's definitions may be contrasted with the
following definitions of "uptake." "We use the term 'uptake'
to denote the incorporation of an exogenous compound into
cells without regard for mechanism" (8). "All the total
uptake values given represent the net uptake as the back-
flux, if any, was not estimated (emphasis author's)" (19).
In the two latter definitions, no stipulations are made
concerning the final state of the substrate transported and
concentration gradients are not considered. But even allow-
ing these definitions to describe uptake, questions
immediately arise as to what should be considered to satisfy
the definitions. The first definition allows one to desig-
nate as uptake a rate limiting conversion of a permeable
substrate into one that is trapped inside the cell. The

second definition allows one to equate uptake with pool size.

Indeed uptake has been defined as the amount of label present in the acid soluble pool at equilibrium (27), which does equate uptake with pool size as seen in the thought experiment above. Some investigators are more careful to qualify their definitions. "The parameter under investigation was not the rate of transport itself, but the maximum amount of amino acid taken up within the cell at a steady state" (32). "The data here reported on the concentrative uptake of amino acids by cultured human cells represent stabilized equilibrium values. The kinetic aspects of amino acid transport by cultured human cells...all remain to be explored" (51).

The following quotes illustrate that the difference between initial and steady state determinations of uptake is understood by some investigators. But even this understanding does not prevent some investigators from continuing to measure uptake during time intervals during which uptake is no longer linear, as shall be shown below. "Uptake of a model amino acid such as aminoisobutyric acid involves an initial rapid influx into the cell followed by a steady state distribution of the amino acid between the intra- and extracellular phases" (48). "Within eight minutes an equilibrium of intra- and extracellular leucine pools was

reached in unstimulated cultures and the radioactivity remained stable for at least two hours" (205).

Turnover of substrate pools in the cell is controlled by influx and efflux from the pool, but no a priori argument suggests a reason why absolute turnover of pool contents should correlate with absolute size of the pool, or indeed why absolute pool size should correlate with rate of influx into the pool. Absolute pool size should remain constant over short periods of time if homeostasis is operant, and rate of influx must match rate of efflux from the pool under these conditions.

Pool sizes cannot be assumed to remain constant between different cell lines, or between cells growing under different conditions. Slowly growing cells have larger pools of several amino acids than rapidly growing cells (61). Phosphate pools in growing cells are five times greater than in contact inhibited cells (66). Normal and transformed cells have differences in amino acid pools (184). Serum decreases the intracellular pool of amino acids (205). An investigator who defines uptake as pool size should therefore find differences in uptake related to the differences cited above, but these differences may have no relation to actual rates of transport as defined by Christensen. In fact, the relation between pools of amino acids and transport may be further complicated by compartmentalization as suggested by the

finding that labeled amino acids are incorporated linearly into protein during a time in which the increase in specific activity of the amino acid pool increases curvilinearly (2).

Amino acid transport in mammalian cells has been shown to satisfy the criterion for active transport (35). However, within the limits of the present study, it will not be possible to demonstrate conclusively that the substrates used were concentrated against a gradient. I therefore attempt to restrict myself to the use of the word "uptake" in reference to data in the present study, maintaining though that were the substrate shown to be concentrated against a gradient and not bound or changed in any way, the method here proposed would yield an accurate rate of active transport. Thus my own definition of uptake is similar to Christensen's definition of transport.

B. CRITERIA FOR EVALUATING METHODS OF TRANSPORT MEASUREMENT

Since the definition of uptake or transport is carried over by the investigator into the method of measuring uptake or transport, these methods bear some analysis. The methods, or the transport assay, are divided as the thought experiment into three parts: input of cells, throughput of environment where the transport takes place, and output of cells carrying some labeled subtrate.

1. The Cells

As soon as the hypothetical realm is left, the experiment no longer deals with individual cells but with groups of cells; for experiments whose goal is the understanding of phenomena that occur only in cells in groups, this stipulation is essential. Two questions immediately arise: What kind of variance exists within this group of cells or between different groups of cells in terms of the characteristics of the cells, and how can we quantitate groups of cells so that each sample contains the same number of cells?

Variance within a sample of cells to be assayed for uptake should average over the sample, since individual cells will not be counted as such. Thus we cannot specify offhand such characteristics of a cell group such as phase of the cell cycle unless they have been synchronized. Between group variance is generally controlled by using parts of the same population for any given experiment. Other types of variance between different populations of cells, such as viral transformation, cell density, and other factors important in cell growth should be controlled by isolating them as possible independent variables.

The unique methodological problem presented by the study of transport in those tissue culture cells not capable of growth in suspension is their requirement of a surface on which to grow. Since these cells exhibit the interesting

phenomenon of contact inhibition only when they are growing attached to surfaces, the measurement of rates of transport should be made while the cells are in their normal state of attachment if transport is to be related to contact inhibition. An additional impetus for measuring transport in cells attached to surfaces comes from a report that showed differences in the kinetics of leucine transport between cells that were suspended and attached to surfaces (143). While the nature of the interaction of cells and surface to which they attach is not clearly understood, and the cells are somewhat particular with respect to the surface to which they attach, it is known that the cells grow on specially treated polyethylene sulfonate, borosilicate glass, and optical quality soda lime glass (42).

The quantitation of number of cells per sample is more difficult than generally appreciated. Using cells in suspension largely circumvents this problem if uniform mixing of the suspension leads to uniform cell number per aliquot. Variance in cell number can always be decreased by increasing sample size. Many investigators who study transport in cells in tissue culture have tended to use large growth surfaces for cells in uptake assays (Table I). The advantages of using many smaller samples of cells over one large sample was first exploited for transport work by Foster and Pardee (59) who adapted the method of Baltimore and Franklin (10) of

TABLE I

GROWTH SURFACE AREA USED BY OTHER INVESTIGATORS FOR UPTAKE ASSAYS*

Surface Area	Total Number of References	References**
Less than 5 cm^2	26	4,6,23,41,58,59,77,78,82,90,91,98,99,112,122, 129,134,145,146,166,176,179,181,183,187,191
5-10 cm^2	13	9,12,16,45,74,75,101,126,143,177,185,203,208
10-20 cm^2	9	24,55,65,83,85,87,186,196
More than 20 cm^2	14	8,11,20,21,60,66,73,79,108,110,174,185,189,195

*Surface areas available for cell growth were calculated from the types of solid surfaces to which cells adhered during the uptake assay. Coverslips and other small surfaces (less than 5 cm^2); Petri dishes and tissue culture flasks of assorted sizes (more than 5 cm^2).

**Tables I through VIII are compilations of methodological differences in several areas among workers in the field of membrane transport. Generally the absence of a referenced study indicates that the parameter under discussion was not explicitly mentioned in the referenced study.

growing cells on coverslips in Petri dishes. A further

advantage of this method is the increased facility with

which the small samples may be manipulated. But those in-

vestigators who have used cells adherent to coverslips have

faced other problems. When coverslips are arranged together

in a Petri dish, the process of moving the dish into and

out of the incubator causes the coverslips to slide over

one another. This sliding may remove a considerable per-

centage of cells randomly from the coverslips, thus increasing

variance in the number of cells per coverslip. That this

problem may have been encountered is suggested by the fact

that some investigators go to the expense and trouble of

placing coverslips in individual compartments (98,99,187,191)

and others use silicon grease (134) or capillary action

(179) to hold the coverslips to the Petri dish. Even using

silicon grease, one of these investigators found it useful

to prelabel the cell protein with [^{14}C]-leucine to standardize

accurately the number of cells per coverslip (134). And

using capillary action, after most of the data had been

gathered, coverslips were changed from conventional thin

coverslips (#2,11x22mm) to one mm thick coverslips (11x25mm)

because of the tendency for "the thin coverslips to slide

over one another during the culturing and subsequent handling"

(179). Mention of this problem is conspicuously lacking in

other studies, especially considering the probability that

variation in cell number should appear as variation in apparent uptake.

2. The Transport Environment

Under the rubric of transport environment are included most of the parameters which are specified by the uptake assay proper. These parameters include such obvious physical characteristics as temperature, pH, and composition of the uptake medium and also such characteristics as duration of incubation and any pretreatment of the cells prior to incubation. In addition, the substrate of interest and the use of an appropriately labeled substrate or labeled substrate analogue must be specified. Also included under the transport environment is its boundary with the output of the assay, labeled cells. This boundary is the separation of cells from environment.

a. Physicochemical Environment

The idea of simplicity of the transport environment has been introduced in the thought experiment. For cells in tissue culture certain minimal criteria are part of a normal environment, and appropriate controls should also be elaborated to maintain the stability of this environment. Cell culture media are defined with respect to the pH, chemical composition, and presence of chemically undefined additives such as serum. In addition the medium is used at

some temperature which allows cell growth, and some means
of humidification is provided to prevent evaporation of
water and concomitant increase in the concentration of other
medium constituents. Simplicity dictates that the transport
environment should mimic the growth environment; following
this dictum is not always simple for the investigator as can
be seen below.

Table II is a compilation of data from many studies
which shows the temperatures at which investigators measure
uptake. Evidently most of the investigators use some means
to warm the incubation medium to 37°C, but a sizable minority
perform their assays at room temperature. Those assays
carried out at room temperature are largely concerned with
the uptake of 3-0-methylglucose or galactose which are
transported quite rapidly relative to the speed of the assay
used. The use of conditions of assay which may decelerate
uptake and allow the use of simpler assay methods is a
theme which is hidden in many transport studies.

Most investigators recognize the need for controlling
pH in the uptake incubation medium to avoid any effect of
pH change on transport (46,92). Indeed effects of pH on
cell growth (28,178) should not be overlooked as possibly
mediating or being mediated by changes in transport; pH has
an effect on the transport of 2-deoxyglucose (8). Control
of pH is generally accomplished by means of a buffered solu-

TABLE II

TEMPERATURES USED BY OTHER INVESTIGATORS FOR UPTAKE ASSAYS

Temperature	Total Number of References	References
25°C	15	3,9,12,36,38,52,55,65,74,103, 134,145,146,179, 195,197
30°C	1	30
37°C	64	3,4,5,7,12,17,18,19,20,21,23,25,26,36,46,59, 63,66,67,69,70,80,81,82,83,85,87,90,91,92,96, 99,101,105,106,107,108,110,111,112,115,121,122, 126,133,134,141,143,158,162,166,167,170,172,174, 179,181,186,189,191,195,196,203,208

tion. The kinds of solutions used are listed in Table III. Only in one fifth of the references cited did the investigator use growth medium for the uptake incubation. Some investigators used HEPES buffer to control pH; aside from general questions of subacute toxicity of this buffer and others of its class (123,200), one study has shown an effect of HEPES buffer on insulin mediated increases in aminoisobutyric acid transport (92). All of the buffer solutions except growth medium lack bicarbonate, which has been shown to be necessary for growth in many cell lines (50,123,200) and to have a marked effect on the transport of glucose and 3-0-methylglucose (3).

At least one group of investigators has been very conscious of the need for a physiologic environment for studies on metabolism and transport (15,22). They describe an imaginative chamber that holds thirty 35 mm plastic Petri dishes in a temperature and humidity controlled, CO_2 gassed environment. Liquid exchange occurs through rubber portals in the apparatus, and medium on the cells can be changed in ten seconds. "Thus we approach closely to the ideal of the steady-state experiment which is to change no physiological condition, and to substitute instantaneously radioactive substrate for unlabeled substrate" (15). In one other study the investigators use a controlled environment chamber of less complex design but to achieve a similarly controlled

TABLE III

SOLUTIONS USED BY OTHER INVESTIGATORS TO CARRY LABELED SUBSTRATES FOR UPTAKE ASSAYS

Solution	Total Number of References	References
PBS	29	4,7,8,12,17,23,32,52,55,59,69,70,90,91, 96,97,102,103,112,145,166,167,170,185,187, 191,195,203
HBSS	15	3,24,79,80,81,82,85,92,115,126,141,172,186, 196,197
HBSS or EBSS with HEPES	10	21,43,106,107,108,110,143,144,158,208
Other	3	8,121,162
Growth Medium	14	19,20,45,76,87,101,102,143,177,179,189,201, 202,208

Abbreviations: Phosphate buffered saline (PBS); Hank's balanced salt solution (HBSS); Earle's balanced salt solution (EBSS); 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES).

environment (20).

The extent to which investigators use nonphysiologic temperatures and incubation media that do not support growth should be apparent from Tables II and III. Yet another technique, preincubation starvation "to allow depletion of intracellular amino acids" (59), has arisen as a normal part of investigating amino acid transport in tissue culture cells. Cells are generally starved for amino acids, serum, and vitamins as part of an incubation in PBS with 0.1% glucose (59). These incubations occur over different periods of time: less than 15 minutes (4,9,170), 15 to 30 minutes (19,30,146,191), 30 to 45 minutes (99,112,195), 45 to 60 minutes (21,23,32,51,58,59,97,117,134,181) and even 18 to 24 hours of starvation for serum and tested substrate alone (166,167). That this starvation might alter the normal physiology of the cell is suggested by the finding of increased transport of "A" system amino acids after starvation for amino acids in chick embryo heart cells (63,64), but these studies are the only ones to systematically investigate starvation for amino acids as a variable in amino acid transport. Two investigators who use the preincubation starvation claim that it had no effect on the magnitude of the rate of uptake (105,170); the claim is also made that results obtained after starvation are more reproducible (98,170). An explanation for the increased reproducibility is never offered. One

review on cancer and transport notes that "it is reportedly essential that the intracellular amino acid pool be reduced by prior incubation in the absence of amino acids to demonstrate differences in amino acid uptake between transformed and non-transformed cells" (155). In fact only recently has the first attempt been made by an investigator of transport to document the oft cited depletion of intracellular pools of amino acids by starvation.

Oxender and co-workers found that after 30 minutes of starvation only the pools of valine, isoleucine, leucine, tyrosine, and phenylalanine were depleted, and less than 50% depletion was found for pools of aspartic acid, glutamic acid, threonine, serine, glycine, and alanine (146). Apparently no one has questioned the validity of making inferences about transport in normal cells from cells whose physiologic balance has been so dramatically altered after starvation in PBS. One investigator has even suggested that PBS may be toxic to the SV-40 transformed 3T3 cells (44,185).

b. Transport Substrate

The issues of temperature, pH, and general features of the incubation medium can be dissected without reference to the substrate whose uptake is the topic of interest. When the substrate of interest is chosen, assumptions about the nature of its role in the uptake assay must be carefully examined. Many researchers have chosen to use analogues of

various substrates (particularly amino acid analogues such as cycloleucine and aminoisobutyric acid and glucose analogues such as 2-deoxyglucose and 3-0-methylglucose). The use of these analogues has several advantages: efflux is initially zero since the analogues are not normally present in the cell, specific activity of the tracer is not changed by dilution because unlabeled analogues are not present in the cell or incubation medium, and some analogues have the fortunate property of not being metabolizable so that concentrative activity of the cell cannot be explained by alteration of the substrate.

It is important to realize that the difficulties circumvented by the use of analogue substrates are not insurmountable by other means. The one specific use of analogues in the analysis of amino acid transport systems as developed by Christensen (33,35) is based on the logic of dissecting out the different components of the transport of naturally occurring amino acids. But even in the opinion of Isselbacher who has used analogues in other experiments (98,99,187), "Data derived from analogue studies are open to question because of the unclear relationship between these compounds and physiological transport substrates" (163). Considering that the analogue aminoisobutyric acid is used twice as frequently as any natural amino acid and analogue sugars are used twice as frequently as natural hexoses (Table IV), this

TABLE IV

SUBSTRATES USED BY OTHER INVESTIGATORS FOR UPTAKE ASSAYS

Substrate	Total Number of References	References
Nucleosides	24	27,28,45,46,55,65,74,101, 120,121,144,151,158,160, 161,162,168,176,177,181, 186,190,202,207
Glucose	8	3,79,82,83,97,118,122,186
Galactose	7	8,36,38,79,97,102,103
Other hexoses	4	16,79,97,182
3-0-methylglucose	14	3,12,36,41,45,52,65,79,97, 108,197,203,204,207
2-deoxyglucose	32	7,8,12,16,24,25,26,36,49, 52,53,69,74,81,82,99,106, 108,113,116,122,126,141, 144,158,160,161,185,186, 196,203,208
Leucine	15	8,19,27,29,30,97,143,144, 145,153,156,166,168,194,205
Glycine	7	4,16,29,30,32,96,145
Glutamic acid	6	9,59,60,71,97,144
Arginine	5	19,59,60,70,97
Phenylalanine	5	4,32,73,74,145
Other amino acids	17	4,9,19,30,32,59,60,71,90, 145,166,167,176,191,205
Cycloleucine	8	36,43,59,97,105,170,194,195
Aminoisobutyric acid	34	6,11,12,48,56,59,60,65,72,74, 92,97,105,109,110,114,115, 116,118,124,131,133,140,152, 153,158,170,174,176,181,186, 187,194,208

assertion about analogue studies, if true, undermines a considerable part of the literature. Let us examine then the means by which the benefits of using analogues can be achieved without incurring their hazards.

The problem of efflux of naturally occurring substrates from cells interfering with the uptake of labeled substrate is dependent on actually incubating the cells in labeled substrate long enough that efflux becomes a significant part of total tracer flux. Insofar as the same problems would be seen with analogues incubated for long time periods, the argument of using analogue substrates to avoid efflux problems is not valid. Dilutional effects of naturally occurring substrate on the label introduced are a real problem, which must be anticipated by determining the amount of substrate in the incubation medium. With defined media, the determination is trivial since concentrations are predetermined. When serum is used in uptake incubation media, the effect of serum proteins on the binding of substrate should be determined. Overestimation of substrate concentration will lead to overestimation of rates of uptake; Damper and Patton (47) have found that pentamidine binding to immunoglobulins plays a significant role in the transport of pentamidine by trypanosomes. The determination of the effect of serum proteins and pH on the effective concentration of amino acids in tissue culture medium was performed as part of the present study (175).

Analogues such as aminoisobutyric acid, cycloleucine, and 3-0-methylglucose, which are not metabolized have been used to circumvent the difficulty of separating the process of transport (where substrate is assumed to be transferred unchanged from one phase to another) from metabolism. In sugar transport, for instance, phosphorylation may be the rate determining step in transport (41,172); the argument could be advanced that the study of transport of amino acids that enter into major metabolic pathways is always contaminated by a complex set of nontransport enzymatic conversions. For this reason the use of amino acids such as leucine or phenylalanine which are not processed through the small molecular weight carbon pool but only through proteins is preferred over glycine or alanine. The problem of protein synthesis contributing to trapping of amino acids can be surmounted by the use of short incubation times. Already between the problems of efflux and metabolism of substrate the importance of short incubation times has become apparent.

Even the purity of the labeled substrate cannot be taken for granted in the uptake assay. I will show in my results the effect of using tracers that are not controlled for purity. Christensen points out that even a one percent contamination (radiolabeled substrates are generally supplied at between 95 and 99% purity) that is taken up or fixed by the cell more rapidly or completely than the sub-

strate of interest could yield very erroneous conclusions in an experiment on the effect of cell density in cell suspension on transport (35). In the light of the seriousness of substrate purity for the interpretation of results of transport data, it is curious that of the referenced studies, all of which were examined for the explicit mention of substrate purity, only one group of investigators actually tested for purity (72) and one group mentioned that the labeled compound was used within one month of purchase (141).

c. Incubation Time.

One of the conclusions from the thought experiment was that the period of time during which transport of a substrate was linear depended on efflux of substrate, transformation of substrate, and the pool size of the substrate. Another conclusion was that only data collected during the period of linear uptake can be validly interpreted as the initial rate of transport of the labeled substrate, which corresponds to the steady state influx of unlabeled substrate. In Table V data from various studies on the period of linearity of uptake for certain substrate classes are compiled. Even considering variation due to cell type and pool size, the discrepancies in reported periods of linear uptake are disconcerting. These discrepancies can not be accounted for by the use of different substrates in the case of the naturally occurring amino acids or hexoses, for similar variation occurs for the analogue substrates. One might attempt to

TABLE V

LINEARITY OF SUBSTRATE UPTAKE REPORTED BY OTHER INVESTIGATORS

Class of Substrate	Time Period (T) During Which Uptake is Linear	Total Number of References	References
Naturally occurring amino acids	$T \leq 2$ min.	12	4,21,30,70,73,74,90,91,145, 153,163,194
	5 min.$\leq T \leq 20$ min.	8	4,8,9,23,51,96,166,191
	$T \leq 20$ min.	3	71,143,145
Analogue amino acids	$T \leq 2$ min.	6	72,118,140,153,163,170
	5 min.$\leq T \leq 20$ min.	6	31,43,60,110,174,208
	$T \leq 20$ min.	7	6,11,74,99,115,170,186
Naturally occurring hexoses	$T \leq 2$ min.	1	118
	5 min.$\leq T \leq 20$ min.	5	8,79,83,122,125
	$T \leq 20$ min.	1	38
3-0-methyl-glucose	$T \leq 2$ min.	4	52,179,197,204
	5 min.$\leq T \leq 20$ min.	1	41
2-deoxy-glucose	$T \leq 2$ min.	1	53
	5 min.$\leq T \leq 20$ min.	10	8,49,106,107,108,116,122,160, 179,208
	$T \leq 20$ min.	5	7,52,69,141,185

26

account for the short periods of linearity by hypothesizing that the investigators in those cases had accelerated uptake by one means or another; however, among the perturbations of physiological conditions such as increased or decreased temperature, pH, or chemical manipulations many perturbations decelerate physiological processes. The safest assumption in any case should lead to the shortest estimate of the period of linearity to guarantee initial rates; this shortest estimate appears to be less than two minutes for most substrates. The distribution of reports on linearity is also interesting in comparing the natural to the analogue substrates; the former are reported to have shorter periods of linear uptake than the latter. If this is true, then periods of linear uptake represent one more area in which the ultimate applicability of analogue studies to the transport of physiologic substrates is called into question.

Consideration of the period of linearity of substrate uptake discussed above should lead one to expect short incubation times in incubation assays. Table VI shows that this expectation is not to be realized. Only ten percent of the studies cited indicated that uptake periods of less than two minutes were routinely used. One third of the studies indicated that time periods of more than ten minutes were used. A comparison of Tables I and VI reveals a possible relationship between the surface area available for cell

TABLE VI

INCUBATION TIMES USED BY OTHER INVESTIGATORS FOR UPTAKE ASSAYS

Time	Total Number of References	References
Less than one minute	7	4,36,53,91,197,203,207
One to two minutes	4	30,119,120,167
Two to five minutes	21	3,5,12,21,26,55,63,73,87,96,97,101,125, 132,133,152,158,160,166,174,177
Five to ten minutes	25	9,24,26,36,37,46,56,66,69,74,80,82,83,85, 105,115,126,141,143,144,161,172,196,202,203
Ten to thirty minutes	19	7,20,25,38,45,52,59,65,92,103,121,134,151, 168,181,189,191,195,201
More than thirty minutes	12	27,28,32,48,68,76,102,112,114,156,190,205

growth in the uptake assay and the time of incubation during which uptake is measured.

Investigators who use a surface area of less than five square centimeters are generally using coverslips to carry their cells. Surface areas greater than five square centimeters generally represent Petri dishes. Technical characteristics of the uptake assay impose lower limits for incubation times which are different for coverslips and Petri dishes. For instance, a coverslip held by the edges with forceps may be dipped into incubation medium, blotted by an edge to filter paper to remove excess medium, and placed into a wash solution all in less than one second. On the other hand, to place five mls of incubation medium into a Petri dish with a pipet, care must be taken not to dislodge cells from the dish with the stream of liquid from the pipet; the incubation medium must then be removed by pouring it off or by aspirating it. These two procedures cannot be reliably accomplished in less than ten seconds. Now these lower limits of duration for each procedure do not guarantee that the duration of one incubation can be successfully matched by another incubation; for incubation times to be duplicated ten second incubations of coverslips and one minute incubations of Petri dishes are more reliable. If we accept the conclusion that linearity of substrate uptake for many important metabolites may be limited to the first minute

or two of uptake, the use of incubation times starting at ten seconds may be deemed indispensable, and therefore coverslip methods are to be preferred. The technical constraints play a large role in determining the longer uptake times seen in Table VI when the investigator concerned used Petri dishes to hold cells in the uptake assay. One investigator explicitly defines the problem, "Although short labeling periods would be required to study initial rates, it was difficult to manipulate experimentally, so _five_ minute periods were chosen as both _convenient_ and _manageable_ as well as being sufficiently long to be more stable and therefore _reproducible_ (emphasis mine)" (73). Another investigator notes the problems associated with studying the transport of 3-0-methylglucose because of its rapid uptake which makes the measurement of initial rates difficult; this author uses coverslips to carry cells but does not exploit the full potential of short labeling times (173).

A recent advance in the methodology of uptake measurements in cells in suspension underlines the inadequacy of most methods used in cells adherent to solid surfaces. The investigators involved used centrifugation of the cells through uptake medium and washing solution to allow a rapid kinetic analysis of thymidine and 3-0-methylglucose transport with points taken at second intervals. These investigators comment "What we encompass within the 18 seconds of a typical run is not

an initial velocity, but the complete attainment of equi-
librium between intra- and extracellular pools." This
degree of rapidity of transport prompts the further warning
"We want on the one hand to emphasize the caution indicated
in the interpretation of transport data gathered at time
scales in excess of these, and, on the other hand, to point
to the potential of rapid kinetic techniques" (208). The
crucial importance of getting genuine initial rates even
if many technical obstacles must be overcome is emphasized
by Kalckar's appraisal of the work on rapid kinetic analysis
"The contrast between uptake data and genuine transport data
could hardly be greater. K_M for transport was found to be
almost one hundred fold greater than that found for the over-
all uptake rates by using slower conventional sampling
techniques" (104).

One might expect from the disagreement on definition of
uptake, (that is, whether uptake represents transport, and if
so whether uptake implies transfer of substrate between two
phases unchanged) that disagreements also arise on the defini-
tion of linearity of uptake. One investigator writes "Leucine
is incorporated into the acid soluble pool. However, it was
observed that the uptake of leucine is linear over a thirty
second interval at 18°C..." (194). Another investigator
writes "(Under the conditions of these experiments) the uptake
of tritiated leucine by attached or suspended 3T3 cells in-

creases linearly up to twenty minutes and ten minutes was chosen for the assay period. During the ten minute incubation, most of the tritiated leucine is incorporated into protein, thus there is very little efflux of labelled leucine from the cells and the uptake assayed is the velocity of leucine influx into the cells" (143). Aside from the startling illogic of concluding that incorporation of counts into protein implies that no efflux occurs, the basic definitions of uptake and linearity of uptake implicit in these two comments are not compatible, showing once more the importance of understanding and defining concepts for the construction of a methodology.

d. Separation of Cells from the Transport Environment

After the cells have been incubated in the uptake medium, the cells must still be separated from labeled substrate from the uptake medium which has not entered the cell. Labeled substrate trapped along with extracellular water or bound to the surface of the cell or to the cell sample but not actually transported will obscure the true rate of uptake of substrate into the cells. The procedure which is used for removing the extracellular fluid differs among investigators and these differences bear examination. Nothing has been written on the effect of different numbers of washes on the amount of label remaining on cells after incubation or on the amount of time the cells should be left

in each washing solution. The constraints in each case involve the implication that more washes of longer duration should remove more nonspecific label, but at the same time efflux of label that had been transported might also occur. Various investigators use between two and four washes for an unspecified amount of time.

In some cases after the use of some washing protocol, the amount of label bound nonspecifically compared to the total amount of label associated with the cell is very small. Frequently however, an effort is made to systematically correct data for this nonspecific binding. One strategy is the use of an incubation whose duration is some small fraction of the test incubation (4,23,59,129), for instance a five second "zero-time" incubation to correct a sixty second uptake incubation. This is theoretically equivalent to the use of more than one time point to draw a line whose slope represents the initial rate (the intercept on the uptake axis then represents "nonspecific" binding). Since the accomplishment of a real zero-time is impossible, the use of any time point close to zero seconds is arbitrary; however, for reasons of limited periods of linearity of uptake, the use of a thirty second "zero-time" to correct a five minute incubation time pulse raises serious objections, unless the substrate can be shown to be transported linearly for five minutes using rigorously controlled methods.

That some of the nonspecific binding can be accounted
for by extracellular water which remains with the cell
sample can be shown by measuring the mannitol or inulin
space of a sample of cells incubated and washed in the same
way as samples used for uptake assays (59,91). A similar
tactic is the use of L-glucose as a control for D-glucose
transport (108). Some investigators apparently believe that
label might be bound to the cell sample in a way that the
label could be competitively removed from the sample using
washes which contain an excess of unlabeled substrate (19,
20,45,121,143,166). Oxender notes that "rapid transport of
an amino acid by exchange can occur in either direction.
For this reason significant losses of accumulated amino
acids will occur if incubations are terminated by diluting
or washing the cells in media containing high levels of amino
acids" (145). An understanding of the exchange transport
phenomenon coupled with the observation that incorporation
of label into protein is linear with time (2) actually
elucidates the conflicting findings discussed above under
the definition of linearity of transport. A more detailed
analysis of the study which contains the finding that leucine
is transported linearly for twenty minutes reveals that the
investigator involved used excess leucine in the wash solu-
tion (143). Indeed this investigator finds that "most of
the tritiated leucine is incorporated into protein."

The problem of nonspecific binding of at least one compound, aminoisobutyric acid, to plastic growth surfaces prompted one group to develop a special washing technique using 48 mM glycylglycine which was claimed not to cause increased efflux (174). A high degree of binding of this analogue to plastic would make any study that used a single time point for rate determinations somewhat suspect.

Other factors contribute as sources of variation in the data from the transport experiment: errors in cell counting, errors in scintillation counting, errors in time measurements. Because these errors are assumed to be random, they can be averaged out over many samples of cells, and these sources are not as worrisome as the systematic errors that have been discussed above.

3. Data Handling

The raw data from an uptake assay that measures the movement of radiolabeled substrate usually take the form of scintillation counts from samples taken at different time intervals. The conversion of these raw data into a usable form and the interpretation of these data constitute the output of the transport experiment. For a method which measures uptake in cells on coverslips, the following flow chart of data conversions can be verified by dimensional check:

$$\frac{\text{CPM}}{\text{coverslip}} \xrightarrow{\quad 1 \quad} \frac{\text{DPM}}{\text{coverslip}} \xrightarrow{\quad 2 \quad} \frac{\text{DPM}}{\text{coverslip}\cdot\text{minute}}$$

$$\Big\downarrow 3$$

$$\frac{\text{picomole}}{10^{6}\ \text{cells}\cdot\text{minute}} \xleftarrow{\quad 5 \quad} \frac{\text{picomole}}{\text{coverslip}\cdot\text{minute}} \xleftarrow{\quad 4 \quad} \frac{\text{DPM}\cdot\text{picomole}}{\text{coverslip}\cdot\text{minute}\cdot\text{micro-liter IM}}$$

$$\Big\downarrow 6 \qquad\qquad \searrow 6$$

$$\frac{\text{picomole}}{\mu\text{l cell } H_2O\cdot\text{minute}} \qquad\qquad \frac{\text{picomole}}{\text{mm}^2\cdot\text{minute}}$$
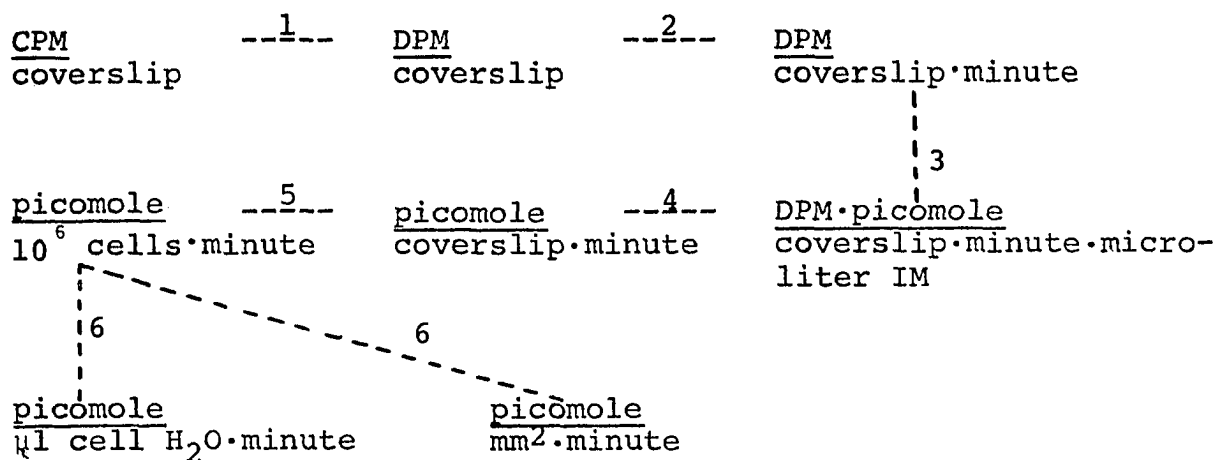
1) Counts per minute (CPM) per coverslip as taken from the scintillation counter data are converted by a channels ratio method to disintegrations per minute (DPM) per coverslip.

2) DPM per coverslip are plotted versus the time of incubation to generate a slope (DPM per coverslip per minute).

This slope represents a rate of transport of a substrate. DPM are converted to picomoles of substrate in two steps. 3) The rate is multiplied by picomoles per microliter incubation medium, a quantity either known or measured separately. 4) The rate is then divided by DPM/microliter transport medium, which is obtained by counting an aliquot of incubation medium in the scintillation counter. 5) The rate, picomoles per coverslip per minute, is divided by cells per coverslip $x10^{-6}$ to yield a rate expressed as picomoles per minute per 10^{6} cells. These data are obtained by measuring the number of cells on a coverslip electronically or with a hemocytometer. In step 6) this rate is converted to picomoles per minute

per unit cell water (128) or unit surface area of the cell

membrane (135) to express concentrative ability or substrate

flux respectively.

This method of data handling is rather uniform up

until the last step. Many investigators omit the second

step of analyzing data from serial time points in favor of

establishing a rate from a single time point. This omission

courts the possibility of measuring uptake in time periods

well in excess of the duration of linear uptake. The third

and the fourth step can be combined when using an analogue

substrate whose specific activity is known.

a. The Use of Cell Protein as an Index for Transport
Measurements

Several investigators have expressed the rate of

transport in terms of an amount of substrate transported

per unit cell protein (4,8,58,99,146,152,181). The rationale

for this approach is "because of variations in cell volume

and surface between (different clones), it is preferable to

relate uptake activity to cell protein rather than cell

number"(4). The applicability of protein as an index depends

on the assumption that cell protein can be related to cell

water or cell surface. Foster and Pardee claim that a linear

relationship exists between cell protein and cell water (59),

but Meisler has pointed out that the former investigators

used two millimolar urea and mannitol in their cell water

measurements which could have changed the cell water by

altering the osmotic properties of the cell's environment
(128). Meisler's own data on cell water as a function of
cell density differ markedly from those of Foster and Pardee,
and cell water does not bear a linear relationship with
cell protein there.

The use of cell protein as an index for transport is
theoretically unsound from another direction. Early work
on the kinetics of growth of tissue culture cells revealed
a lag phase before the cells initiated logarithmic growth
(180,193). These studies showed that in this phase cell
protein, DNA, and RNA doubled before the cells had increased
in number, which would confirm that cell protein and perhaps
cell volume may not be strictly related to cell number.

In addition, cells export glycosaminoglycans and pro-
teins into the medium and into the extracellular matrix
(39,171). It is difficult to see how the increase in
protein per coverslip due to protein in the extracellular
matrix would be reflective of changes in cell volume.

Romano (172) has been a vocal critic of the use of
2-deoxyglucose uptake as an index of glucose transport because
of the question of rapid phosphorylation of this analogue.
He states the case against using cell protein as a basis for
quantifying transport, "Almost all measurements (of transport)
are expressed in terms of nanomoles of substrate taken up
per mg cell protein. Since transport is essentially the

movement of a solute from a solution outside the cell into an intracellular pool of fluid, it would be more logical to express transport measurements in terms of nanomoles per unit volume intracellular fluid. The relationship between cellular protein and intracellular fluid volume is not necessarily constant, especially in comparisons between untransformed and transformed cells. There is often a change in cell geometry that affects surface area to volume relationships" (172).

In any case, no one argues against the use of cell water as a basis of expressing transport, and these measurements have been made using double label isotope techniques (128,191) and electronic sizing (189). Romano compares the interpretations of data expressed in terms of cell protein and cell volume "On the basis of cell protein, transport of 3-θ-methylglucose by the transformed cells was greater than 3T3 cells by a factor of 1.4. When based on intracellular volume, transport in all three cell types was essentially the same. When one considers that many claims of increased transport in transformed cells, based on cellular protein, are based on a factor of two, this consideration could be significant" (173). If we take Romano's argument a little farther, and assume that cell volume may vary under different conditions other than transformation versus control, the data compiled in Table VII become more interesting. In reports of differences in transport rates between two condi-

TABLE VII

MAGNITUDE OF DIFFERENCES BETWEEN EXPERIMENTAL AND CONTROL

UPTAKE RATES REPORTED BY OTHER INVESTIGATORS

Magnitude of Difference	Total Number of References	Reference Followed by Substrate*
One to twofold difference	7	12(DOG),30(Ala,Leu),76(PO$_4$),116(DOG), 181(AIB),185(DOG),189(K)
Two to threefold difference	10	5(Glc),29(Gly),45(PO$_4$,Uri),52(DOG), 99(AIB),112(Rb),152(AIB),176(Gln),185 (DOG),196(DOG)
Three to fivefold difference	4	20(AA),39(Gly),185(DOG),201(PO$_4$)
More than fivefold difference	5	24(DOG),36(HEX),125(Glc),185(DOG),203(OMG)
Any change reported in the kinetics of substrate uptake		
1) maximal velocity (Vmax)	17	24(DOG),25(DOG),46(NUC),83(Glc),96(Gly), 99(AIB,C-leu),101(PO$_4$),108(DOG),110(AIB), 115(AIB),116(AIB,DOG),160(DOG,Uri),170(AIB), 174(AIB),176(AIB),197(OMG),203(OMG)
2) substrate concentration at half-maximal velocity (K_M)	8	25(DOG),79(HEX),81(DOG),83(Glc),122(Glc), 143(Leu),170(C-leu),185(DOG)

*Abbreviations: amino acids (AA); aminoisobutyric acid (AIB); alanine (Ala); cycloleucine (C-leu); 2-deoxyglucose (DOG); glucose (Glc); glutamine (Gln); glycine (Gly); hexoses (HEX); potassium ion (K); leucine (Leu); nucleosides (NUC); 3-0-methylglucose (OMG); phosphate (PO$_4$); rubidium ion (Rb); uridine (Uri).

tions, one to threefold differences in rates are twice as common as differences of greater than threefold. Furthermore in changes reported in the kinetics of transport, differences in Vmax are twice as common as differences in K_M. Because Vmax is a velocity, it is expressed as a rate per unit protein if the rates which contribute to the calculation are expressed per unit protein. K_M, being simply a concentration, is independent of the units of the rate. For the purposes of the present study, it is especially significant that in Table VII differences in the kinetics of amino acid transport are four times as common in Vmax as in K_M. In this light one must also question the puzzling occurrence in the transport literature of Lineweaver-Burke plots that have their intercepts at the origin (17,134). One explanation could be that as sometimes happens the concentration range used for the kinetic determinations was not in the same range as the K_M (25,41,83). A less favorable explanation would point to the possibility that rates of transport are not really being measured; for instance if pool size were measured, it might be linear with increasing external concentrations of substrate, and not be saturated like a typical enzyme controlled reaction.

b. Variation in Results

Although many investigators do not provide data on the variation in uptake results which they report, some investigators

use error bars in their time points (67,153,158) and some give standard deviations of the mean for typical experiments. Most of those investigators who admit to some variation claim that their standard deviations are less than 15% of the mean (23,43,46,141,185,191). Kalckar has reported standard deviations of 50% (37), 20% (195), and 30% (105) of the mean. Interestingly, the variation in one study was 2% of the mean for sparse transformed cells and 20% for confluent cells (195). If problems of variation in cell number per sample in the coverslip methods are as serious as indicated in that discussion, one would expect that other investigators would find wider variations in their uptake data. Indeed, a common observation among transport investigators is variability between experiments (17,58,59,92,133, 145,167,185,191). How much of this variability can be explained by problems within the method is unknown, and the dirth of data on variability within experiments does not help. Yet another indication that variability is a problem is the appearance of two studies on the computer analysis of transport data (138,206). As will be seen in this study, data that fit a line with a correlation coefficient of 0.99 do not need to be analyzed by computer to draw a line.

C. ANALYSIS OF A PUBLISHED STUDY

Once all is said and done in establishing a method and interpreting the results, a variety of different questions

are approachable. Different investigators have studied the effect of cell density on transport (18,68), the effect of cell cycle phase on transport (19,43,142,181,190,194), different systems for different amino acid transport (33,35), the efflux of amino acids (54,75), temperature sensitive mutants for transport (126), and competition between different amino acids for transport (60,110,143,145,167,170).

The maintenance of rigorous controls is still necessary in this kind of study. Specificity of effects of a given stimulus must be checked by giving a neutral stimulus, as pointed out in a study where uridine uptake was stimulated by serum, but also stimulated to the same extent by salt solution (28). Another group finds an increase in aminoisobutyric acid transport specific for insulin and not hydrocortisone, but they find that "the increased transport requires the presence of natural amino acids in the incubation medium. Apparently the presence of natural amino acids, which compete with the non-metabolizable amino acids for the transport site, is important in assessing the increase in the level of amino acid transport reflected by aminoisobutyric acid uptake" (114).

A study by Foster and Pardee (59) illuminates the problems of methodology and interpretation that have been discussed above. This study has received much attention, both for its methods and its results. One of the more

interesting aspects of this study is the fact that its
results have been interpreted in contradictory ways by
different investigators who cite the study to support their
own data (Table VIII). In fact, more investigators appear
to disagree than agree with the conclusion stated plainly
enough by the authors themselves. These contradictions can
be explained using the information we have amassed thus far
to analyze the sources of ambiguity in this study by Foster
and Pardee.

Foster and Pardee (59) set out to describe "transport
of amino acids by confluent and nonconfluent 3T3 and polyoma
virus-transformed 3T3 cells growing on coverslips." In
their summary, they state that "aminoisobutyric acid and
cycloleucine were accumulated about 30% less rapidly by
confluent than by nonconfluent 3T3 cells." Since these
authors base their transport measurements on cell protein,
this 30% apparent decrease in transport could also be
accounted for by an increase in extracellular protein during
confluency. But they also state "rates of accumulation of
metabolizable amino acids such as glutamic acid, glutamine,
and arginine were not lower in confluent than in nonconfluent
3T3 cells" (59). Thus depending on which result one feels
is important, either claims of differences or no differences
in transport as a function of cell density have been made.
Christensen (34) has pointed out that while aminoisobutyric

TABLE VIII

CONCLUSIONS DRAWN BY OTHER INVESTIGATORS FROM FOSTER AND PARDEE (59)*

Conclusion	Total Number of References	References
Transformation of tissue culture cells results in increased uptake of amino acids.	13	8,69,112,137,145,146,150,154,157,164, 174,195,204
Transformation of tissue culture cells results in increased uptake only of analogue amino acids.	7	38,98,121,141,155,185,198
Proliferating cells exhibit increased uptake over density inhibited cells.	12	11,46,54,67,72,100,143,153,158,182,187, 194
No differences are seen between proliferating cells and density inhibited cells with respect to amino acid uptake.	8	27,40,57,60,162,198,201,202

*The authors themselves state, "The growth regulation apparently exemplified by density-dependent inhibition of growth in vitro seems not to be due to general alterations in membrane permeability and transport" (59).

acid is transported by the A system (alanine preferring),
glutamic acid, glutamine, and arginine are not transported
by this system. This difference makes the understanding
of appropriate use of amino acid analogues even more criti-
cal. Foster and Pardee (59) further note twofold increased
transport of aminoisobutyric acid, cycloleucine, and
glutamine due to transformation, with no change in glutamic
acid or arginine transport. Here questions of cell volume
and also the questions raised above must be answered in
attempting to interpret this data.

Foster and Pardee (59) measured uptake using glass
coverslips, twenty-one of which were arranged in a 100-mm
plastic tissue culture dish. "Cells were seeded as a sus-
pension in growth medium...the cells were allowed to settle
onto the coverslips and after about fifteen minutes they
adhered sufficiently so that moving the dish to an incubator
did not cause the cells to wash about (which can result in
uneven distribution of the cells)" (59). No mention is made
of the problem mentioned by others of coverslips sliding
over one another, even though the method used by these
authors involves feeding the cells with fresh medium twice
before the uptake assay, which means that a minimum of two
transfers of medium and four transfers of coverslips (bench
or hood to incubator) is being carried out.

Cell number determinations were performed using a

hemocytometer, which is usually only accurate to $\pm 15\%$ (1).
This may explain some of the preference for basing transport
measurements on cell protein, which is more easily and less
tediously performed. Foster and Pardee use [^{14}C]-labeled
amino acids, thus avoiding some of the problems associated
with tritium exchange with water which will be discussed
below. They use a preincubation starvation period of one
hour, and incubate the cells for an uptake period of ten
minutes to one hour, using a 30 second incubation to correct
for extracellular isotope not removed by rinsing. The
difficulties of interpreting results based on methods using
preincubation starvation, long uptake intervals, and long
incubations to correct for nonspecific binding have been
discussed in detail above.

Foster and Pardee attempt to show that the amino acid
transport they are measuring is actually active transport.
They show inhibition of aminoisobutyric acid uptake by
omission of glucose, addition of cyanide or iodoacetate, and
by temperatures of 0-1°C (59). Christensen (35) criticizes
this kind of approach: "Occasionally the term active trans-
port is applied on the equivocal basis that a dependence
has been shown on an energy-yielding metabolic process.
This observation cannot be considered a fully reliable indica-
tion that the transport is uphill. Conceivably the energy-
yielding processes serve only to generate a mediating structure
or even a binding agent, which may then account for the

accumulation of the solute. To be proved active, the transport should be capable of establishing a thermodynamic gradient."

Referring back to the claim that aminoisobutyric acid was transported 30% less rapidly by confluent than nonconfluent cells, one must consider the results of the experiments "in which the incubation time was extended to three hours (which) showed that the level at which accumulation of aminoisobutyric acid stopped was about 30% lower with confluent than nonconfluent cells" (59). Here is possibly the best substantiation of suspicions that many claims of increased transport are really dependent on differences in pool size.

This study is representative of others also in its findings of changes in Vmax without change in K_M and in its finding of variability of results between experiments, the significance of which has already been discussed. Considering all of the difficulties involved in this method and the interpretation of its results, it is perhaps surprising that so large a number of other investigators have adopted it essentially without change or criticism (6,13,16,17,30,41, 58,78,90,142,176,183,187), but this provides an even greater impetus for the development of new methods.

II

MATERIALS AND METHODS

A. GENERAL

1. Routine Cell Culture

All tissue culture work was performed in an Edgegard Horizontal Flow Hood (Baker Co., Biddeford, Maine). Mouse embryo fibroblast cells (3T3) and their SV-40 transformed derivatives (SV-3T3) were grown at 37°C in a 10% CO_2 (Air Products) humidified incubator (Wedco, Silver Springs, Md.). Cells were routinely carried in 75 cm^2 plastic tissue culture flasks (Falcon, Oxnard, Ca.) in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (both from Grand Island Biological Company, N.Y.) without antibiotics. Stock cultures were shown to be mycoplasma free using culture, RNA electrophoresis, and uracil/ uridine uptake ratio. Cultures were periodically discarded and replaced with stock cultures. These cell culture procedures have been described in detail (128).

2. Cell Counting and Cell Water Determinations

Cell layers were washed once with sterile Tris buffer (NaCl 138 mM, KCl 5.0 mM, Na_2HPO_4 0.7 mM, Tris

49

(hydroxymethyl) aminomethane 25 mM, pH 7.5) and then covered

with 0.1% trypsin in EBSS for 15 minutes at 37°C. Trypsin-

ization was terminated by the addition of an equal amount

of DMEM with calf serum. Cells were dispersed with a

Pasteur pipet or an 18 gauge needle on a ten ml syringe.

This suspension was diluted to five or ten mls with Tris

buffer, and the sample was counted in triplicate on a

Celloscope Particle Counter (MKS Scientific, Lancaster,

N.Y.) equipped with a 100 micron orifice. Coverslips

monitored under phase contrast microscopy (Tiyoda, Tokyo,

Japan) after trypsinization showed better than 95% removal

of cells.

The methods for determination of cell water for both

cell types over a wide range of densities were as previously

described (128).

3. Scintillation Counting

Counts per minute (CPM) were measured in a Mark I

liquid scintillation spectrometer (Nuclear Chicago,Ill.)

and converted to disintegrations per minute (DPM) by a

Channels Ratio method using an external standard. Tritium

counting efficiency was approximately 33%. The scintillation

fluid was composed of one part Triton X-100 (Emulsion

Engineering, Elkgrove Village, Ill.) and two parts of a

solution of 0.25 grams per liter POPOP and 8.25 grams per

liter PPO (Amersham/Searle, Arlington Heights, Ill.) in

toluene.

4. Reagents

All inorganic reagents were from Mallinckrodt (St. Louis, MO.). All radiolabeled chemicals were from New England Nuclear (NEN, Bedford, Mass.) unless otherwise noted. The routine biochemicals such as amino acids were from Nutritional Biochemicals. Methionine sulfoxide was from Sigma.

[^{35}S]-methionine was stored in small aliquots at -140°C and used within two months of purchase. Tritiated phenylalanine and leucine were stored at 0°C and used within one month of purchase. Initial purity of methionine was greater than 90% and of phenylalanine and leucine greater than 99%.

5. Cell Culture on Coverslips

Fifteen millimeter diameter, number two thickness, optical glass coverslips (Corning Glass, N.Y.) were washed for two days in sulfuric acid dichromate solution (Chromerge, Manostat, N.Y., N.Y.). They were then rinsed thoroughly for two hours in running distilled water, washed five times in double distilled water, and three times in 95% ethanol, and stored in 95% ethanol. Coverslips were flame sterilized and were transferred, 65 coverslips to each 150 mm plastic Petri dish (Falcon). They were covered with a cell suspension of 4×10^5 cells in 25 mls of growth medium and were placed in dishes in the incubator. The next day the medium

was changed on all of the dishes except those to be used on that day.

This procedure for cell culture on coverslips was modified after the minimal perturbation studies (Methods, B4) to decrease variation in cell number per coverslip. Automatic pipets (MLA precision pipets, Mt. Vernon, N.Y.) were tested for their accuracy and precision by measuring the weights of successive aliquots on a Mettler balance. Automatic pipets were fitted with sterile cotton plugged one inch lengths of rubber tubing which connected the non-sterile automatic pipet with the sterile disposable pipet tip. These pipets were used to deliver fixed volumes of cell suspension onto each coverslip. A volume of 300 micro-liters was found to be convenient as it spread over the whole coverslip but was easily maintained within the bounda-ries of the coverslip by surface tension. Using this modi-fied method, no more than 40 coverslips were placed in one 150 mm dish to prevent the cell suspensions from adjacent coverslips from touching one another. The cell suspensions from such coverslips spilled off into the dish.

Cell suspensions were prepared as follows: medium from cells growing in 75 cm^2 tissue culture flasks was aspirated. The cell layer was washed once with 10 mls of warmed 0.1% trypsin (Difco, Detroit, Mich.) in Earle's Balanced Salt Solution (EBSS, GIBCO), and then incubated in 5 mls of this solution for 15 minutes at 37°C. Five mls of growth medium

were added to the flask, and the cells were removed from the surface of the flask and made into a monodispersed suspension of cells by passing the cell suspension ten times through a 20 gauge needle on a 10 ml syringe. Cell concentration was determined (Methods, A2) and the suspension was diluted to an appropriate concentration with growth medium. The diluted suspension was transferred to a plastic Petri dish. The cells were kept uniformly suspended and monodispersed during the plating procedure by gently rocking the Petri dish and by passing the whole suspension through an 18 gauge needle on a 30 ml syringe between each set of 30 to 40 coverslips.

Cell density was varied by adjusting the cell concentration of the suspension used for plating. Cells on coverslips were used for uptake assays two days after plating, thus avoiding the necessity for changing the medium on the coverslips. In the experiments on cells starved for a particular amino acid, coverslips were removed from the Petri dish, washed once in ten mls of medium lacking that amino acid, and arranged in a new 150 mm Petri dish containing 25 mls of the deficient medium. These starved cells were incubated in the deficient medium for four hours prior to the uptake assay.

B. TRANSPORT ENVIRONMENT

1. Medium pH during Cell Growth

Cells were inoculated into duplicate sets of 60 mm
plastic Petri dishes. The following day (zero time) the
medium was aspirated and replaced with exactly four mls of
fresh medium. At each subsequent time point, medium was
rapidly aspirated into a syringe containing one ml of min-
eral oil and then slowly expelled into a test tube without
bubble formation. The pH was then measured using a long
stemmed combination electrode (Radiometer). This method
gave pH values identical to those determined without opening
the incubator door, using the same apparatus rigged inside
the incubator. Since small but definite pH changes occurred
as a function of repeated opening and closing of the incu-
bator during the day, control plates containing medium but
no cells were placed in the incubator and used to correct
pH determinations in the experimental plates.

2. Binding of Amino Acids to Serum Proteins

DMEM containing 10% calf serum was prepared containing
an isoosmotic quantity of NaCl (2.58 grams per liter) in
place of the standard $NaHCO_3$ (3.7 grams per liter). To 200
mls of medium 4 microcuries of ^{14}C -labeled amino acid
(Schwarz-Mann) or 100 microcuries of a tritiated amino acid
were added for each experiment. The use of the isotopes

simultaneously in the quantities indicated above gave the same results as the use of the isotopes singly and shortened the time required to construct reproducible calibration curves. However, all amino acids were tested both singly and in combination.

. Thirty 6 ml aliquots of this labeled medium were titrated with 0.15 N NaOH or 0.15 N HCl to the pH range determined in the previous section. The volume of all samples was adjusted to 6.25 mls using 0.15 N NaCl. Eight additional samples containing no serum were also prepared and were used to determine amino acid retention by the filter. Samples were allowed to equilibrate in the cold overnight. The samples were brought to room temperature and the pH of each one measured using a Radiometer pH Meter 26. One hundred microliters were removed to determine the content of radioactive amino acid in the medium. Filtration was then performed in a 10 ml ultrafiltration device (Amicon, Lexington, Mass.) using UM2 filters (Amicon) at 60 psi of nitrogen.

Filtrations were performed serially without washing or dismantling the apparatus. The first 200 microliters collected from each sample were discarded to allow for equilibration between the pH of the new sample and the pH of the filter, and to remove the void volume of approximately 50 microliters in the tubing. The next 250 microliters were

collected and two 100 microliter aliquots were used for counting radioactivity. The retention due to the filter alone was estimated using the samples containing no serum (blanks), four prior to the experiment and four at the conclusion. This correction due to retention by the filter was essential to the procedure since it was found to vary from 20 to 30% depending upon the filter lot, but did not vary with pH or the labeled amino acid. No difference in filter retention was observed between blanks counted at the start and conclusion of the experiments.

The quantity of radioactivity in DPM per 100 microliters was compared with the radioactivity in the medium prior to filtration. The amount of radioactivity bound to the serum in the medium could then be calculated. Using the above procedure, standard binding curves such as those shown in the Results could be constructed showing the relationship between the pH of the medium and the fraction of the total amino acid bound to serum.

Since the ultrafiltration described above is carried out in nitrogen, the determination of binding under experimental conditions in a CO_2 atmosphere required the determination of the relationship between the pH of the medium inside and outside of the incubator. Therefore, aliquots of medium from growing cells were removed from the incubator and the pH was measured before and after a 3 hour equilibration with room air.

3. Basic Uptake Assay

All manipulations were carried out at 37°C. Cells growing on coverslips in growth medium were removed from their Petri dish using forceps to hold each coverslip by its edge. The coverslip was blotted by its edge to filter paper. When the incubation medium differed from the growth medium by more than the presence of a labeled substrate, the coverslip was washed in a solution which was identical to the incubation medium except that it lacked the substrate whose transport was tested. The coverslip was then gently immersed in the incubation medium for an interval determined by a timer (Precision Scientific) to the nearest tenth of a second. Two to three seconds before the required interval had elapsed, the coverslip was removed from the incubation medium, quickly blotted, and immersed in a wash solution exactly when the required time interval had elapsed. The coverslip was then blotted and washed once more, blotted again and finally dropped into an empty scintillation vial. Any combination of three or more time points of less than one minute was used, and each time point was performed at least in quadruplicate. Several fifty microliter aliquots of the incubation medium were taken before and after the experiment and measured for radioactivity. This basic uptake assay has been described in detail (129).

4. Uptake Assay Modified to Insure Minimal Perturbation of Cells

Preliminary experiments on amino acid transport were performed in a room maintained at 37°C. The incubation medium consisted of a solution of amino acids at their concentration in growth medium in a solution of 0.1% glucose in Tris buffer. While the pH of the incubation medium was thus assured to remain constant, the pH of the cell growth medium became increasingly basic in room air during the course of the experiment. Transfer of the cells to a buffer solution prior to the experiment, achieving a similar effect to a preincubation starvation, was avoided for reasons described above. This consideration led to the construction of a special box which could be maintained thermostatically at $37 \pm 1°C$, gassed with 10% CO_2 (pH of fresh medium 8.04 ± 0.10) and humidified to 100% relative humidity (seen as condensation on the sides of the box). The top five sides of the box were constructed out of ¼ inch plexiglas with three large holes in the front fitted with isoprene gloves through which coverslips were manipulated with forceps. The dimensions of the box were two feet long, one foot high, and one foot wide. Heating was accomplished by means of a 25 watt heater attached to a thermostatic element (Honeywell).

Cells were grown on coverslips as described above, 65 coverslips per 150 mm dish plated from a cell suspension

which covered the dish. For each experiment, the appropriate dish with coverslips was removed from the incubator and immediately placed in the box, which was flooded with 10% CO_2 in air at six liters per minute for one to two minutes; the box was thereafter gassed at 1.2 to 1.4 liters per minute for fifteen minutes to allow complete equilibration. The pH of the medium was measured through a small hole at the top of the box with a combination electrode.

Because these experiments were designed to determine the rate of transport of amino acids as the cells grow in the incubator, the incubation medium consisted of exactly the same medium in which the cells had been growing. Therefore, 5 mls of medium were removed from each of two 150 mm dishes with coverslips and combined in a 60 mm Petri dish. One hundred microcuries of tritiated amino acid were added to this medium. Nine coverslips were removed from each dish for determination of cell number per coverslip.

The uptake assay was performed as described above. However, since the incubation medium did not differ from the growth medium, coverslips were not passed through an intermediate wash, but simply removed from their 150 mm dish, blotted, and then immersed in the incubation medium for 10, 20, 30, or 40 seconds. These time points were taken two at a time in seven series to randomize any systematic differences in ambient temperature, pH, or cell number per coverslip. Fourteen coverslips from each of two dishes were used

at each time point; thus 112 coverslips were used in the determination of each rate.

After the incubation, coverslips were washed in Tris buffer, blotting between each wash. Aliquots of the incubation medium taken before and after the experiment were not different with respect to radioactivity.

The use of medium in which the cells were growing necessitated the determination of the amino acid concentrations of the medium, since this concentration could change as a function of cell growth in the medium. Six mls of medium were removed from each dish after the experiment and allowed to equilibrate with room air at least three hours or overnight at 0-4°C. The pH of the equilibrated medium was then measured. The sample was ultrafiltered as described above and the total quantity of a given amino acid was determined by amino acid analysis on a Beckman amino acid analyzer, Model 120C. Using the calibration curve constructed above, the fraction of amino acid bound to protein was estimated at the pH of the equilibrated medium, and the total amount of amino acid present in the original medium was determined from the quantity in the filtrate (unbound amino acid). This total amount of amino acid in the medium was then used to determine the specific activity of the amino acid whose uptake was studied under the assumption that radioactive amino acid added to the medium would equilibrate rapidly

with the bound unlabeled amino acid. The effective free amino acid concentration at the pH of the medium in which the cells grow in the incubator could then be determined directly from the total concentration of amino acid in the medium if the pH of the medium in the incubator were known, or alternatively the pH of the medium in the incubator could be determined from the pH of the equilibrated medium.

5. <u>Refinements of the Uptake Assay</u>

Although the uptake assay as described in the last section yielded reliable data fairly consistently, the execution of the method was quite tedious due to the use of 112 coverslips to determine a single uptake velocity. Means were therefore sought to decrease the variation in radioactivity per coverslip and reduce the nonspecific radioactivity per coverslip (as approximated by the intercept on the uptake assay of a line drawn through the data). One refinement consisted of decreasing the variation in cell number per coverslip as described above.

All subsequent experiments were performed in a larger controlled environment box (Labconco Corp., Kansas City, Mo.) the volume of which was five to six times that of the small box in Part 4. The sides and back of the box were made of an opaque synthetic material; a one foot high three foot long viewing panel was located in the front of the box. In the course of the first trial with this box, temperature

fluctuations as a function of position in the box were noted. These fluctuations were corrected by increasing the forced circulation of air in the box with a shaded pole blower (Dayton Electric Manufacturing Co., Chicago, Ill.).

Prior to each experiment the box was gassed with 10% CO_2 in air at 0.6 liters per minute for two hours. The atmosphere thus created produced a pH of 7.8 in DMEM with calf serum, and this pH was stable for at least two days. Transfer of material from inside to outside or vice versa was accomplished through an air lock which was gassed with 10% CO_2 in air at 1.5 liters per minute for 15 minutes. The flow of 10% CO_2 in air was always through an inlet near the floor of the box and the outlet was near the roof of the box since CO_2 is heavier than air. Humidification was accomplished using a tray of water with 0.1% benzalkonium chloride. Humidification was judged adequate if condensation could be seen on the front panel of the box. Since the box was designed for continuous operation, this condensation was minimized during the times when uptake assays were not in progress by laying a sheet of heavy paper over the front panel. This sheet of paper apparently creates a layer of warm air between it and the viewing panel. In addition, the paper prevents the entry of light onto cells growing inside the box, thus avoiding the production of toxic photoproducts of riboflavin, tryptophan, and tyrosine (192,199) in the

growth medium. Future versions of this box should be equipped with a viewing panel with a heating element to prevent fogging.

Handling of coverslips was greatly facilitated by the use of triceps (Universal Technical Products Incorp., Forest Hills, N.Y.) instead of forceps. Used properly the triceps allowed rapid pickup of coverslips without scratching cells from the coverslip, and the problems of losing coverslips into the incubation medium or washes and breakage of cover-slips associated with the use of forceps were avoided.

At least some part of the high nonspecific binding of labeled substrate associated with the basic uptake assay was hypothesized to reflect the washing procedure. Thus different washing procedures using Tris buffer and silicon oil (SF-96, 107 centistokes, sp.gr. 0.968, General Electric, Waterford, N.Y.) in different combinations were evaluated. Tritiated mannitol was used as a marker for extracellular water remaining on coverslips after these washing procedures, on the assumption that a decrease in extracellular water per coverslip would lead to a decrease in nonspecific binding.

6. Uptake Assay Modified to Determine Kinetics of Amino Acid Transport

Preliminary experiments on the kinetics of amino acid transport using DMEM with amino acids as the uptake incubation medium gave highly anomalous results. The uptake of

labeled substrate did not seem to be suppressed by increasing the concentration of unlabeled substrate in the uptake incubation medium.

Therefore, DMEM without amino acids (GIBCO) was finally chosen for the experiments to determine the kinetic properties of amino acid transport. An appropriate quantity of labeled substrate (1.5 microcuries per ml of [^{35}S]- amino acid or 5 microcuries per ml of tritiated amino acid) was added to produce the basic uptake incubation medium. The concentration of unlabeled substrate was adjusted in this medium to vary between 2.88 and 0.04 millimolar by a serial dilution keeping the concentration of labeled substrate constant. DMEM without amino acids or vitamins (DMEM buffer) was chosen as the wash solution to maintain nearly physiologic conditions but avoid the problems of exchange transport described above. Buffers without bicarbonate such as Hank's or Earle's Balanced Salt Solutions were found to be unsatisfactory as wash solutions because in a 10% CO_2 atmosphere the pH of these buffers is between 5.0 and 5.5. Coverslips were washed three times in DMEM buffer with less than five seconds elapsing between the end of the uptake incubation and the end of the last wash.

Tritiated leucine was analyzed for the presence of tritiated water and other impurities. Presence of tritiated water was determined by radioactivity remaining after evaporation and other impurities were detected by their presence as

minor spots on chromatography. Two batches of tritiated leucine, both from New England Nuclear, were tested, one which was purchased two years and the other which was purchased two months before the time of testing for purity. The effect of methionine sulfoxide, the main contaminant of [^{35}S]-methionine, on methionine uptake was tested by adding unlabeled methionine sulfoxide in varying concentrations to incubation medium containing [^{35}S]-methionine and 200 micromolar methionine.

The uptake of methionine was studied to determine the limits of linearity of transport and the maximum amount of time that the cells could be washed without causing significant efflux. Since transport of methionine and the other amino acids investigated appeared to be linear for at least one minute, and three washes appeared to reduce nonspecific binding to a very low percentage of total uptake without causing significant efflux, kinetic parameters were determined from uptake data on coverslips incubated at different concentrations of substrate for fifty seconds.

C. CALCULATIONS

The format for the calculations leading to an initial rate in the form of picomoles per microliter of cell water per minute have been described above. The data needed for these calculations and the source of the data are as follows:

1) CPM per coverslip- Obtained directly from the

scintillation counter.

2) DPM per coverslip- Channels ratios were obtained directly from the scintillation counter. These ratios were converted to counting efficiencies using a quench curve. Slope and intercept for the quench curve were obtained by linear regression analysis of the relevant portion of the curve. A short program was written on an Olivetti Programmable calculator which accepted the CPM and ratio as input and gave DPM as output.

3) DPM per coverslip per minute- DPM per coverslip from different time points were plotted versus time and a line was drawn by eye. The data were also analyzed by linear regression. Results from the two methods did not differ by more than 5%. In the latter experiments on kinetic parameters of uptake, several coverslips were incubated for 50 seconds. In that case mean DPM per coverslip and standard deviations were calculated using another program, and DPM per coverslip per minute were obtained by multiplying by 6/5.

4) Picomoles per microliter incubation medium- measured by amino acid analysis and corrected for binding in the minimal perturbation studies and varied systematically in the kinetic studies.

5) DPM per microliter incubation medium- derived from CPM per microliter incubation medium in the same way as for coverslips.

6) Cells per coverslip- derived from cells per ml from cell counter by multiplying by the volume in which cells were suspended.

7) Cell water per cell- derived from a standard curve which has been published elsewhere (128).

In the experiments on the kinetics of uptake, rates were obtained at different substrate concentrations. The rates were plotted versus rate/substrate concentration in an Eadie-Hofstee plot. Slope ($-K_M$) and intercept (Vmax) were computed by linear regression analysis. This program also gave Σx^2, Σx, and Syx which were used to calculate standard deviations of the slope:

$$\text{S.D.} = k \ (\text{Syx}) \quad \text{and} \quad k = \sqrt{\frac{n/n-2}{\Sigma x^2 - ((\Sigma x)^2/n)}} \quad (188).$$

RESULTS

A. CELL CULTURE ON COVERSLIPS

The contribution of variation in cell number per coverslip to overall variation in uptake results was not originally appreciated. For the minimal perturbation studies, cells from three coverslips were trypsinized together and the suspension counted. Thus, standard deviations of these cell counts, which ranged from 5% to 10% of the mean of four to eight sets of three coverslips, were not truly representative of the larger variation of cell number per coverslip in a population of individually counted coverslips.

Although the variation in cell number per coverslip and other sources of variation contributed to the large variance of the minimal perturbation method as described, the use of large numbers of coverslips made possible the collection of reliable data. For the determination of the kinetic parameters of uptake, however, a less laborious method was needed. An attempt was made, therefore, to devise a method of cell plating as described above that would reduce the variation in cell number.

68

Since aliquots of cell suspension contain numbers of cells whose variance is due to errors in aliquot volume and uniformity of suspension, the number of cells on cover-slips plated as individual cultures using such aliquots should reflect the same degree of variance. These aliquots were held in place on the individual coverslips by surface tension. A volume of 150 microliters was just sufficient to cover the surface of the coverslip, where-as a volume of 500 microliters could be maintained only tenuously by surface tension on the coverslip. In practice, a volume of 250 or 300 microliters was chosen as a convenient aliquot size. The 250 microliter automatic pipet used was shown to deliver 250 ± 2 microliters, and when the sterile tube connector was attached, pipet delivery was decreased to 234 ± 2 microliters (Fig. 1). In the experiments below in which cells were plated with automatic pipets, sterile tube connectors were used. This method of cell plating has been subsequently carried out without the sterile tube connector without contamination (134).

Forty-four coverslips were plated by this technique and the cells on these coverslips were trypsinized two days later. Coverslips were incubated individually. The distribution of cell counts is shown in Fig. 2. The mean cell count was $157.8 \times 10^3 \pm 13.9 \times 10^3$ cells per coverslip (8.6%). The median cell count was 157.4×10^3 and the modal

cell count was 157.5×10^3 cells per coverslip.

The variations in volume per aliquot from the automatic pipet and in cell number per coverslip may both be overestimated. Some of the variation in volume per aliquot may be due to error in weighing. Some of the variation in cell number per coverslip is certainly due to less than complete removal of cells from the coverslip, incomplete dispersion of the cells into a monocellular suspension, errors of dilution, and errors in the cell counter itself. In many smaller series of coverslips plated by this technique, variation in cell number at different densities was always between 1% and 5% of the mean.

B. TRANSPORT ENVIRONMENT

1. pH during Cell Growth

The importance of pH in cell growth and transport has been discussed above (Criteria for Evaluating Methods of Transport Measurement). The change in medium pH as the cells grow is indicated with methyl red. Indeed the first impetus to the development of a transport assay in a controlled environment was the observation that the growth medium on cells in room air at 37°C became deep red to purple within fifteen to twenty minutes. This change is especially marked in dense populations of SV-3T3 cells, whose

Fig. 1.   Accuracy and precision of automatic pipet delivery.

A 250 microliter automatic pipet was used to de-

liver sixteen successive aliquots into a tared

aluminum foil cup on a Mettler balance.   Read-

ings in milligrams were taken after each aliquot.

The distribution of the weights obtained from the

same pipet with (unshaded) and without (shaded)

a sterile tube connector (Methods) is plotted as
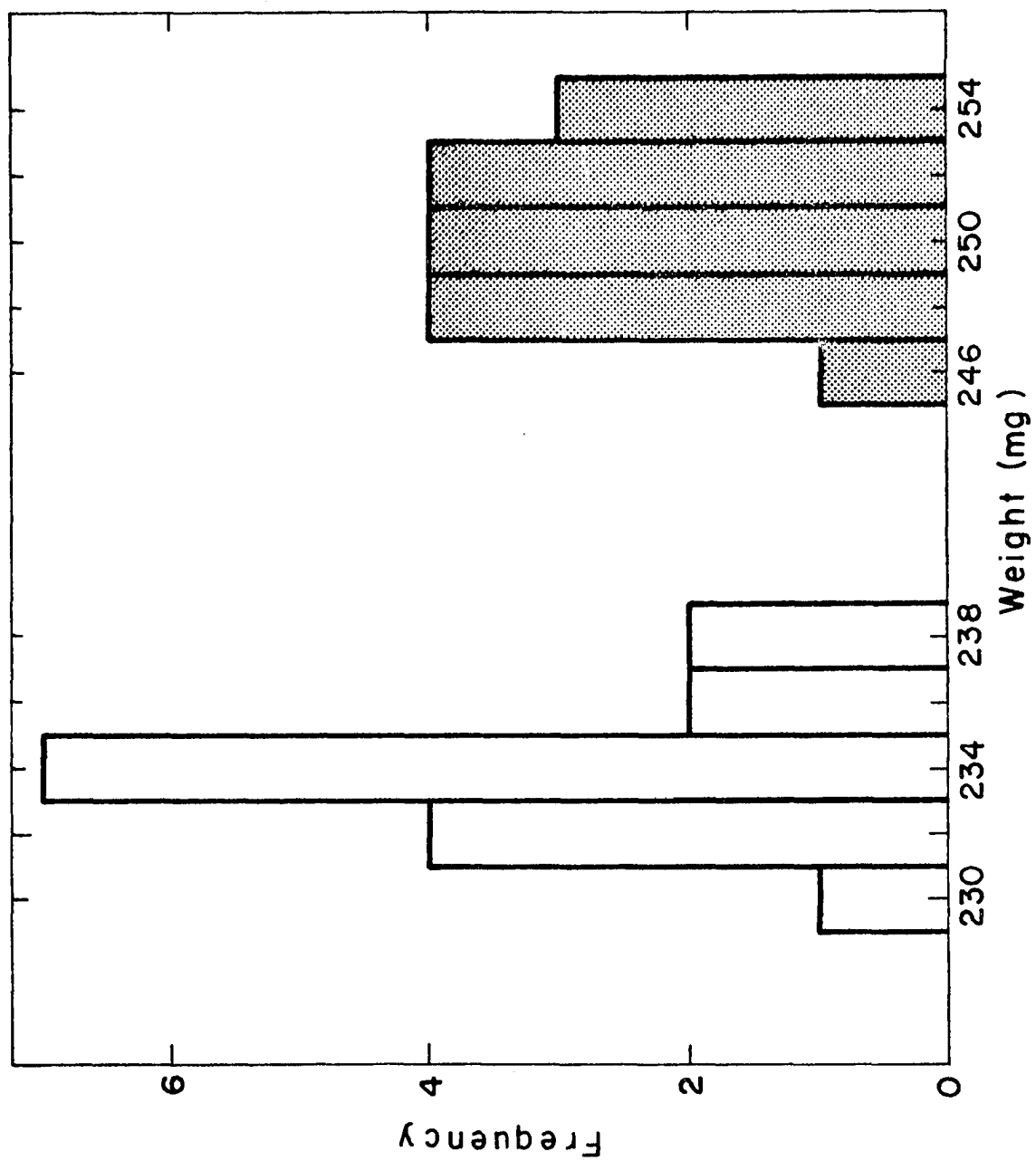
a frequency histogram.

Fig. 2.   Precision of automatic pipet delivery of cell sus-

pensions. A 300 microliter automatic pipet with a

sterile tube connector was used to aliquot a cell

suspension onto forty-four coverslips. Cells on

coverslips were allowed to grow for two days, and

then the coverslips were incubated individually

in 0.1% trypsin and each cell suspension was

counted (Methods). The distribution of the

cell counts obtained is plotted as a frequency

histogram.

Cells/coverslip ($\times 10^{-3}$)

Frequency
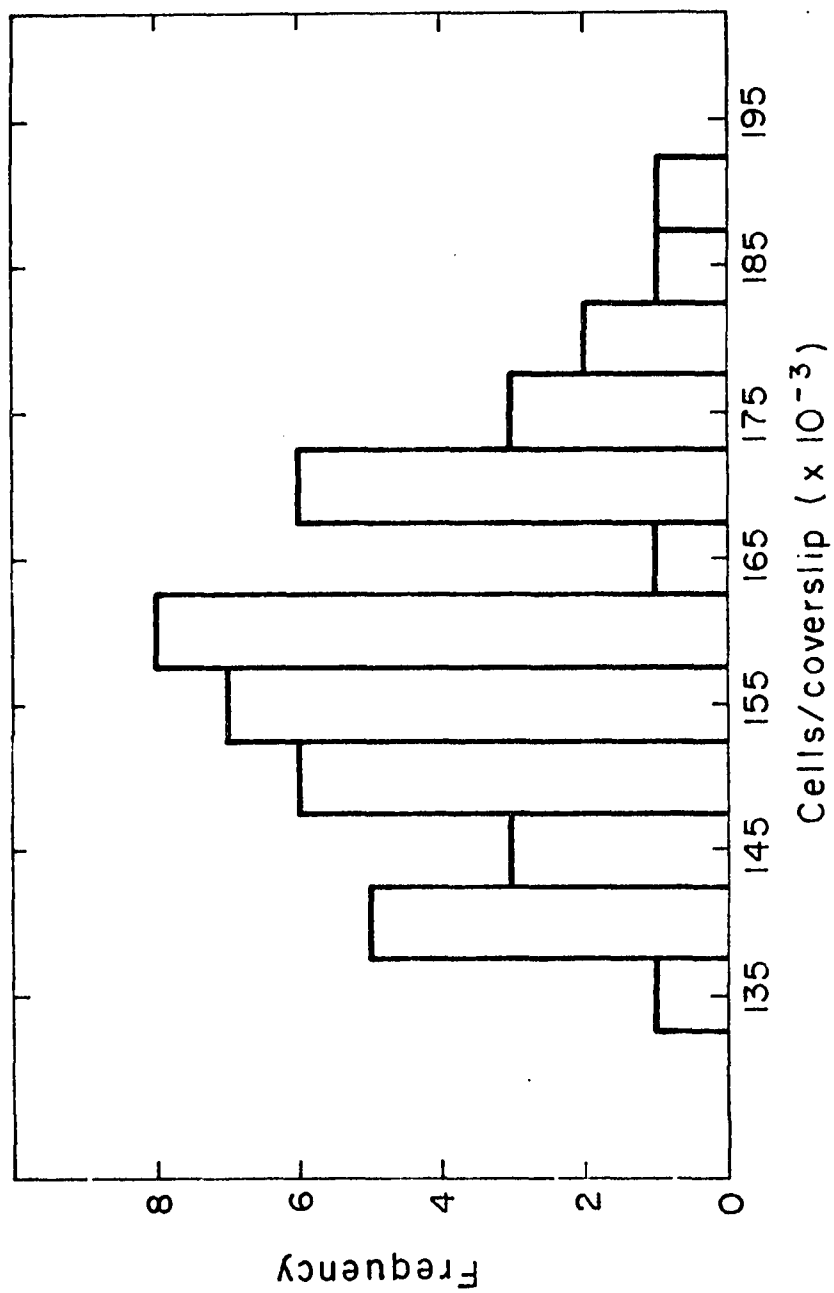
growth medium is almost completely yellow in the incubator, and then progresses through orange to red as the culture is left in the room air.

Since the minimal perturbation studies were designed to measure uptake in the cells' own environment, the pH was constantly changing through the duration of a series of uptake assays at different cell densities. Amino acid concentration might also be changing, and if amino acids were bound to serum proteins, the binding might change as a function of pH. For all of these reasons, a systematic measurement of the pH of growth medium on cell cultures of 3T3 and SV-3T3 was carried out.

The results of these measurements are shown in Fig. 3. The range of pH encountered was 7.6 to 8.0 for 3T3 and 7.0 to 8.0 for SV-3T3. That these pH values are reproducible is seen from the results of the two separate experiments in Fig. 3. Lower pH values are probably encountered for higher densities of 3T3 and SV-3T3, but these cultures were not routinely studied due to the large number of floating and necrotic cells.

2. Amino Acid Binding to Serum Proteins

The total quantity of a particular amino acid in the growth medium had to be known in the minimal perturbation studies in order to convert the rate of uptake from DPM to

Fig. 3. pH during cell growth. The pH of medium from duplicate sets of cultures of 3T3 (A) and SV-3T3 (B) was measured each day for four days at different cell densities. Each point is the mean of triplicate determinations on the same aliquot of medium. Open and closed circles represent two separate experiments.
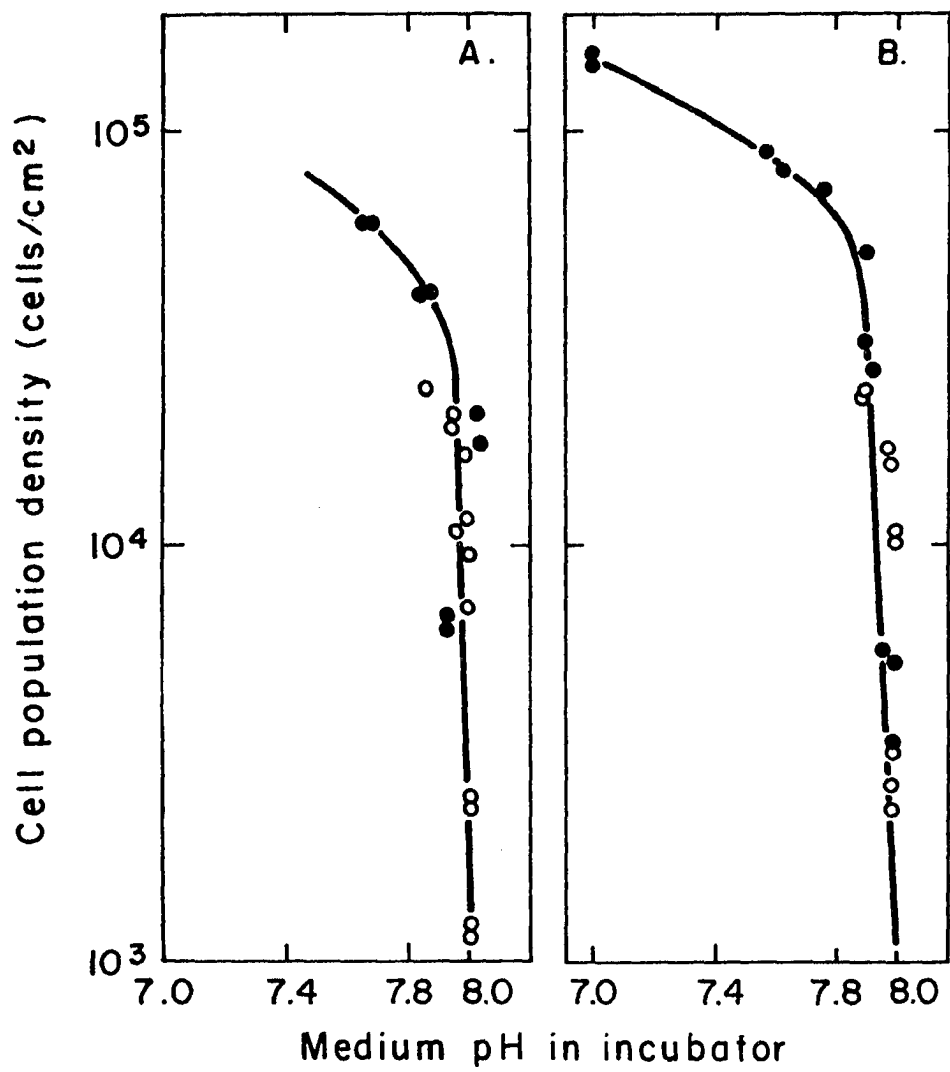
picomoles substrate. Since binding of some amino acids to serum protein was expected, the manner in which to extract the total amount of free amino acids from the medium was not clear. Acid precipitation would certainly disturb the relationship of bound to unbound amino acid, and recovery of amino acid after three acid extractions was different for arginine, histidine, and isoleucine (172).

Ultrafiltration was chosen for the determination of amino acid binding to serum proteins and for the processing for amino acid analysis of samples of growth medium used in uptake assays. In this way, the pH of the medium would not be changed, and only the concentration of macromolecules in the medium would be increased. Even this change involved only an 8% change in concentrations, since only 450 microliters out of 6 mls were filtered.

Eight of the ten essential amino acids were studied for binding to serum proteins as a function of pH (Fig. 4). All of the binding studies were performed at least twice with excellent agreement. The binding of several of these amino acids undergoes a significant change in the range of pH values encountered during cell growth. The binding of arginine and tryptophan decreases fivefold from 25% to 5% from pH 8.0 to 7.0. Histidine binding increases three fold from 5% to 15% in the same pH range. The binding of arginine, lysine, and tryptophan increase with

Fig. 4. Amino acid binding to serum proteins. Aliquots of growth medium were titrated with 0.15 NaOH or HCl to the pH range 6.4 to 8.8. These aliquots, to which the indicated labeled amino acid had been added, were filtered in an ultrafiltration device under nitrogen, and the filtrate was counted for radioactivity. Results were corrected for filter retention and were divided by the amount of radioactivity in the medium prior to filtration to determine binding to serum components.
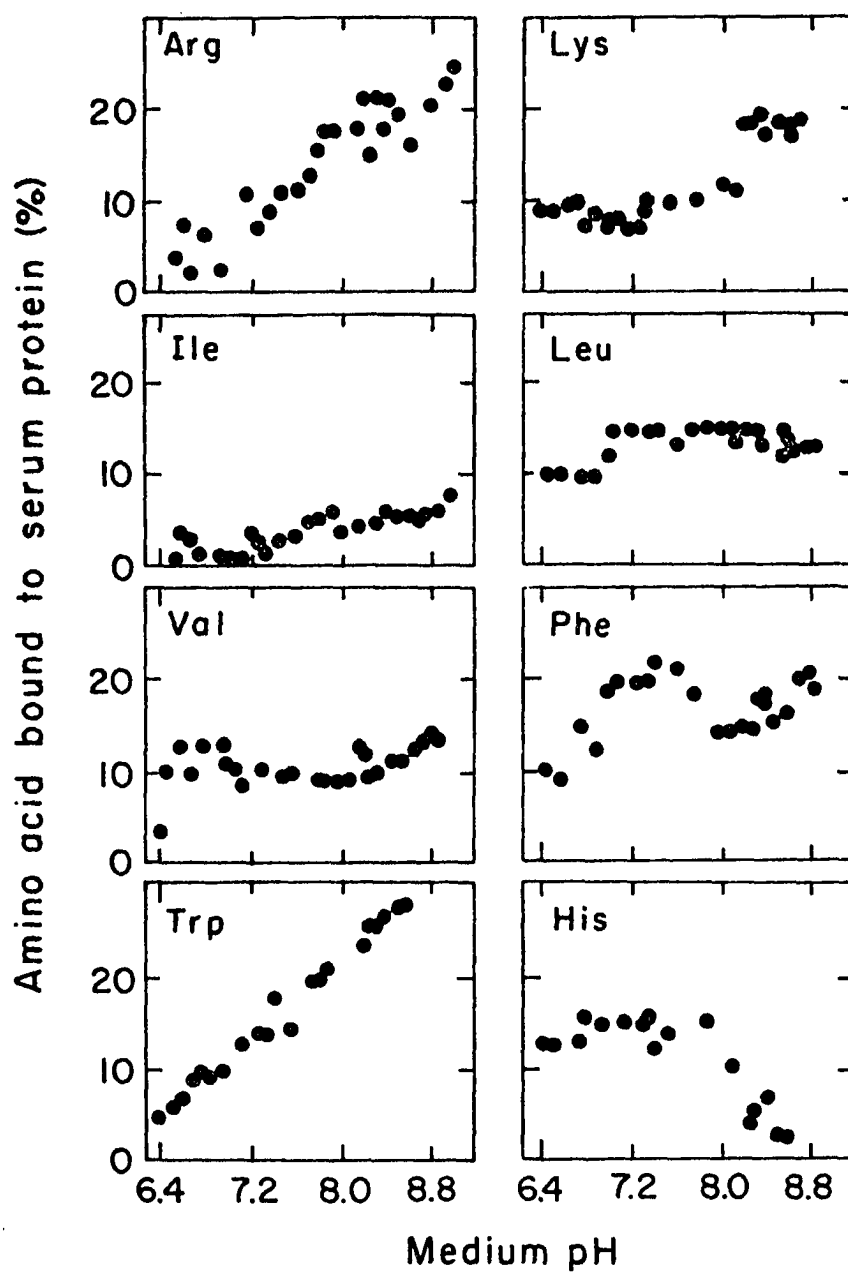
increasing pH, isoleucine and valine show small increases, and leucine, phenylalanine and histidine show a complex relationship with pH.

The specificity of this binding has not been studied exhaustively, but in experiments in which the total concentration of unlabeled leucine was increased tenfold, the subsequent binding of labeled leucine and phenylalanine were both reduced almost to zero. Tenfold increased unlabeled valine similarly reduced the binding of labeled valine and phenylalanine. Other combinations have not been tested, so binding might be specific for other amino acids. This binding was shown to be reversible, since dialysis against Tris buffer (pH 7.4) for 24 hours removed all bound radioactive amino acids from the medium components retained in the dialysis bag.

3. Relationship of Medium pH Inside Incubator to pH After Equilibration with Room Air

The pH of medium after equilibration with room air as a function of pH in the incubator is shown in Fig. 5. The relation appears to be linear in the pH range studied. The pH of fresh medium in the incubator was 8.0 $\pm$ 0.1 and rose to 8.7 $\pm$ 0.1 after equilibration. The small variation is probably due to opening and closing of the incubator throughout the day.

Fig. 5. Relationship of medium pH inside incubator to pH
after equilibration. The pH of medium was
measured from aliquots of growth medium from cell
cultures at different densities before and after
equilibration with room air for three hours. The
points were fitted to a line by linear regression
analysis.

Medium pH in incubator (vertical axis, 8.0, 7.5, 7.0)

Medium pH after equilibration with air (horizontal axis, 9.0, 8.5, 8.0, 7.5)
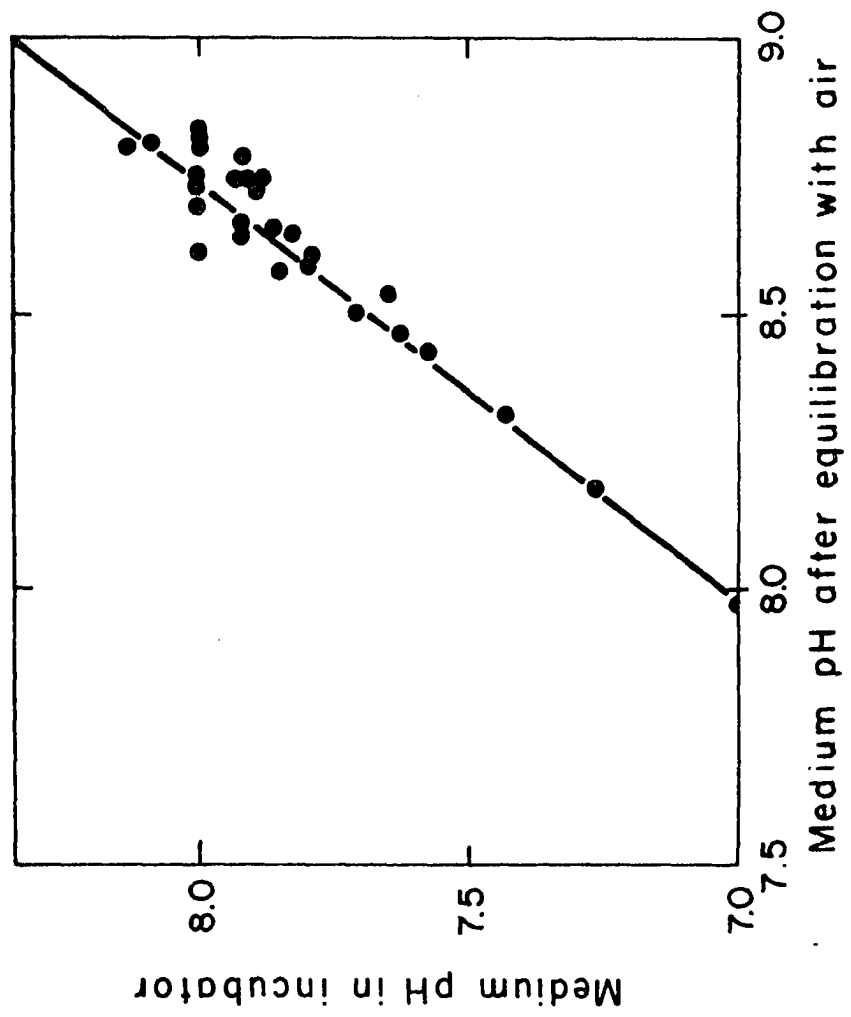
4. Minimal Perturbation Studies

Two kinds of questions can be asked about transport: what is the rate of transport of a substance into a cell, and what are the characteristics of the transport of a substance into a cell. These questions are quite different in their intentions. For instance, one might like to know if transformation of a cell is effected or affected by changes in transport. If it were possible to show definite changes in the rate of transport of some substrate after transformation, tentative confirmation would be available for this hypothesis, but information concerning the mechanism of this change would be wholly lacking. Conversely, information about the kinetic parameters K_M and Vmax for a transport system might suggest the potential for transport differences under certain specifiable but physiologically unattainable conditions, yielding a mechanism but no proof of the existence of a change in transport.

The answer to the question concerning the rate of transport under steady state conditions requires a method of assay that guarantees minimal perturbation of the cells. In Figs. 6 through 9 are shown the results of four series of experiments. The transport of leucine and phenylalanine was assayed in 3T3 and SV-3T3 cells using the minimal perturbation method. Thus, the uptake incubation medium consisted of the medium in which the cells were growing to

Fig. 6.   Steady state measurements of phenylalanine trans-

port by SV-3T3 at different cell densities.   In

figures six through nine the transport of

phenylalanine and leucine was studied by the

first method (Methods).   Each point represents

the mean and standard deviation of determinations

from 26 to 28 coverslips.   Day one, cell density

0.44×10^4 cells/cm^2 (●——●); day two, cell den-

sity 1.04×10^4 cells/cm^2 (▲——▲); day three, cell

density 3.77×10^4 cells/cm^2 (○——○); day four,

cell density 5.87×10^4 cells/cm^2 (◇——◇)

Fig. 7.   Steady state measurements of leucine transport

by SV-3T3 at different cell densities.   Day one,

cell density 0.81×10^4 cells/cm^2 (●——●); day

two, cell density 1.15×10^4 cells/cm^2 (▲——▲);

day three, cell density 2.12×10^4 cells/cm^2

(○——○); day four, cell density 4.23×10^4

cells/cm^2 (◇——◇); day five, cell density 4.99×10^4

cells/cm^2 (□——□); day six, cell density 7.29×10^4

cells/cm^2 (■——■)

Fig. 8.   Steady state measurements of phenylalanine trans-

port by 3T3 at different cell densities.   Day one,

cell density 0.22×10^4 cells/cm^2 (●——●); day two,

cell density 0.47×10^4 cells/cm^2 (▲——▲); day

three, cell density 0.73×10^4 cells/cm^2 (O——O);

day four, cell density 0.56×10^4 cells/cm^2 (◇——◇);

day five, cell density 1.39×10^4 cells/cm^2 (□——□)
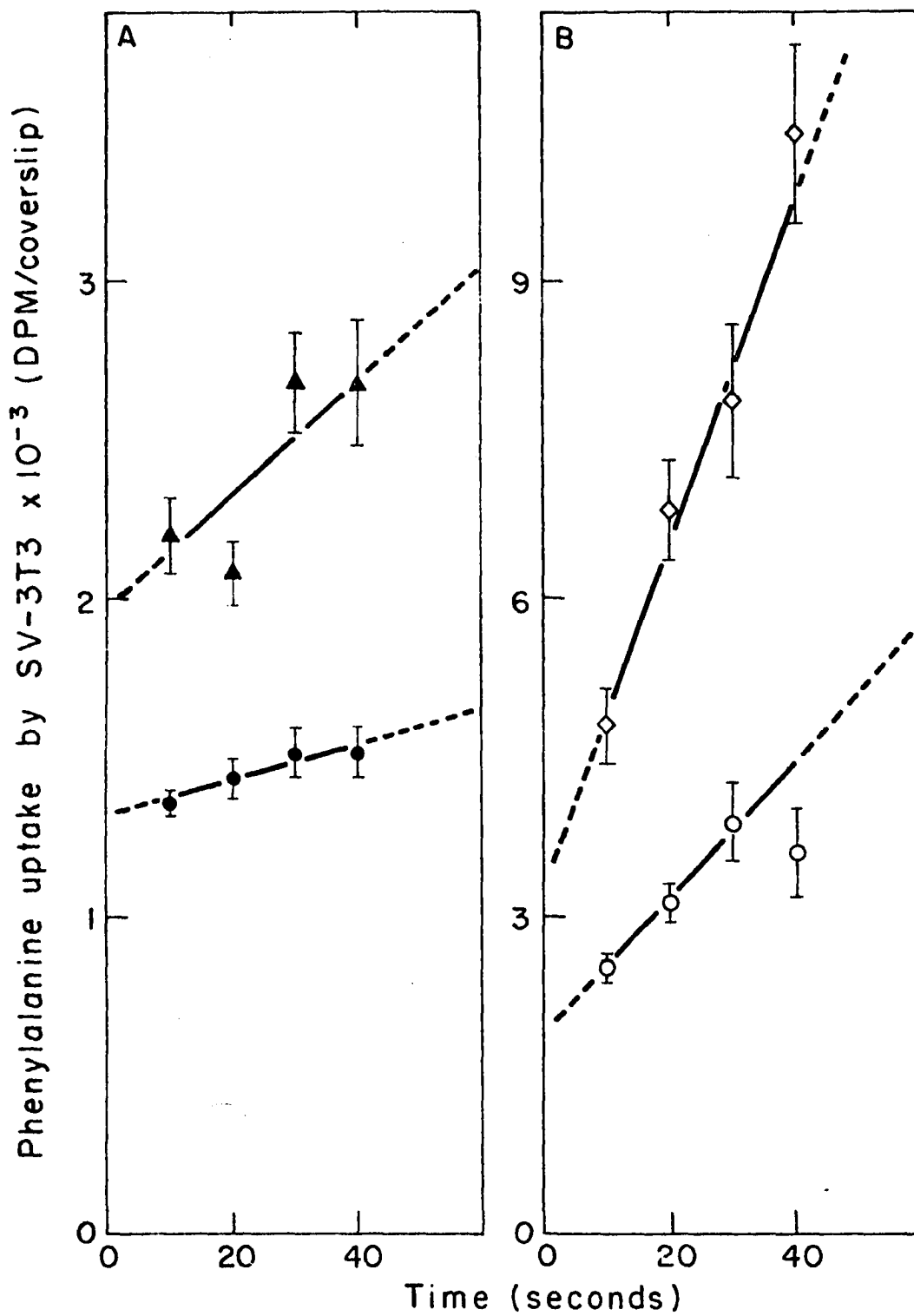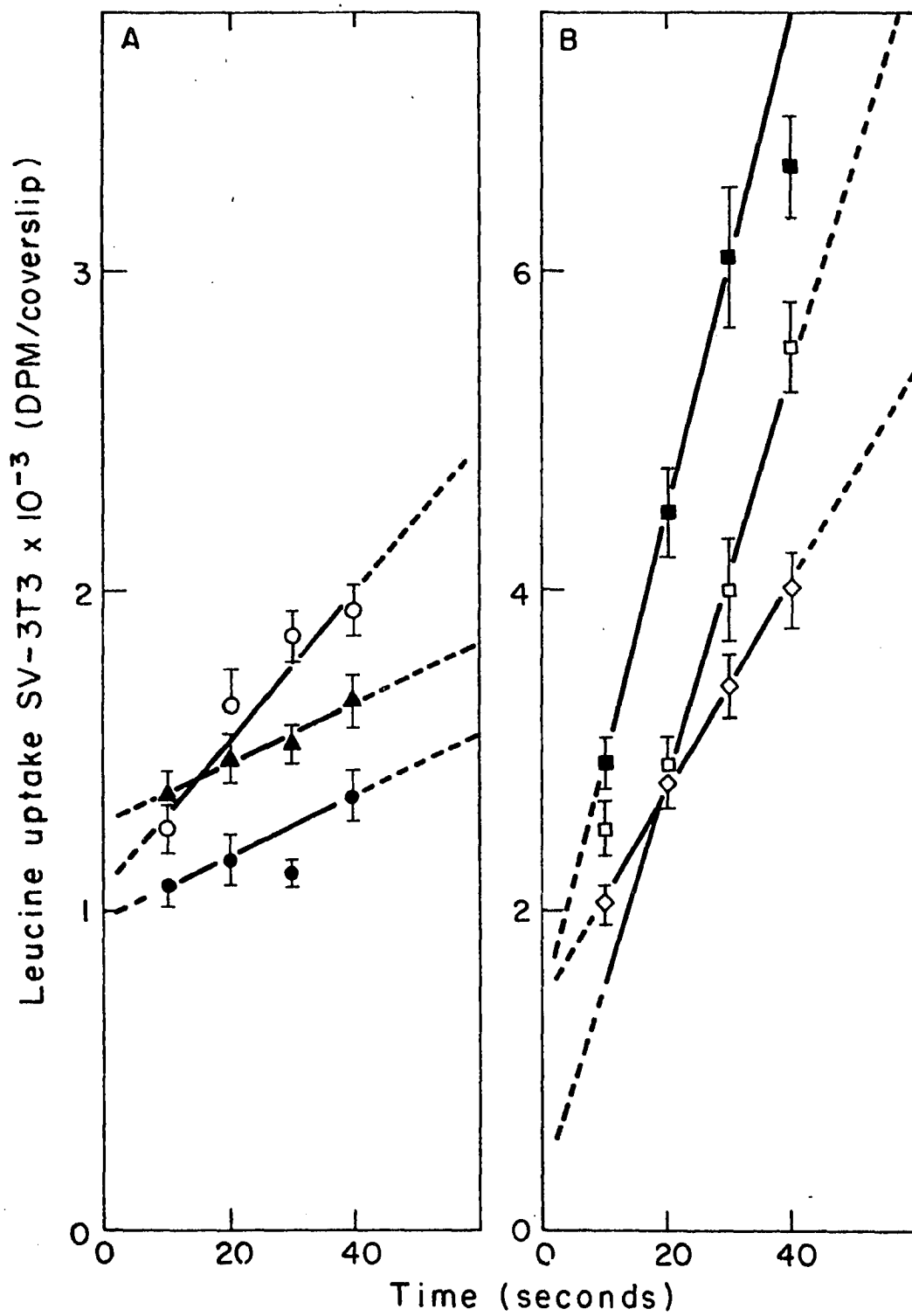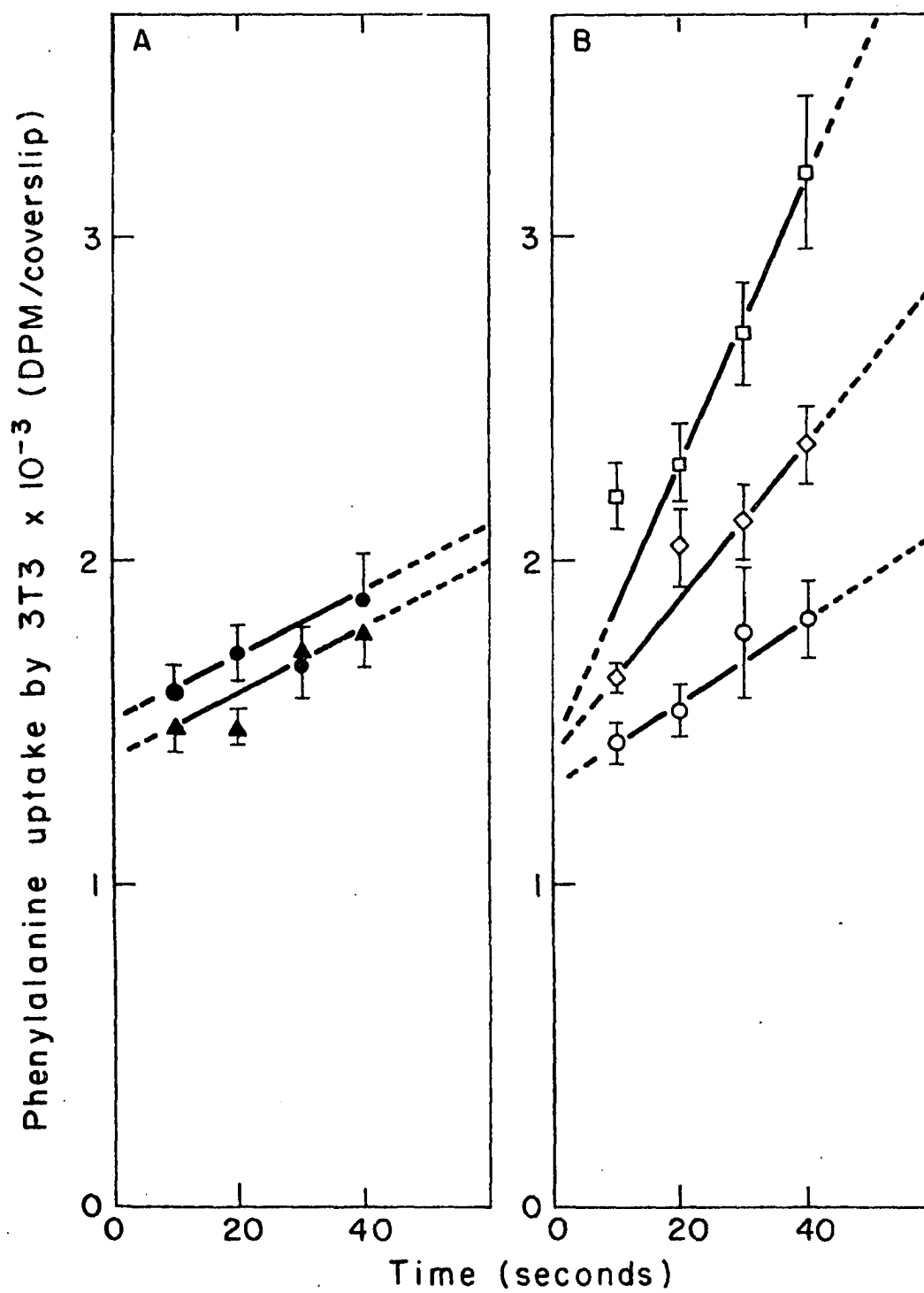
Fig. 9. Steady state measurement of leucine transport by 3T3 at different cell densities. Day one, cell density 0.28×10^4 cells/cm^2 (●——●); day two, cell density 0.67×10^4 cells/cm^2 (▲——▲); day three, cell density 0.62×10^4 cells/cm^2 (○——○); day four, cell density 1.11×10^4 cells/cm^2 (◇——◇); day five, cell density 2.18×10^4 cells/cm^2 (□——□); day six, cell density 4.03×10^4 cells/cm^2 (■——■)
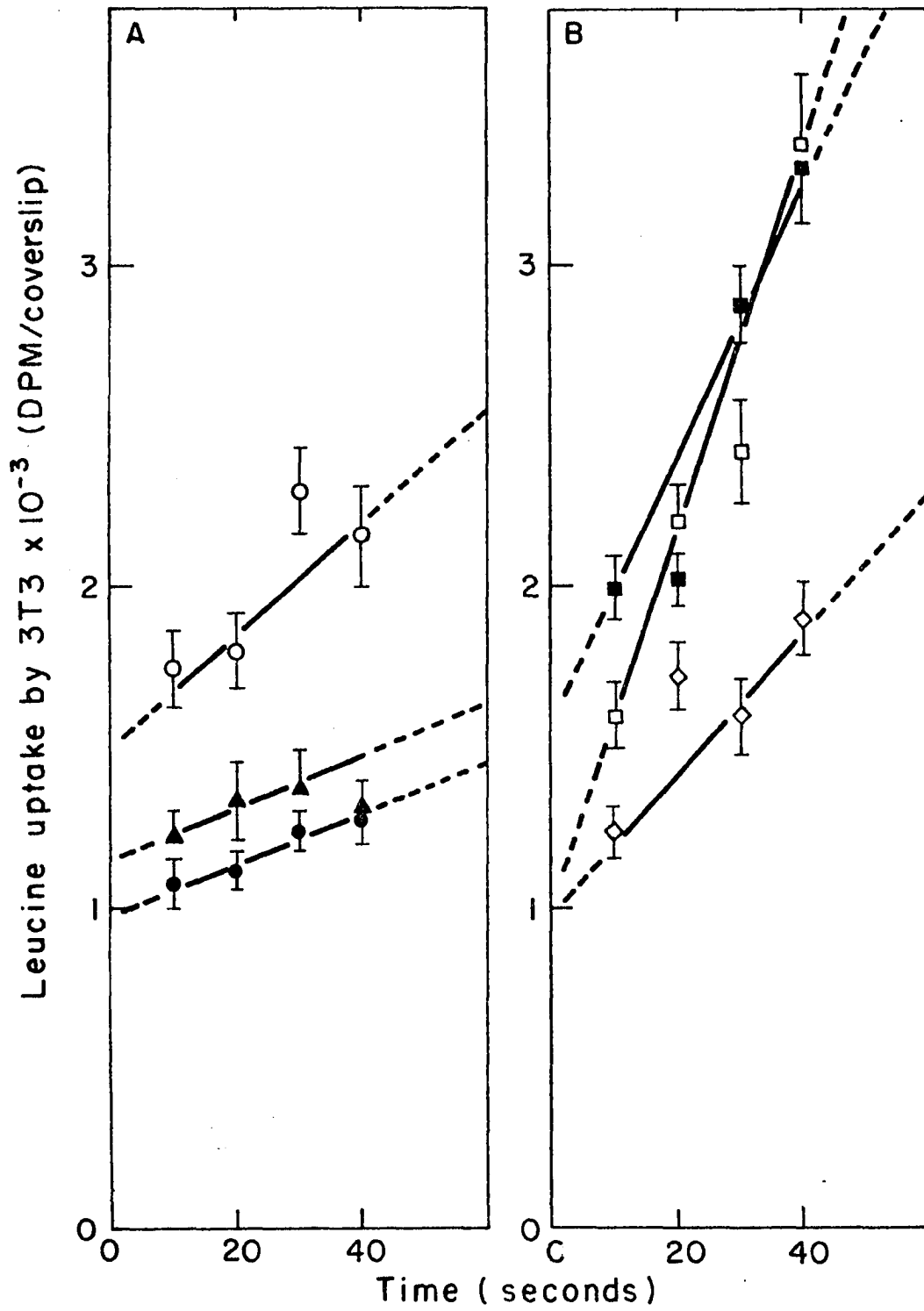
which enough labeled amino acid was added so that DPM per coverslip were always greater than 1000. Using enough labeled amino acid was especially important for measurements on sparse cultures such as day one in Fig. 6. There the differences between successive points are not significant, but the line as a whole can be seen to fit the points quite well.

For almost all of the lines drawn in Figs. 6 through 9, the dotted line which is extrapolated towards the y-axis intersects the axis between 1000 and 2000 DPM per coverslip. This intercept reflects the nonspecifically bound label if and only if the extrapolation is correct, that is if the lines truly represent initial rates. Non-specifically bound label can be considered noise, and may contribute to the overall variance if the noise is variable. With some exceptions the noise appears to increase with increasing cell number per coverslip. Since in these experiments labeled substrates were present at a concentration of 10,000 to 20,000 DPM per microliter, if the non-specifically bound label were present mostly in trapped extracellular water, then the trapped extracellular water would amount to between 0.1 and 0.2 microliters.

When the variation in DPM/coverslip for any given time point is considered with respect to the total DPM per coverslip, the standard deviation is usually less than 5%

of the mean. This appearance of small variance actually obscures the problem of a much higher percentage of standard deviation over specific uptake. For instance, in Fig. 6, day one, the uptake at 20 seconds is 1430 ± 60 DPM per coverslip, which gives a standard deviation of 4% of the mean. If specific uptake is total uptake minus the extrapolated intercept, in this case 1330 DPM per coverslip, then specific uptake is 100 DPM per coverslip, and the standard deviation of 60 is 60% of the mean. The rates finally calculated from Fig. 6 through 9 should be taken then as indications of the magnitude of actual rates and differences between rates, and not as absolute rates.

The rates as calculated in picomoles per microliter cell water per minute are shown in Table IX. Phenylalanine transport by SV-3T3 cells appears to increase with increasing cell density. 3T3 cells show a similar pattern if the difference between the transport at 0.22 and 0.47 x 10^4 cells per cm^2 is disregarded; the measurement of transport by this method at such low densities is difficult for reasons mentioned above. SV-3T3 cells transport leucine at a threefold increased rate from low to high densities, although the decrease in transport between 2.12 and 4.23 x 10^4 cells per cm^2 may be significant, whereas 3T3 cells transport leucine at about the same rate at all densities, with a questionably significant increase in transport at intermediate

TABLE IX

SUMMARY OF THE STEADY STATE MEASUREMENTS OF SUBSTRATE UPTAKE AT DIFFERENT
CELL DENSITIES

Cell type: substrate	Cell density $\times 10^{-4}$	$\dfrac{DPM}{(cs)(min)}$	$\dfrac{DPM}{\mu l\ IM}$	$\dfrac{\mu l\ cell\ H_2O}{10^6\ cells}$	$\dfrac{picomoles}{(\mu l\ cell\ H_2O)(min)}$
SV-3T3: phenylalanine	0.44	305	12400	6.80	184
	1.04	800	13300	5.11	256
	3.77	3800	13700	2.67	624
	5.87	10400	14300	3.89	720
SV-3T3: leucine	0.81	550	15300	6.80	294
	1.15	630	13200	4.39	428
	2.12	1380	14400	2.19	928
	4.23	3853	20500	3.03	662
	4.99	7060	15300	3.57	1173
	7.29	9474	14700	4.00	1002
3T3: phenylalanine	0.22	608	18400	7.43	444
	0.47	688	20000	7.43	222
	0.73	760	12500	7.43	253
	0.56	1416	14700	7.43	526
	1.39	2640	13700	2.88	1082
3T3: leucine	0.28	407	23500	7.43	371
	0.67	495	15200	7.43	293
	0.62	998	25600	7.43	382
	1.11	947	25400	2.71	560
	2.18	3268	20600	6.51	506
	4.03	2668	22000	4.55	297

Cell density (cells/cm^2) multiplied by surface area per coverslip (1.77 cm^2) yields cells per coverslip. DPM per coverslip (cs) per minute is derived from the data plotted in Figures 6-9. DPM per microliter incubation medium (IM) divided by the substrate concentration in the incubation medium (0.4 millimolar phenylalanine and 0.8 millimolar leucine) yields the specific activity.

cell densities. The transport of phenylalanine in 3T3 cells
was not tested at confluence because the cells plated ini-
tially at a very low density.

The rates calculated above were not corrected for
amino acid binding to serum proteins, because these trans-
port experiments and the amino acid binding experiments
were being conducted during the same period of time. The
amino acid binding data were not yet reliable enough to use
in correcting other data, but the principle behind this
correction remains valid.

5. Refinements of the Uptake Assay

a. Control of Temperature

The use of the controlled environment box as initially
constructed for the minimal perturbation studies was adequate
for small scale experiments. More material in the form of
cells on coverslips in Petri dishes was required for the
kinetic studies, so a larger controlled environment box
was obtained. Optimally cells are grown in the controlled
environment box, so that movement of the coverslips once
the cells are plated is reduced to a minimum. In initial
experiments when cells were plated in the box, growth was
unpredictable, and several times the cells detached from
the surface. The box was equipped with a thermostatic ele-
ment, the sensor of which was located toward the front of

the box. A Saybolt viscosity thermometer was used to check the temperature readings from the thermostatic sensor, and the results were found to differ. Twenty eight test tubes with one ml of water were then set up in the box, and temperatures were recorded from all locations in the box (Table X). A high power shaded pole blower was used to correct the temperature gradient seen in Table X, but this blower was not designed for continuous operation. Thus cells were grown in the incubator and had to be transferred to the box.

b. Washing Procedure

Part of the nonspecific binding that became problematic in the minimal perturbation studies was thought to be due to trapped extracellular water. The efficacy of the washing procedure was therefore studied in an attempt to minimize the amount of trapped extracellular water without exposing cells too long to solutions in which efflux of labeled substrate would occur. Various combinations of Tris buffer and silicon oil were tested; the use of silicon oil was prompted by the assumption that efflux would only occur into an aqueous solution.

From Table XI it can be seen that coverslips washed three times in buffer retained only 0.1 microliters of extracellular water as determined by mannitol space, and a

TABLE X

MAP OF TEMPERATURES VERSUS AREA ON BOX FLOOR

Left						Right
Back 41.1°	40.4°	39.2°	37.7°	36.5°	36.5°	36.5°
41.5°	40.2°	39.1°	37.3°	36.7°	36.4°	36.4°
41.0°	40.6°	39.7°	38.3°	37.4°	36.7°	36.3°
Front 38.6°	38.7°	37.6°	36.7°	35.9°	35.6°	35.7°

Day one

Back 41.6°	39.8°	38.8°	37.2°	36.4°	35.9°	35.7°
40.3°	39.4°	38.4°	36.6°	36.1°	35.6°	35.6°
39.6°	39.5°	38.9°	37.7°	36.9°	36.1°	35.6°
Front 37.6°	37.8°	37.0°	36.2°	35.9°	35.1°	35.1°

Day five

Back 38.2°	38.1°	37.9°	37.8°	37.6°	37.6°	37.5°
37.9°	37.8°	37.6°	37.5°	37.6°	37.6°	37.5°
37.9°	37.8°	37.6°	37.5°	37.5°	37.5°	37.5°
Front 37.7°	37.6°	37.5°	37.5°	37.5°	37.5°	37.4°

With extra fan

*Twenty eight test tubes with one ml of water were arranged in a grid in test tube racks on the floor of the controlled environment box. Temperatures were measured serially with a Saybolt viscosity thermometer (0.1°Cgradations). The thermometer was allowed to equilibrate one minute for each measurement. On day one the heater and fan ( ) were connected in series and the apparatus was run continuously for four hours at the same setting before temperatures were measured. The apparatus was allowed to run until day five when the measurements were repeated. A high power shaded pole blower ( ) was then installed and the original heater and fan connected in parallel, and the temperatures were measured after four hours of operation. Floor area was approximately eight square feet, and box volume approximately sixteen cubic feet.

fourth wash did not remove more of this extracellular water. Silicon oil alone was not effective; the combination with buffer was only slightly more effective than buffer alone for one wash in buffer, and not different at all for two washes in buffer. Different densities of silicon oil were not tested, and the extracellular water retained at different cell densities was not determined, since only rough comparisons of the effectiveness of the washing protocols were required. Three washes were subsequently used for all transport assays.

c. Uptake of Labeled Leucine

The effect of the new plating methods and the improved washing procedure on the results of the uptake assay was evaluated by performing several uptake assays with tritiated leucine in SV-3T3 cells. Uptake was linear for at least one minute, and the line of best fit passed through or close to the mean of each set of time points (Figure 10). The fit of the data to a line was as good or better in this method using 25 to 30 coverslips per rate determination than the fit of the data in the minimal perturbation studies using 112 coverslips. As familiarity with the new method increased, variation in the results from coverslips at the same time point decreased, and the ratio of background radioactivity to total uptake decreased. This method has been used

TABLE XI

MANNITOL SPACE AFTER DIFFERENT WASHES*

Washing condition	DPM/coverslip (means $\pm$ S.D.)	μl extracellular water/coverslip (means $\pm$ S.D.)
Washed once in buffer	2019 $\pm$ 208	0.723 $\pm$ 0.074
Washed twice in buffer	545 $\pm$ 54	0.195 $\pm$ 0.019
Washed three times in buffer	295 $\pm$ 37	0.105 $\pm$ 0.013
Washed four times in buffer	396 $\pm$ 48	0.141 $\pm$ 0.017
Washed once in silicon oil	14267 $\pm$ 1182	4.72 $\pm$ 0.39
Washed twice in silicon oil	8304 $\pm$ 761	2.75 $\pm$ 0.25
Washed once in buffer and once in silicon oil	1764 $\pm$ 144	0.583 $\pm$ 0.048
Washed twice in buffer and once in silicon oil	661 $\pm$ 103	0.218 $\pm$ 0.034

*Coverslips with attached cells were incubated for one hour at 37^0C with 2.5 microcuries per ml tritiated mannitol in their growth medium. Six coverslips were washed in each of eight different ways after this incubation. Extracellular water was calculated from the DPM/coverslip and DPM/μl incubation medium (obtained from six aliquots of 50 microliters). Coverslips contained SV-3T3 cells at a density of 8×10^4 cells/cm^2.

TABLE XI

MANNITOL SPACE AFTER DIFFERENT WASHES*

Washing condition	DPM/coverslip (means $\pm$ S.D.)	µl extracellular water/coverslip (means $\pm$ S.D.)
Washed once in buffer	2019 $\pm$ 208	0.723 $\pm$ 0.074
Washed twice in buffer	545 $\pm$ 54	0.195 $\pm$ 0.019
Washed three times in buffer	295 $\pm$ 37	0.105 $\pm$ 0.013
Washed four times in buffer	396 $\pm$ 48	0.141 $\pm$ 0.017
Washed once in silicon oil	14267 $\pm$ 1182	4.72 $\pm$ 0.39
Washed twice in silicon oil	8304 $\pm$ 761	2.75 $\pm$ 0.25
Washed once in buffer and once in silicon oil	1764 $\pm$ 144	0.583 $\pm$ 0.048
Washed twice in buffer and once in silicon oil	661 $\pm$ 103	0.218 $\pm$ 0.034

*Coverslips with attached cells were incubated for one hour at 37° C with 2.5 microcuries per ml tritiated mannitol in their growth medium. Six coverslips were washed in each of eight different ways after this incubation. Extracellular water was calculated from the DPM/coverslip and DPM/µl incubation medium (obtained from six aliquots of 50 microliters). Coverslips contained SV-3T3 cells at a density of 8×10^4 cells/cm^2.

Fig. 10. Tritium label uptake by SV-3T3. Cells growing on coverslips were incubated for the times indicated in medium without serum with tritiated leucine (old batch). Results are from two separate experiments, each conducted in the controlled environment box. Cell densities were 2.5×10^4 cells/cm^2 (A) and 5.0×10^4 cells/cm^2 (B). DPM/coverslip (●——●); DPM/coverslip, two overlapping points (○——○); mean DPM/coverslip, experiments 1A and 1B only (◇——◇)

[³H] - Label uptake by SV-3T3 (DPM/coverslip)

Time (seconds)
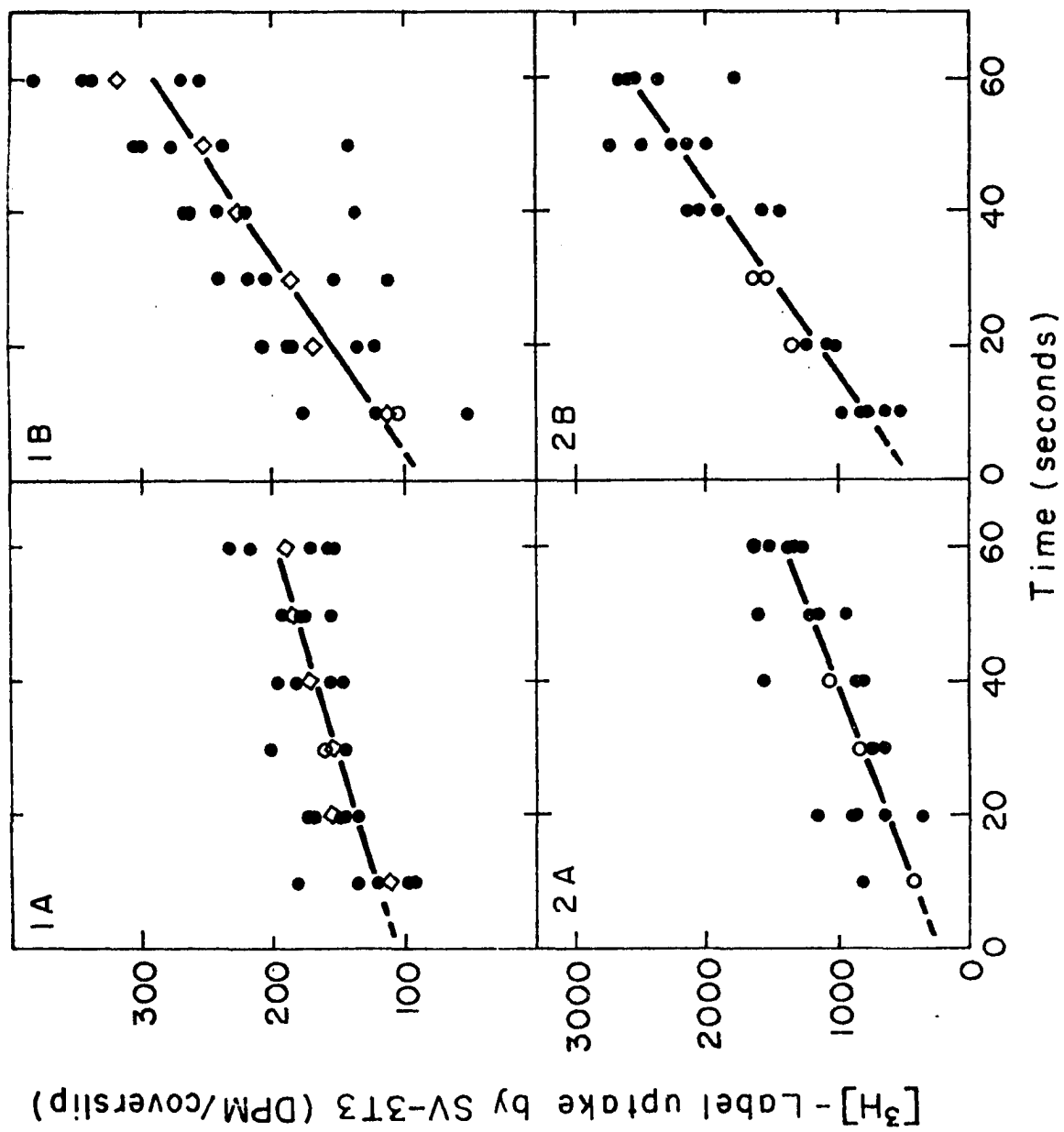
subsequently by other investigators (136) with the same ex-
cellent fit of data to a line and low nonspecific binding
(Fig. 11).

6. Uptake Assay Modified to Determine Kinetics of Amino Acid Transport

a. Kinetics of Uptake of an Old Batch of Labeled Leucine

Preliminary experiments on the kinetics of leucine
uptake were carried out to estimate the range of concen-
trations that should be used to determine the kinetic
parameters of transport (Fig. 12). Since the concentration
of labeled leucine was the same in all of the uptake incu-
bation media, the only difference among the media was the
concentration of unlabeled leucine. The expected result
of suppression of uptake by increasing concentration of
unlabeled substrate was not supported by the data, and the
data fit the lines closely enough that factors other than
errors of measurement were suspected.

These results generated four hypotheses: a) If the
concentration range of unlabeled substrate greatly exceeded
the K_M for transport, labeled substrate might be forced into
the cell by diffusion at a rate proportional to substrate
concentration. b) If the concentration range of unlabeled
substrate were greatly exceeded by the K_M for transport, then
the Michaelis-Menten equation would be reduced to v=c S
where c=Vmax/K_M. c) If other amino acids present in the

Fig. 11. Leucine uptake. These data were kindly provided

by Dr. K. Muirhead (136), who used the method

described in this study (Methods). Cells grow-

ing on coverslips were incubated for the times

indicated in medium without serum with tritiated

leucine (recent batch) in the controlled environ-

ment box. Four or five coverslips were used for

each time point determination for which the mean

and standard deviation are shown. The correlation

coefficient of all the points for each line is bet-

ter than 0.99. 3T3 cells were growing at a den-

sity of 4 x 10^4 cells/cm^2, SV-3T3 cells at a

density of 8 x 10^4 cells/cm^2.
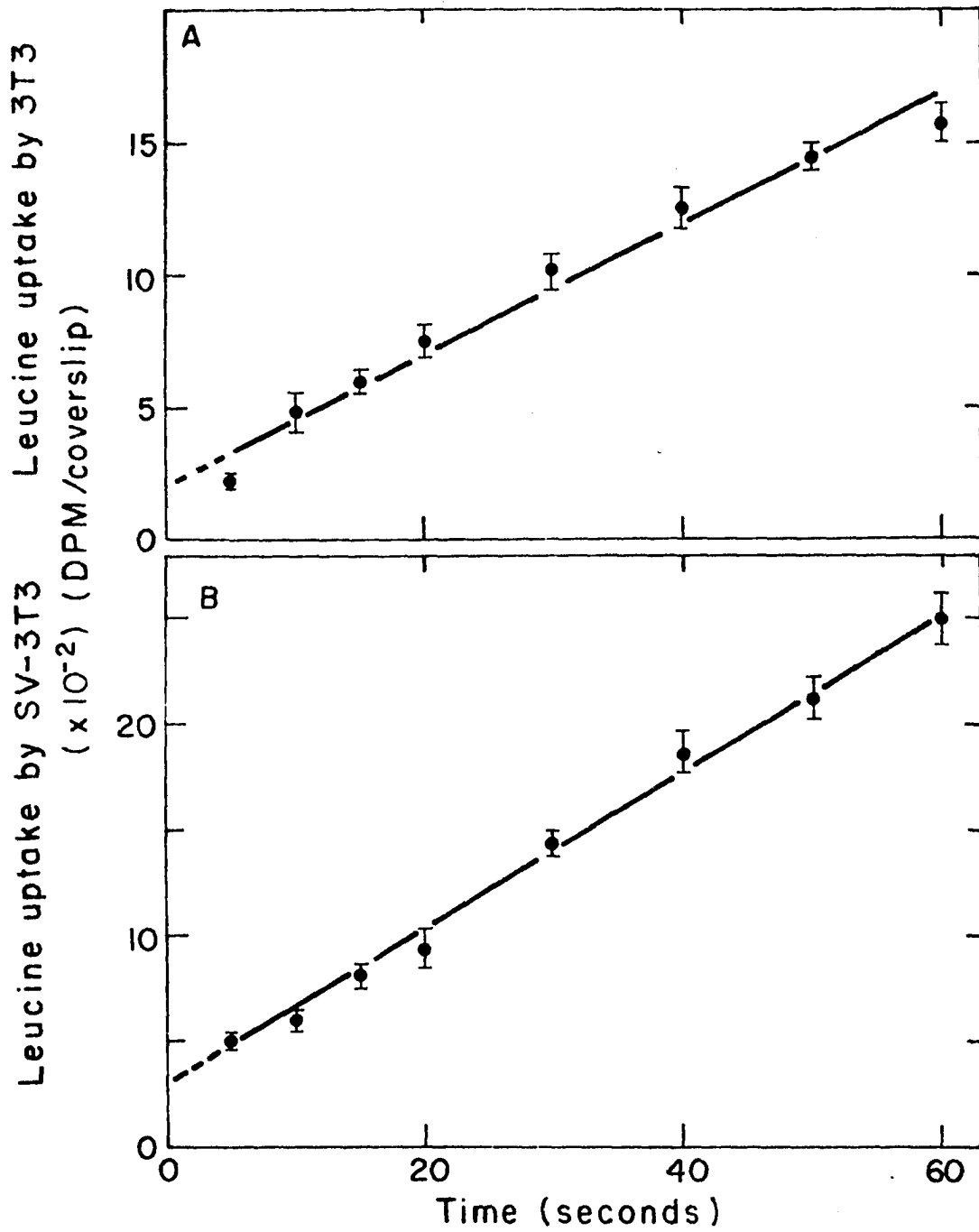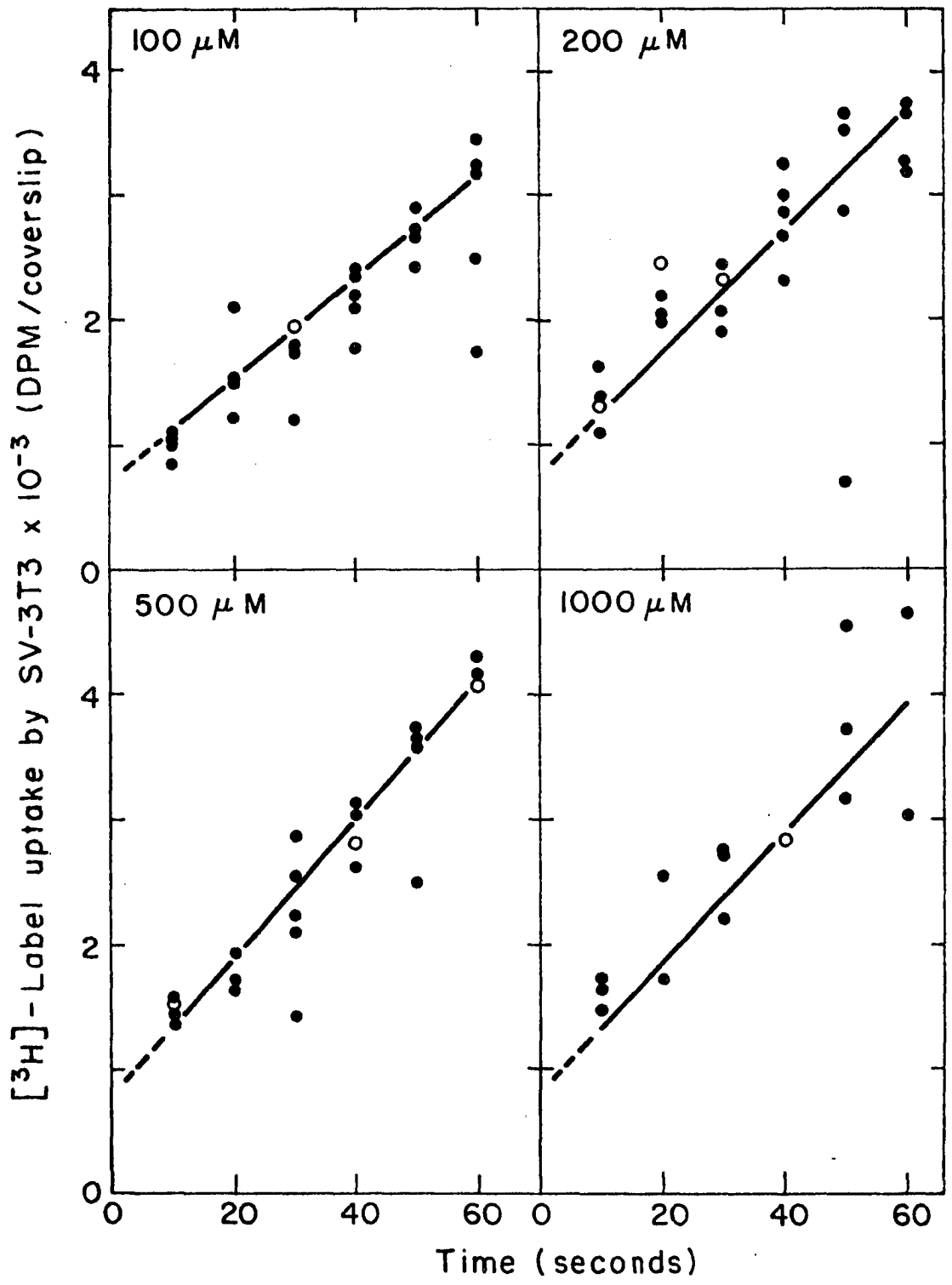
Fig. 12. Kinetics of uptake of the tritium label by SV-3T3. Cells growing on coverslips were incubated for the times indicated in medium containing no serum or leucine to which different amounts of unlabeled leucine and the same amount of labeled leucine (old batch) were added. Cell density was 6.0×10^4 cells/cm^2. DPM/coverslip (●——●); DPM/coverslip, two overlapping points (O——O)

medium competed for transport with the amino acid being
tested, then the amount of unlabeled amino acid added in
determining the kinetic parameters might be insignificant
compared to the total amount of competing amino acid.

d) If the label were not actually in the substrate, but
rather in water or other impurities the transport of label
would not be suppressed by the addition of unlabeled sub-
strate. The latter two of these possibilities were investi-
gated by determining the effect of different uptake incuba-
tion media on the apparent kinetics of uptake of amino acids
and by determining the purity of labeled substrate.

b. Purity of Labeled Leucine

The labeled leucine used in the experiments described
in Figs. 10 and 12 was purchased in 1975, two years before
its use in the experiments. Rapid exchange of tritium on the
leucine with water such as might occur with tritium on the
carboxyl or amino groups was not expected since the leucine
was nominally labeled in the 4,5 positions which are methyl
groups. But the presence of some tritiated water or other
tritiated impurities was tested for (Table XII). The re-
sults seen in this table, that more than 90% of the tritium
label was evaporable with water, were repeated several times.
A batch of leucine that was purchased two months before test-
ing did not contain any tritiated water detectable by this
technique. This result in itself militated against the use

TABLE XII

LEUCINE PURITY*

		Tritiated leucine (old batch)		Tritiated leucine (new batch)	
		Neat	Dried	Neat	Dried
	1)	145,000	11,000	32,000	36,000
DPM/sample	2)	146,000	11,000	32,000	32,000
	3)	146,000	12,000	37,000	33,000
		Chromatography spots		Chromatography spots	
Percent	a)	5.4%	6.0%	1.2%	1.3%
total	b)	33.1%	33.5%	1.9%	1.8%
counts	c)	52.0%	55.9%	95.9%	96.0%
applied	d)	9.4%	4.7%	0.8%	0.8%

*Two batches of tritiated leucine were tested for the presence of tritiated water or tritiated impurities. The first batch was purchased in 1975 and the second in 1977, two years before testing and two months before testing respectively. The presence of tritiated water was detected by placing aliquots of the labeled leucine onto a number of filter paper disks, half of which were immediately put into vials and covered with scintillation counting solution(neat), the other half of which were air dried with a blower before counting (dried). Other tritiated impurities were detected by ascending paper chromatography on Kodak paper #13179 in a solvent of butanol:acetic acid:water (12:3:5) with unlabeled leucine added as carrier. Spots were visualized with ninhydrin spray, and each strip was cut into four blocks, eluted in one ml of 0.2M KCl, 0.1M HCl for one hour, and counted in scintillation counting solution (chromatography by Dr. Katharine Muirhead).

109

of any batch of labeled substrate that had not been recently tested. Subsequent analysis of old and new batches revealed that even the tritium remaining after evaporation was not all contained in leucine in the old batch. Almost half of the tritium was not found in leucine in the old batch, compared to less than 4% in the more recent batch (136).

c. Linearity of Methionine Uptake, Efflux of Methionine, and Effect of Methionine Sulfoxide on Methionine Uptake

The problems encountered in measuring the kinetics of leucine uptake due to the presence of tritiated label prompted the use of $[^{35}S]$-methionine which would not be subject to the problem of exchange of label with water. Linearity of leucine uptake had been established to be at least one minute (Fig. 11), but the period of linear uptake of methionine and the limits of this period of linearity for any amino acid had not been determined. In addition, efflux of amino acid during the washing procedure had not been investigated. Thirdly, labeled methionine was known to be contaminated with between 5 and 10% methionine sulfoxide. Competition of methionine and methionine sulfoxide for a common transport site would distort the kinetics of methionine transport, so the extent of this competition was determined.

In Fig. 13A the results of the competition experiment are shown. The concentrations of labeled and unlabeled

methionine in the uptake incubation medium were kept con-
stant, and all incubations were for 50 seconds. Under
conditions where methionine sulfoxide was in fivefold ex-
cess over methionine, no inhibition of methionine uptake
was seen.

Methionine uptake was linear for 75 seconds (Figure
13B). The apparent decrease in counts per coverslip at 100
seconds in probably not real, and the counts per coverslip
at 125 seconds are only 75% of the counts expected from
extrapolation of a line drawn through the first three points.
Coverslips incubated in the same medium for 50 seconds and
then incubated in the buffer used for washing showed no
change in counts retained until after 20 seconds of incuba-
tion in the buffer. Efflux appeared linear but this process
was not studied systematically. In any case the use of
three washes performed in less than five seconds seemed
safe with respect to minimal loss of substrate by efflux.

d. Kinetics of Methionine Uptake in DMEM Containing Amino Acids

Having determined that the methionine uptake was lin-
ear for at least one minute, that efflux was not significant
in the washing time used, and that competition between
methionine and methionine sulfoxide would not be problematic,
the kinetics of methionine uptake by SV-3T3 cells were
studied for the same purposes described in Results B6a.

Fig. 13.   Effect of methionine sulfoxide on methionine up-

take, linearity of methionine uptake, and efflux

of methionine. SV-3T3 cells growing on cover-

slips were incubated in DMEM buffer with 200

micromolar methionine and [^{35}S]-methionine. In

"A" varying amounts of methionine sulfoxide were

added to the incubation medium, and coverslips

were incubated for 50 seconds. In "B" the cover-

slips were incubated for the times indicated, and

some coverslips were removed (open circles) after

50 seconds and incubated for the remaining time

in the wash solution. Cell density was 26x10^4

cells/cm^2.
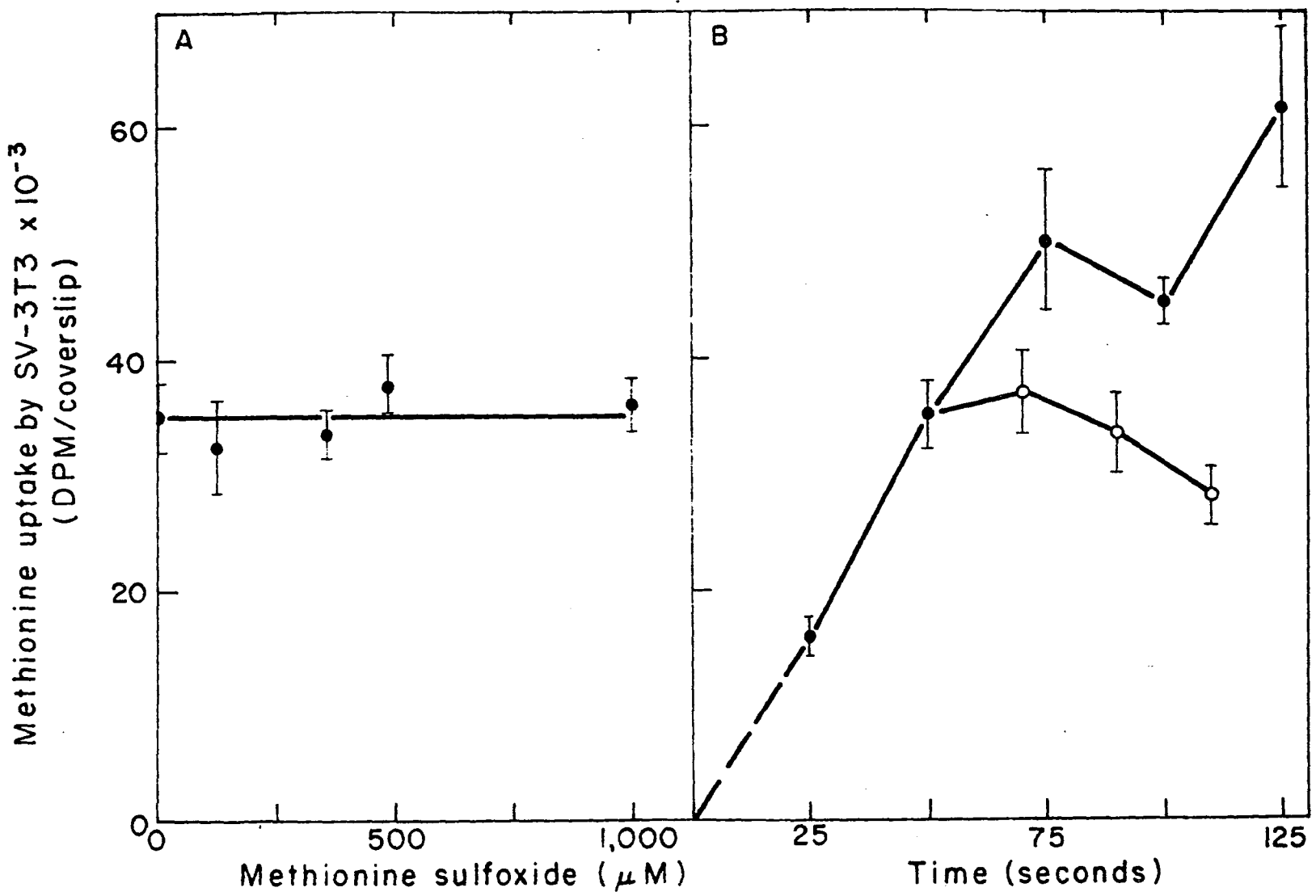
Using the same concentration range as in the experiments on leucine, the results of this experiment on methionine were disturbingly similar (Figure 14). The tenfold increase in concentration from 100 to 1000 micromolar methionine suppressed the uptake of label only 10%. Assuming that the concentration range used was not an order of magnitude higher or lower than the K_M, and that the label was associated with methionine, the conclusion was drawn that other amino acids in the medium were competing with methionine for transport.

e. Kinetics of Methionine Uptake in Incubation Media Lacking Amino Acids

The above experiments on the kinetics of methionine transport were repeated using Tris buffer (Figure 15A) and DMEM buffer (Figure 15B) as the uptake incubation media. The results using Tris buffer confirmed the suspicion that the presence of other amino acids in the uptake medium interfered with the measurement of transport kinetics, a finding which has been cited by others (60,110,143,145,167, 170). Subsequent to this experiment the pH of Tris buffer was found to be 5.5 in the 10% CO_2 atmosphere, so DMEM buffer was chosen as an appropriate uptake incubation medium (Figure 15B).

C. KINETICS OF AMINO ACID UPTAKE

The uptake assay described above in Methods (B4-5) was used to determine the kinetic parameters for the transport

Fig. 14. Kinetics of methionine uptake by SV-3T3 in DMEM.

Cells growing on coverslips were incubated for

the times indicated in medium containing no

serum or methionine to which different amounts

of unlabeled methionine and the same amount of

$[^{35}S]$-methionine were added. Cell density was

28×10^4 cells/cm^2. DPM/coverslip (●——●); DPM/

coverslip, two overlapping points (○——○)
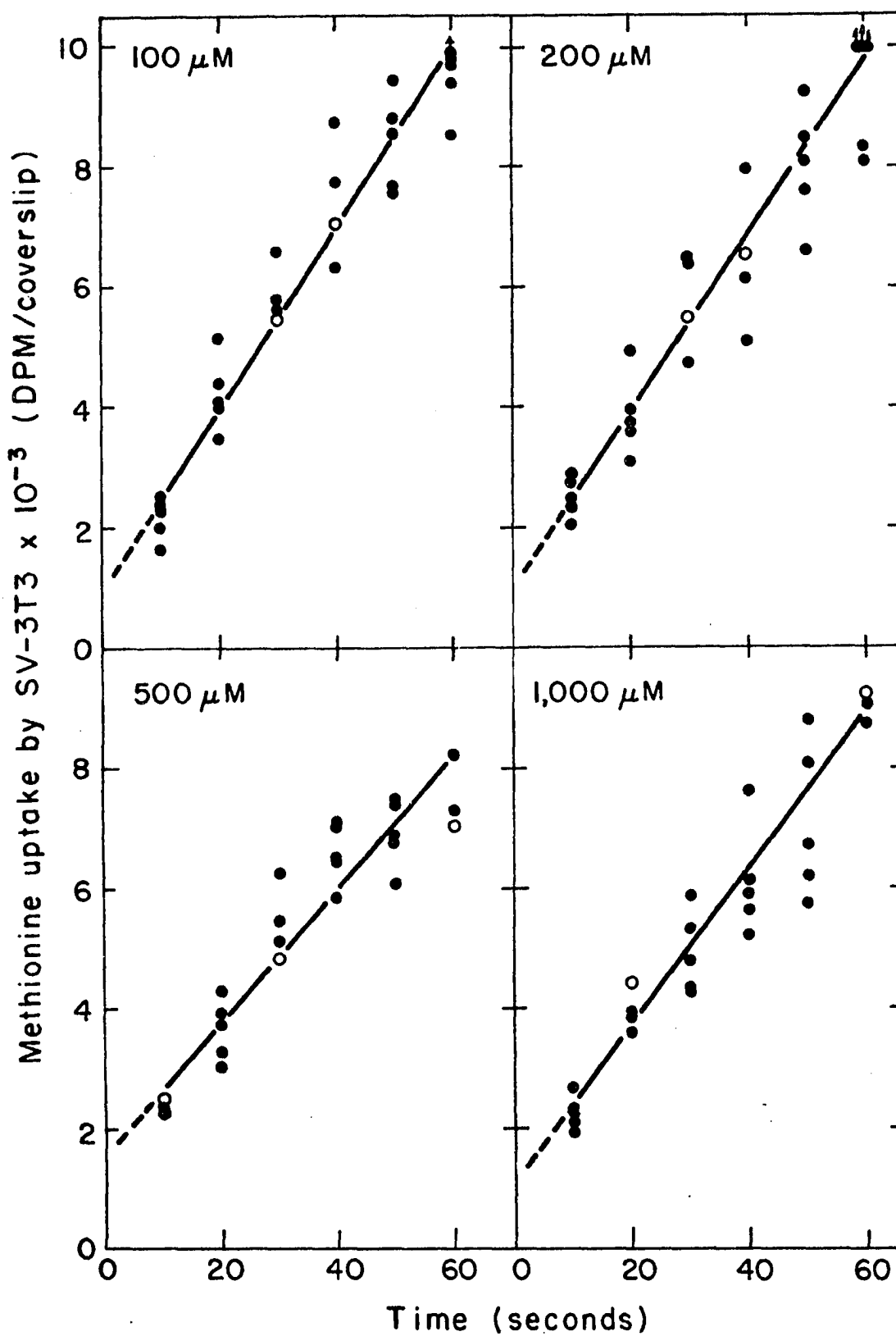
Fig. 15. Kinetics of methionine uptake by SV-3T3 in medium

lacking amino acids. Cells growing on coverslips

were incubated for 20 to 40 seconds in Tris

buffer (A) or DMEM buffer (B) to which different

amounts of unlabeled methionine and the same

amount of $[^{35}S]$-methionine were added. Cell

densities were 17×10^4 cells/cm^2 (A) and 13×10^4

cells/cm^2 (B). Mean and standard deviation (when

greater than the width of the point) are shown

for eight coverslips per time point.
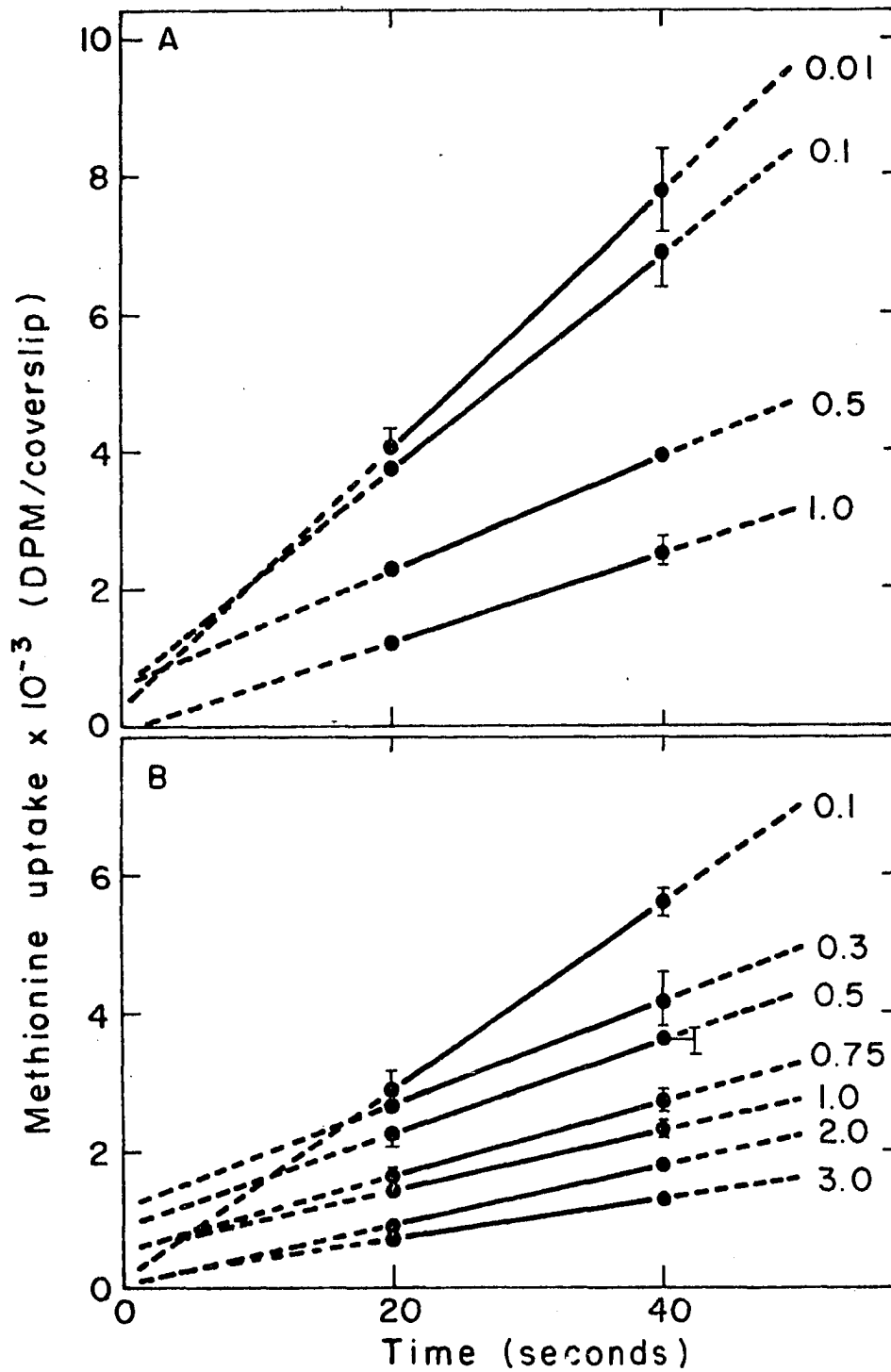
of several amino acids in 3T3 and SV-3T3 cells at different densities and under different conditions. Since these experiments were designed only to estimate the values of Vmax and K_M, the concentration range does not always overlap with K_M; further studies are necessary for a more exact determination of these values.

1. Kinetics of Leucine Uptake by SV-3T3

A new batch of labeled leucine was used for this experiment (Fig. 16). Vmax appears as the intercept on the y-axis as 2039 picomoles per microliter cell water per minute. This value is of the same order of magnitude as the rates of leucine transport in SV-3T3 seen in Table VII. K_M is the absolute value of the slope of the line, which is 245 micromolar.

2. Kinetics of Methionine Uptake by SV-3T3 and 3T3

SV-3T3 cells were assayed for uptake under control and starved conditions at high density, and 3T3 cells were assayed under control conditions at high density (Fig. 17). No significant difference is seen between Vmax or K_M for control versus starved SV-3T3 cells. 3T3 cells however have a threefold lower Vmax and threefold lower K_M than the SV-3T3.

3. Kinetics of Phenylalanine Uptake by SV-3T3 and 3T3

The effect of cell density on the kinetics of phenylalanine uptake by 3T3 cells is shown in Fig. 18.

Fig. 16. Kinetics of leucine uptake by SV-3T3. Cells
growing on coverslips were incubated for 15,
30, and 45 seconds in DMEM buffer containing
tritiated leucine (new batch) and varying
amounts of unlabeled leucine (75 to 5000 micro-
molar). Cell density was 13×10^4 cells/cm^2.
$K_M = 245 \pm 26$ micromolar and Vmax=2039 $\dfrac{\text{picomoles}}{\text{microliter} \cdot \text{min}}$

Fig. 17. Kinetics of methionine uptake by SV-3T3 and 3T3.

Cells growing on coverslips were incubated for

50 seconds in DMEM buffer containing [^{35}S]

-methionine and varying amounts of unlabeled

methionine (40 to 2880 micromolar). Cell density

for SV-3T3 was 18x10^4 cells/cm^2 and for 3T3 was

8x10^4 cells/cm^2. In "A" the SV-3T3 cells were

tested under control (open circles) and starved

(closed circles) conditions. In "B" the 3T3

cells were treated in the same way as the control
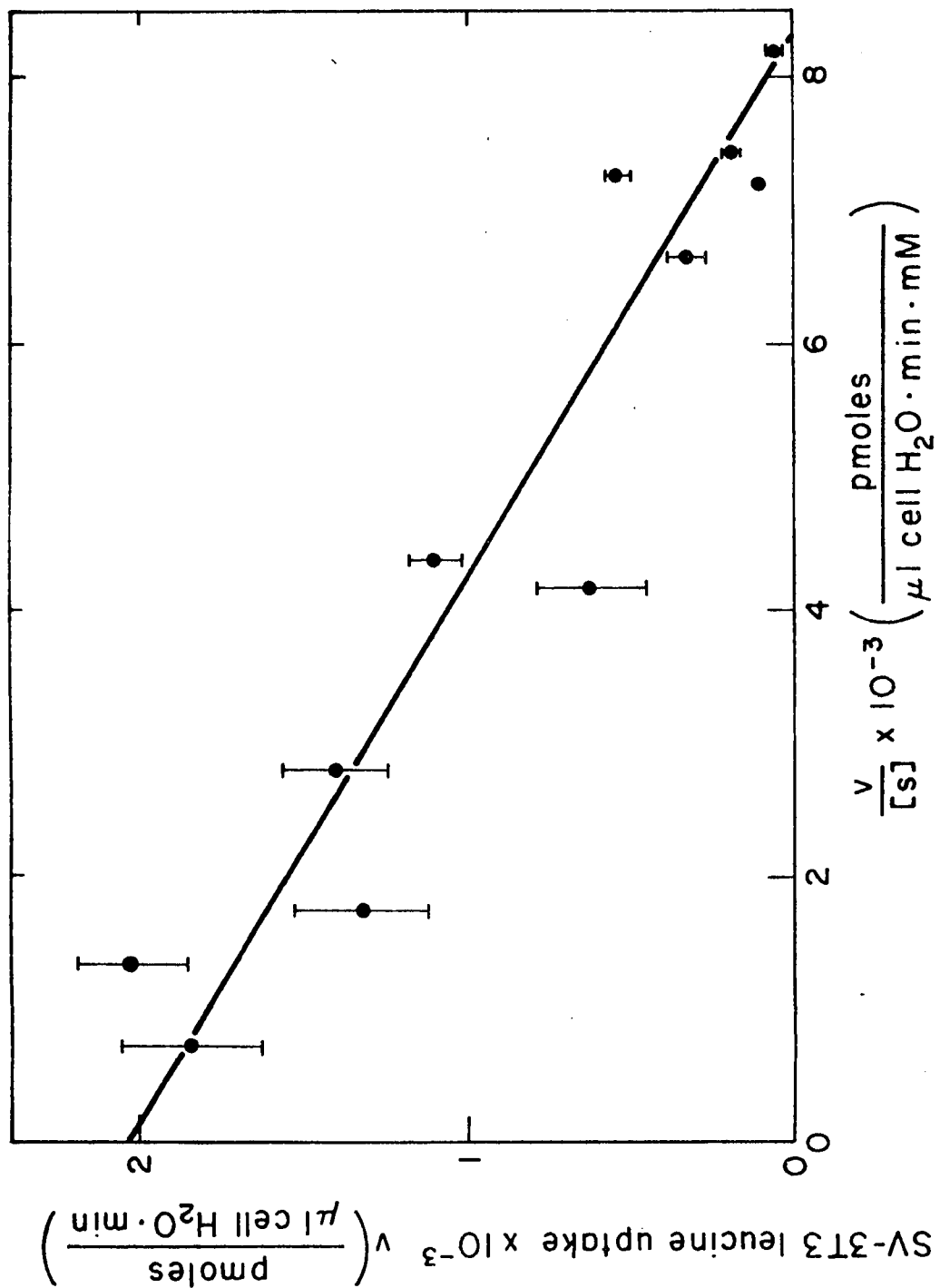
SV-3T3 cells.

Fig. 18.   Effect of cell density on the kinetics of

phenylalanine uptake by 3T3.   Cells growing on

coverslips were incubated for 50 seconds in DMEM

buffer containing tritiated phenylalanine and

varying amounts of unlabeled phenylalanine (40

to 2880 micromolar).   In "A" subconfluent 3T3

cells were at a density of 4×10^4 cells/cm^2 and

in "B" confluent cells were at a density of

7×10^4 cells/cm^2.
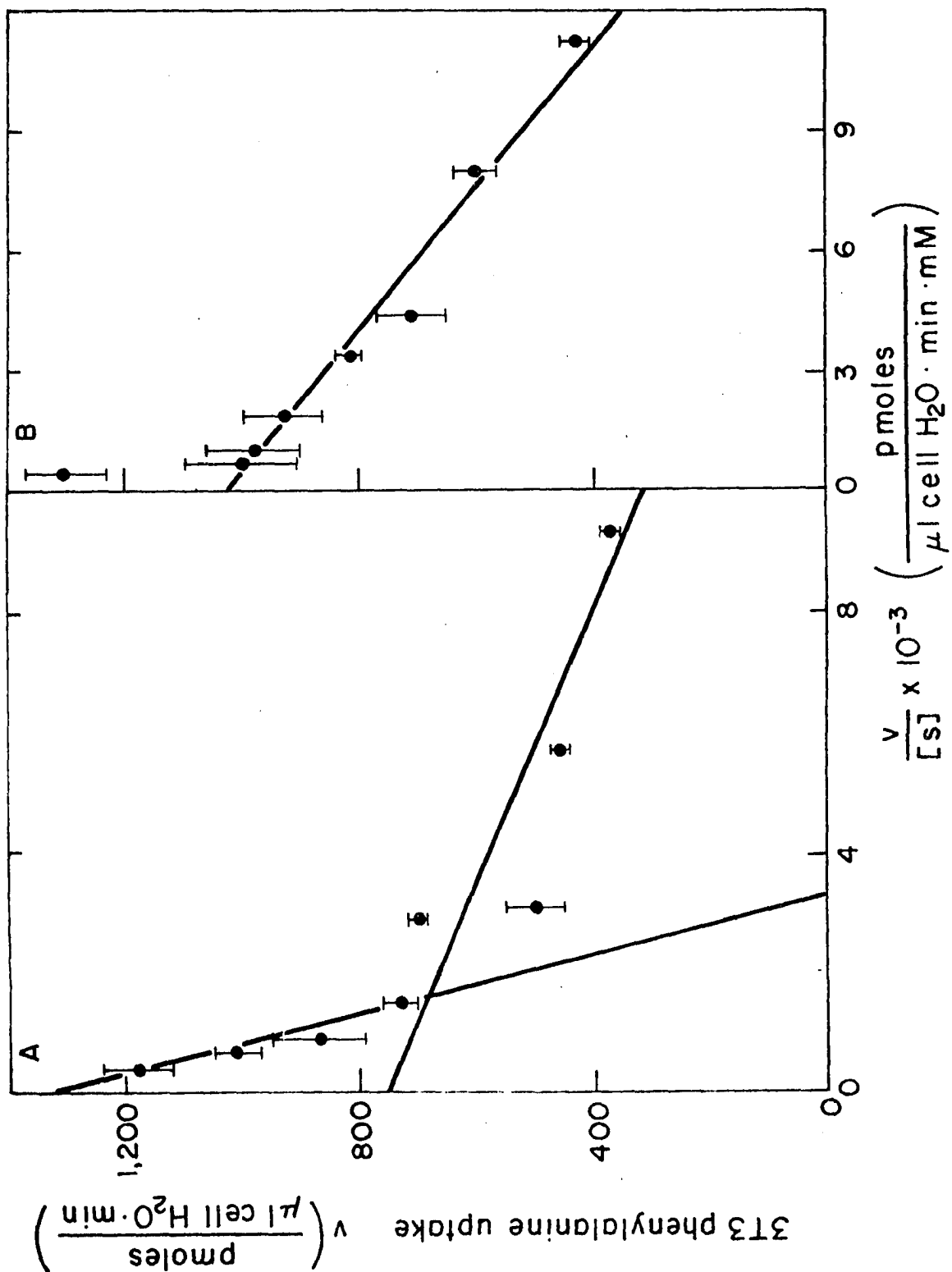
Subconfluent cells show a two component system for uptake, one component with a high Vmax (1300 picomoles per microliter per minute) and high K_M (400 micromolar) and one component with a low Vmax (750 picomoles per microliter per minute) and low K_M (44 micromolar). The confluent cells have only one system with high affinity (K_M=56 micromolar) and a Vmax intermediate between the two systems in the subconfluent cells (Vmax=1000 picomoles per microliter per minute).

In Fig. 19 the effect of starvation for phenylalanine on the kinetics of phenylalanine transport in SV-3T3 is seen to be remarkably similar to the effect of low density on the transport of phenylalanine in 3T3. The starved SV-3T3 cells have a high Vmax (1100 picomoles per microliter per minute), high K_M (2110 micromolar) component and a low Vmax (160 picomoles per microliter per minute), low K_M (49 micromolar) component in a two component system. The control cells have only one component with a Vmax of 1300 picomoles per microliter per minute and a K_M of 290 micromolar.

Fig. 19. Effect of phenylalanine starvation on the
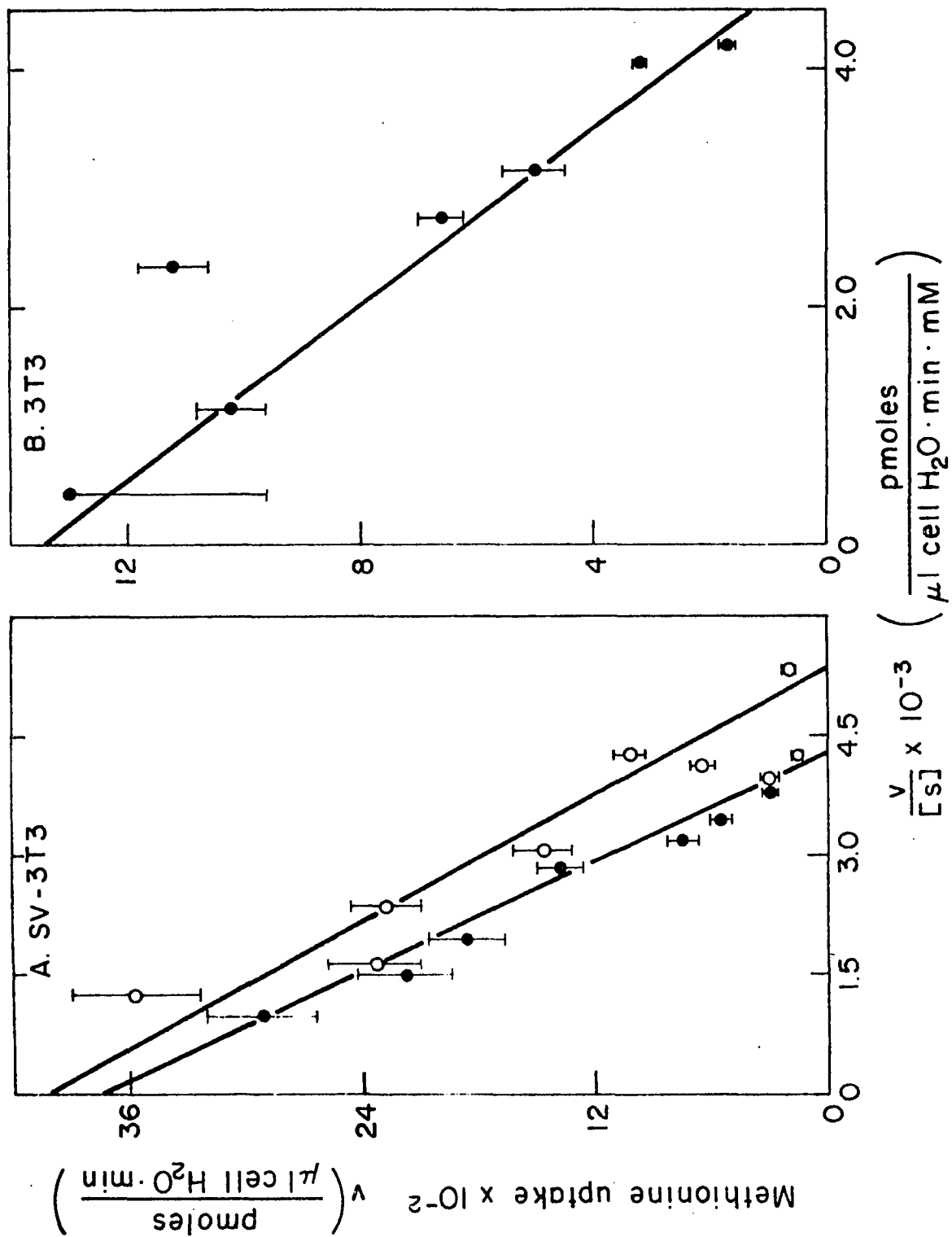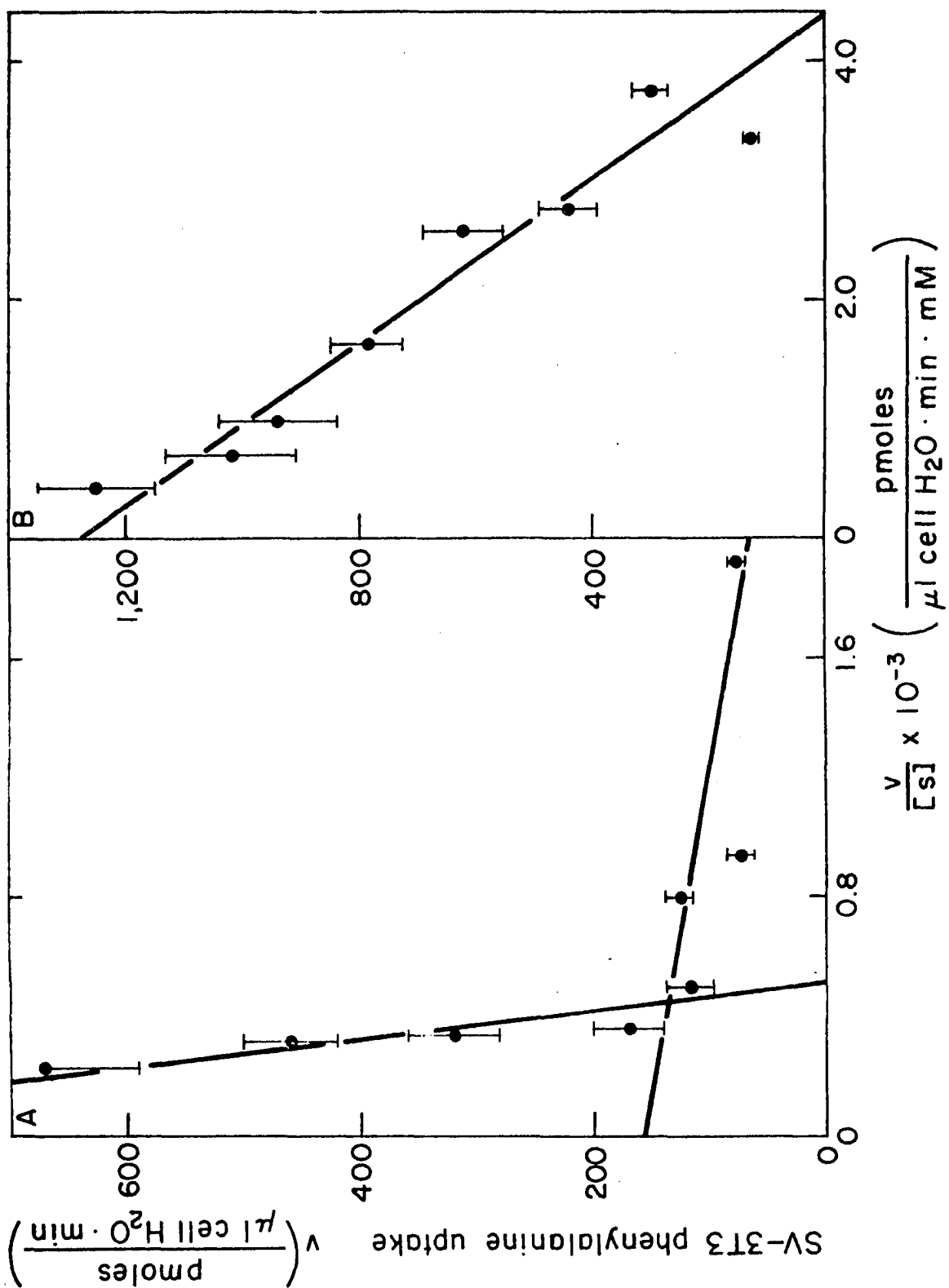
kinetics of phenylalanine uptake by SV-3T3.

Cells growing on coverslips were incubated for

50 seconds in DMEM buffer containing tritiated

phenylalanine and varying amounts of unlabeled

phenylalanine (40 to 2880 micromolar). SV-3T3

cells were tested under starved (A) and control

(B) conditions at a cell density of 21×10^4

cells/cm^2.

IV

DISCUSSION

In this study I have attempted to formalize the methodology of the measurement of transport in cells in tissue culture. More specifically, the format of an ideal transport assay has been outlined, the definition of "uptake," "transport," and "active transport" have been clarified with respect to the assays used, aspects of the transport environment which must be controlled during the assay have been examined in detail, calculation and interpretation of results have been critiqued, and a frequently cited study has been dissected according to the criteria thus developed. Methods for measuring the steady state level of amino acid transport, including methods for determining levels of effective substrate concentration, and methods for measuring the kinetics of amino acid transport have been constructed; these methods adhere as closely as possible to the requirements of the ideal assay. A method for plating cells on coverslips and a cautionary note on label purity were included in the elucidation of these methods. Preliminary data using these methods were shown for several amino acids in normal and

129

transformed cell lines under different conditions.

In the ideal transport assay, the transfer of substrate is measured in the single cell. All assays as practiced to date use large populations of cells. The form of the population of cells has implications for the practicability of the transport assay. Tissue slices may be used for transport assays, but they present numerous difficulties. Cells may be injured by the cutting instrument, multiple layers of cells create a diffusion barrier and thus prevent equal access of the incubation medium to all cells, and tissues are heterogeneous with respect to cell type. In addition, the problems of trapping of extracellular water and quantitation of the number of cells in tissue samples are far more difficult in tissue slices than either cell suspensions or cells in tissue culture. Vertebrate tissue cells which normally grow in suspension in vivo or in vitro are easily used in transport assays (62, 63, 64, 124, 165, 190, 207). Blood cells, protozoa, and bacteria can also be studied in suspensions. Compared to tissue slices, suspension cultures do not suffer the trauma of isolation, the diffusion barrier, or the heterogeneity of cell type when the suspension has been well prepared. Trapping of extracellular water and quantitation of cell number are trivial problems compared to the corresponding problems of tissue slices. However, cells in suspension

do not exhibit as clearly the interesting forms of regulation by cell density that are seen in cells that adhere to solid surfaces.

The problems in studying cells on solid surfaces have been discussed in detail above. Some investigators have attempted to circumvent these difficulties by using plasma membrane vesicles isolated from tissue culture cells (72, 118, 120, 140, 153, 162, 163). In drawing conclusions about the behaviour of the membranes of cells as they function as part of the cell from the behaviour of membrane vesicles, these investigators assume that the process of isolation of the membrane has not changed the functional properties of the membrane. More importantly, the interaction of the membrane with the cell and its metabolic regulation is uncoupled by the isolation process. The most appropriate use of membrane vesicles is the measurement of the kinetics of transport, with the caveat always in mind that the kinetics may only be representative of the actual cellular process.

The cellular process of interest in this study has been the transport of substrate into the cell, but this process is only one of many processes that determine the total metabolic balance in the cell. Efflux of substrate is equally important in determining the net flow of substrate, but it has not received the amount of attention given to

uptake (54, 75). Qualitative differences in protein synthe-
sis (61) and differences in levels of protein degradation
(88) between two groups of cells could contribute to over-
all differences in amino acid metabolism.

The manner in which transport and related words have
been defined by different investigators has determined to
a large degree the kinds of methods used. The need for
rigor has been emphasized by Christensen as follows:
"Occasionally the exceedingly naive assumption is made
that, if a labeled substance at a low external concentration
enters a cell or tissue that already contains a higher con-
centration of the unlabeled form of this substance, then
active transport has occurred. The same behaviour would be
shown by an inert dialysing membrane. A labeled solute at,
say, 10^{-3} M will migrate across a really inert membrane
quite as rapidly into a 10^{-1} M solution as into a 10^{-3} M
solution. Active transport cannot be shown by measuring a
single, one-way flux of the solute" (35).

The methods developed in this study are not suitable
without further modification for the proof that the uptake
measured represented active transport. The contention is
made, however, that if the uptake were shown to operate
against a thermodynamic gradient, the method as described
would be appropriate for the measurement of initial rates
under physiologic conditions. The salient features of the

method are the cell plating procedure, the controlled environment box, the use of physiologic substrates and maximally pure radiolabels, the use of a physiologic uptake incubation medium and short incubation times, and the expression of results in terms of cell water.

The advantages of using coverslips to grow cell samples for transport assays have been described above. Especially with the help of the triceps, the coverslips are easily manipulated and uptake can be measured rapidly. Coverslips plated individually in the manner described provide a group of cell populations that differ by less than 5% in cell number. The materials required are simpler and less expensive than the multiple well dishes that have been used by others (98, 99, 187, 191).

This method is the first one to use cells growing on coverslips in an uptake assay in a controlled environment. The necessity for maintaining a controlled environment stems from considerations of temperature, pH, and the kind of buffer used. The effect of temperature on transport has been most systematically studied in terms of possible mechanisms for this effect in E. coli mutants requiring exogenous fatty acids for growth. The fatty acid composition of the membrane was modified by growth in fatty acids of different lengths and saturation. Cells grown in each fatty acid had a unique characteristic for the

induced transport of beta galactosides, and this character-

istic was the temperature at which a transition occurred

in the degree of temperature dependence on transport.

The transition temperature varied from 7°C for linoleic

acid to 30°C for elaidic acid (35). The possibility of

phase changes occurring at temperatures as high as 30°C

is certainly one incentive for measuring uptake rates at

physiologic temperatures. Although the mechanism of the

effect of pH or buffer solution on transport has not been

worked out in such depth, the necessity for bicarbonate in

cell growth media with few exceptions (50, 123, 200) sug-

gests that transport should be measured in an uptake incuba-

tion medium containing bicarbonate at a physiologic pH.

The difficulties encountered in maintaining temperature at

a constant level over a large area have been shown in the

Results.

Physiologic substrates have been used throughout this

study. Two of the amino acids used, leucine and phenylal-

anine, are not metabolized in the small molecular weight

carbon pool, but are only aminoacylated onto transfer RNA

and synthesized into protein. Less than 5% of these amino

acids is present in acid insoluble material after two min-

utes of uptake (130). Methionine is converted into S-

adenosylmethionine which is used in many metabolic path-

ways; this process is currently under study (31). For

phenylalanine and leucine, and probably methionine, the uptake observed is not due to trapping of these molecules by metabolic conversion. The linearity of uptake observed during the first minute suggests that efflux of labeled material is not significant during this time.

Purity of labeled substrate has also been examined as a contributor to uptake results. An incidental advantage of the coverslip technique and the use of several samples for a rate determination is that preferential uptake of an impurity should be evident from decreasing uptake of total label by successive portions of cells (35). Such a pattern was looked for but never seen in the data shown here. The use of radiolabeled material which is old may result in anomalous results as seen above. Such material should be periodically examined before use.

In measuring steady state levels of amino acid transport, the logic of using an uptake incubation medium which was similar to the growth medium has been discussed. For the measurement of kinetic parameters, the use of an uptake incubation medium containing amino acids other than the one whose uptake is assayed obscures the kinetics of uptake, presumably due to competition of different amino acids for the same transport site. The arguments in favor of using physiologic temperatures, pH and buffer still are valid; therefore, DMEM buffer was used in the kinetic

experiments, and the assays were carried out in the controlled environment box.

The most important result of using the coverslip method is that the amount of time in which transport can be measured has been decreased by an order of magnitude. Reliable data can be obtained for a five second incubation, although such short incubations are rarely needed. The small possibility still exists that the only linear transport occurs in the first 0.1 seconds of incubation, and what has been seen as nonspecific binding is actually very rapid transport. This possibility is less likely after considering the decrease of nonspecific binding to very low levels after using three washes, improved plating techniques, and linearity of transport in the first minute.

Converting the results of the uptake assay from DPM per coverslip to picomoles per microliter cell water per minute involves two intermediate calculations which are not in general use. When the uptake incubation medium has been used to support cell growth and contains serum, the determination of the total substrate concentration is complicated by the influence of pH on the binding of amino acid to serum protein. The calibration curve constructed in this study can be used for this determination. The other calculation involves the conversion of uptake data in terms of cell number to cell water, using calibration

curves established elsewhere (128).

The speculation that transformation and malignancy are related to changes in transport has its adherents and detractors. Unfortunately the many studies in this area are so riddled with the problems described in the Criteria for Evaluating Methods of Transport Measurement that interpretation of results may not prove fruitful, and inconsistencies in interpretation of studies such as that of Foster and Pardee (59) are one proof of this assertion. Still one group of investigators would like to use "hexose transport as an ideal index of the physiologic and sociologic response of nontransformed cells to environmental variation"(84). Transformed cells are seen as "outlaw, sociopathic cells" which "gobble all available glucose," while "normal cells grow on austerity." Holley asserts somewhat less colorfully, "The crucial change in a malignant cell is an alteration in the cell surface that results in increased internal concentration of nutrients that regulate cell growth" (93). Tomkins coined the term "pleiotypic response" to explain much the same relationship between transport and cell proliferation (89, 113). The serious question that arises when such elegant hypotheses are generated before reliable data are available is whether the relationship between cell growth and transport may be much more subtle than preliminary experiments would suggest.

The minimal perturbation studies indicate that transport changes do occur at different cell densities and across cell lines. Many factors could conceivably be involved in the change observed; changes in medium pH, concentration of nutrients or growth factors, cell density, time in culture, and so forth. These transport changes in themselves are significant, with three to fourfold differences across cell density and cell line; the mechanism of these differences can only be studied in terms of analysing the separate factors individually for their contribution to the change and in terms of the kinetics of transport.

The kinetics of methionine transport in 3T3 cells were shown to be quite different than their transformed derivatives. The 3T3 cells showed a threefold decrease in Vmax, usually interpreted as a decrease in the number of sites for transport, but also showed a threefold decreased K_M, an increase in the affinity of the sites for the substrate. Such a change is quite consonant with the differences between a well regulated differentiated cell and an unregulated wildly proliferating transformed cell. The SV-3T3 cells starved for methionine might be expected to revert to some of the characteristics of controlled growth, but this expectation was not borne out in the results of methionine transport from methionine starved cells.

The effect of cell density on the kinetics of phenylalanine transport gives further insight into possible control mechanisms operant in transport and cell proliferation. Subconfluent cells which are still actively dividing show a two component system for phenylalanine transport. One component is a high capacity, low affinity system which would be expected to perform best during bountiful supply of substrate in the medium, and the other component is a low capacity, high affinity system which would be better suited during more austere conditions. Interestingly, at high cell density only the high affinity component is apparent, and the capacity of this component has increased.

SV-3T3 cells under control conditions show a K_M for phenylalanine which is 75% of the low affinity component of the subconfluent 3T3. The Vmax for the SV-3T3 cells is the same as that of the subconfluent 3T3. This result points strongly to a relationship between transport and cell proliferation per se, and not necessarily between transport and transformation. When the SV-3T3 cells are starved for phenylalanine for four hours prior to assay, a two component system for phenylalanine transport is seen. The low affinity component has a K_M which is much larger than the corresponding K_M in 3T3 cells, but the Vmax is similar to both the 3T3 and control SV-3T3 cells. The high affinity to component has a K_M similar to, and a Vmax much

lower than, the corresponding values in the subconfluent 3T3 cells. Here the SV-3T3 cells do appear to revert to the characteristics of a more differentiated state, albeit a differentiated state in which growth is still active.

From the data gathered thus far, the generalization can be made that normal and transformed cells differ with respect to transport, and the mechanism of this difference appears to be related to the process of cell proliferation rather than transformation itself. The kinetic parameters indicate that differentiated cells and cells that revert to this state have a high affinity but low capacity for their substrate, while transformed cells have a lower affinity but higher capacity for the substrate. Actively growing normal cells may regulate transport using a two component system, the high capacity low affinity component of which is inactivated during the approach to cell confluence.

V

BIBLIOGRAPHY

1. Absher, M, 1973, in Tissue Culture Methods and Applications, ed. Kruse, PF and Patterson, MK, Academic Press, N.Y., p. 395.

2. Adamson, LP, Herington, AC, and Bornstein, J, 1972, Biochim. Biophys. Acta, 282:352.

3. Amos, H, Christopher, CW, and Musliner, TA, 1976, J. Cell Physiol., 89:669.

4. Archer, EG, Breakefield, XO, and Sharata, MN, 1977, J. Neurochem., 28:127.

5. Asodourian, H, Chu, L, Lau, K, and Amos, H, 1975, Biochem. Biophys. Res. Commun., 64:1142.

6. Ayala, E and Canonico, PG, 1975, Proc. Soc. Exptl. Biol. Med., 149:1019.

7. Bader, JP, 1972, J. Virol., 10:267.

8. Bader, JP, Lew, MA, and Brown, NR, 1976, Arch. Biochem. Biophys., 175:196.

9. Balcar, VJ, Berg, S, and Mande, P, 1977, J. Neurochem., 28:87.

10. Baltimore, D, and Franklin, RM, 1962, Proc. Nat. Acad. Sci., 48:1383.

11. Baran, DT, Lichtman, MA, and Peck, WA, 1972, J. Clin. Invest., 51:2181.

12. Barnes, D and Colowick, SP, 1976, J. Cell Physiol., 89:633.

13. Bartsocas, CS and Erbe, RW, 1973, Acta Paediat. Scand., 62:615.

14. Baserga, R, Costlow, M and Rovera, G, 1973, Fed. Proc., 32:2115.

141

15. Bassham, JA, Bissell, MJ, and White, RC, 1974, _Anal. Biochem._, 61:479.

16. Benke, PJ, Erbstdeszer M, and Pitot, HC, 1972, _Lancet_, 1:182.

17. Benke, PJ, 1973, _Biochem. Med._, 8:309.

18. Bhargava, PM, Szafarz, D, Bornecque, CA, and Zajdela, F, 1975, _J. Membr. Biol._, 22:347.

19. Bhargava, PM, Allin, EP, and Montagnier, L, 1976, _J. Membr. Biol._, 26:1.

20. Bhargava, PM, Allin, EP, and Montagnier, L, 1976, _J. Membr. Biol._, 26:19.

21. Birckbichler, PJ, Whittle, WL, and Dell'Orco, RT, 1975, _Proc. Soc. Exptl. Biol. Med._, 149:530.

22. Bissell, MJ, White, RC, Hatie, C, and Bassham, JA, 1973, _Proc. Nat. Acad. Sci._, 70:2951.

23. Booth, CW and Nadler, HL, 1975, _Proc. Soc. Exptl. Biol. Med._, 148:272.

24. Bose, SK and Zlotnick, BJ, 1973, _Proc. Nat. Acad. Sci._, 70:2374.

25. Bradley, WEC and Culp, LA, 1974, _Exptl. Cell Res._, 84:335.

26. Brownstein, BL, Rozengurt, E, Jiminez de Asua, L, and Stoker, M, 1975, _J. Cell Physiol._, 85:579.

27. Cass, CE, 1972, _Exptl. Cell Res._, 73:140.

28. Ceccharini, C and Eagle, H, 1971, _Proc. Nat. Acad. Sci._, 68:229.

29. Cecchini, G, Quay, SC, Dick, TE, and Oxender, D, 1976, _Fed. Proc._, 35:1357.

30. Cecchini, G, Lee, M, and Oxender, DL, 1976, _J. Supramol. Struct._, 4:441.

31. Centrella, MA and Meisler, AI, 1978, _J. Biol. Chem._, submitted for publication.

32. Chowdhury, TK, 1975, _Adv. Exptl. Med. Biol._, 55:187.

33. Christensen, HN, de Cespedes, C, Handlogten, ME, and Ronquist, G, 1973, Biochim. Biophys. Acta, 300:487.

34. Christensen, HN, 1973, Fed. Proc., 32:19.

35. Christensen, HN, 1975, Biological Transport, second edition, W.A. Benjamin, Inc., Reading, Mass., 514 pages.

36. Christopher, CW, Colby, WW, and Ullrey, D, 1976, J. Cell Physiol., 89:683.

37. Christopher, CW, Ullrey, D, Colby, W, and Kalckar, HM, 1976, Proc. Nat. Acad. Sci., 73:2429.

38. Christopher, CW, Colby, WW, Ullrey, D, and Kalckar, HM, 1977, J. Cell Physiol., 90:387.

39. Cohn, RH, Cassiman, J, and Bernfield, MR, 1976, J. Cell Biol., 71:280.

40. Colby, C, and Edlin, G, 1970, Biochem., 9:917.

41. Colby, C and Romano, AH, 1975, J. Cell Physiol., 85:15.

42. Coriell, LL, 1973, in Tissue Culture Methods and Applications, ed. Kruse, PF and Patterson, MK, Academic Press, N.Y., p. 671.

43. Costlow, M and Baserga, R, 1973, J. Cell Physiol., 82:411.

44. Culp, LA and Black, PH, 1972, Biochem., 11:2161.

45. Cunningham, DD and Pardee, AB, 1969, Proc. Nat. Acad. Sci., 64:1049.

46. Cunningham, DD and Remo, RA, 1973, J. Biol. Chem., 248:6282.

47. Damper, D and Patton, CL, 1976, Vth Int. Congress of Proto-zoology, p. 390.

48. Dickson, JA, 1970, Exptl. Cell Res., 61:235.

49. Dolberg, DS, Bassham, JA, and Bissell, MJ, 1975, Exptl. Cell Res., 96:129.

50. Eagle, H, 1959, Science, 130:432.

51. Eagle, H, Piez, KA, and Levy, M, 1961, J. Biol. Chem., 236:2139.

52. Eckhart, W, and Weber, MJ, 1974, _Virology_, 61:223.

53. Edstrom, A, Kanse, M, and Walum, E, 1976, _Exptl. Cell Res._, 97:6.

54. Eilam, Y, and Vinkler, C, 1976, _Biochim. Biophys. Acta_, 433:393.

55. Eilam, Y, and Cabantchik, I, 1976, _J. Cell Physiol._, 89:831.

56. Elsas, LJ, Wheeler, FB, Danner, DJ, and DeHaan, RL, 1975, _J. Biol. Chem._, 250:9381.

57. Engelhardt, DL, 1971, _J. Cell Physiol._, 78:333.

58. Fletcher, DS and Lin TY, 1973, _Clin. Chim. Acta_, 44:5.

59. Foster, DO, and Pardee, AB, 1969, _J. Biol. Chem._, 244:2675.

60. Frank, W, and Ristow, HJ, 1970, _Eur. J. Biochem._, 49:325.

61. Frindel, E, Baserga, R, and Brigham, P, 1970, _Cell Tissue Kinet._, 3:143.

62. Gazzola, GC, Franchi, R, Saibene, V, Ronchi, P, and Guidotti, GG, 1972, _Biochim. Biophys. Acta_, 266:407.

63. Gazzola, GC, Gazzola, RF, Ronchi, P, and Guidotti, GG, 1973, _Biochim. Biophys. Acta_, 311:292.

64. Gazzola, RF, Gazzola, GC, Ronchi, P, Saibene, V, and Guidotti, GG, 1973, _Biochim. Biophys. Acta_, 291:545.

65. Gerschenson, LE and Berliner, JA, 1976, _J. Steroid. Biochem._, 7:159.

66. Gray, PN, Cullum, ME, and Griffin, MJ, 1976, _J. Cell Physiol._, 89:225.

67. Greenberg, DB, Barsh, GS, Ho, T, and Cunningham, DD, 1977, _J. Cell Physiol._, 90:193.

68. Griffiths, JB, 1972, _J. Cell Sci._, 10:515.

69. Grimes, WJ, and Schroeder, JL, 1973, _J. Cell Biol._, 56:487.

70. Groth, U, and Rosenberg, LE, 1972, _J. Clin. Invest._, 51:2130.

71. Hakala, MT, Suolinna, EM, Kenny, LN, and Tritsch, GL, 1976, _Biochim. Biophys. Acta_, 338:1.

72. Hamilton, RJ, and Nilsen-Hamilton, M, 1976, Proc. Nat. Acad. Sci., 73:1907.

73. Hare, JD, 1967, Cancer Res., 27:2357.

74. Hare, JD, 1975, Arch. Biochem. Biophys., 170:347.

75. Hare, JD, Marinetti, GV, Meisler, AI, and Tometsko, AM, 1976, Biochim. Biophys. Acta, 443:485.

76. Harel, L, Jullien, M, and Blat, C, 1975, Exptl. Cell Res., 90:201.

77. Harris, JF, and Whitmore, GF, 1974, J. Cell Physiol., 83:43.

78. Hassell, JA, Colby, C, and Romano, AH, 1975, J. Cell Physiol., 86:37.

79. Hatanaka, M, Huebner, RJ, and Gilden, RV, 1969, J. Nat. Cancer Inst., 43:1091.

80. Hatanaka, M, Todaro, GS, and Gilden, RV, 1970, Int. J. Cancer, 5:224.

31. Hatanaka, M, Augl, C, and Gilden, R, 1970, J. Biol. Chem., 245:714.

82. Hatanaka, M, and Hanafusa, H, 1970, Virology, 41:647.

83. Hatanaka, M, Gilden, RV, and Kelloff, G, 1971, Virology, 43:734.

84. Hatanaka, M and Graff, JC, 1973, Bibl. Haematol., 40:621.

85. Hatanaka, M, 1973, Proc. Nat. Acad. Sci., 70:1364.

86. Hatanaka, M, 1974, Biochim. Biophys. Acta, 355:77.

87. Hatanaka, M, 1976, J. Cell Physiol., 89:745.

88. Hendil, KB, 1977, J. Cell. Physiol., 92:353.

89. Hershko, A, Mamont, P, Shields, R, and Tomkins, GM, 1971, Nature New Biology, 232:206.

90. Hillman, RE, 1974, Biochim. Biophys. Acta, 367:81.

91. Hillman, RE, and Otto, EF, 1974, J. Biol. Chem., 249:3430.

92. Hollenberg, MD, and Cuatrecasas, P, 1975, J. Biol. Chem., 250:3845.

93. Holley, RW, 1972, Proc. Nat. Acad. Sci., 69:2840.

94. Holley, RW, 1975, Nature, 258:487.

95. Hopfer, U, 1976, Horiz. Biochem. Biophys., 2:106.

96. Hume, SP, and Lamb, JF, 1976, J. Physiol., 259:83.

97. Inbar, M, BenBassat, H, and Sachs, L, 1971, J. Membr. Bio., 6:195.

98. Isselbacher, KJ, 1972, N. Engl. J. Med., 286:929.

99. Isselbacher, KJ, 1972, Proc. Nat. Acad. Sci., 69:585.

100. Jainchil, JL, and Todaro, GJ, 1970, Exptl. Cell Res., 59:137.

101. Jiminez de Asua, L, Rozengurt, E, and Dulbecco, R, 1974, Proc. Nat. Acad. Sci., 71:96.

102. Kalckar, HM, Ullrey, D, Kijomoto, S, and Hakamori, S, 1973, Proc. Nat. Acad. Sci., 70:839.

103. Kalckar, HM, and Ullrey, D, 1973, Proc. Nat. Acad. Sci., 70:2502.

104. Kalckar, HM, 1976, J. Cell Physiol., 89:503.

105. Kalckar, HM, Christopher, CW, and Ullrey, D, 1976, J. Cell Physiol., 89:765.

106. Kletzien, RF, Perdue, JF, and Springer, A, 1972, J. Biol. Chem., 247:2964.

107. Kletzien, RF, and Perdue, JF, 1973, J. Biol. Chem., 248:711.

108. Kletzien, RF, and Perdue, JF, 1974, J. Biol. Chem., 249:3366.

109. Kletzien, RF, Pariza, MW, Becker, JE, and Potter, VR, 1975, Nature, 256:46.

110. Kletzien, RF, Pariza, MW, Becker, JE, Potter, VR, and Butcher, FR, J. Biol. Chem., 251:3014.

111. Kletzien, RF, and Perdue, JF, 1976, J. Cell Physiol., 89:723.

112. Kimelberg, HK, and Mayhew, E, 1975, J. Biol. Chem., 250:100.

113. Kram, R. Mamont, P, and Tomkins, GM, 1973, Proc. Nat. Acad. Sci., 70:1432.

114. Kravitt, EL, Baril, EF, Becker, JE, and Potter, VR, 1970, Science, 169:294.

115. Kuroki, T, and Yamakawa, S, 1974, Int. J. Cancer, 13:240.

116. Kuroki, T, and Yamakawa, S, 1974, Int. J. Cancer, 14:32.

117. Leffert, HL, and Paul, D, 1973, J. Cell Physiol., 81:113.

118. Lever, JE, 1976, J. Cell Physiol., 89:779.

119. Levinson, C, 1972, J. Cell Physiol., 79:73.

120. Li, CC, and Hochstadt, J, 1976, J. Biol. Chem., 251:1175.

121. Lingwood, CA, and Thomas, DB, 1974, J. Cell Biol., 61:359.

122. Lust, WD, Schwartz, JP, and Passonneau, JV, 1975, Mol. Cell. Biochem., 8:169.

123. McLimans, WF, 1972, in Growth, Nutrition, and Metabolism of Cells in Culture, vol. I, ed. Rothblat, GH and Cristofalo, VJ, Academic Press, N.Y., p. 155.

124. Mahoney, MJ, and Rosenberg, LE, 1970, Biochim. Biophys. Acta, 219:500.

125. Marineau, R, Kolbacher, M, Shaw, SN, and Amos, H, 1972, Proc. Nat. Acad. Sci., 69:3407.

126. Martin, GS, Venuta, S, Weber, M, and Rubin, H, 1971, Proc. Nat. Acad. Sci., 68:2739.

127. Martonos, A, 1975, Biochim. Biophys. Acta, 415:311.

128. Meisler, AI, 1973, J. Cell Sci., 12:847.

129. Meisler, AI, 1973, J. Cell Sci., 12:861.

130. Meisler, AI, 1977, unpublished observations.

131. Mendelson, J, Skinner, A, and Kornfeld, S, 1971, J. Clin. Invest., 50:818.

132. Merryman, P, Fernandez, A, and Jaffe, IA, 1976, Biochem. Pharmac., 25:547.

133. Michaelis, FB, and Dalgarno, L, 1971, Exptl. Cell Res., 65:43.

134. Miller, MS, and Wallach, DFH, 1975, Cancer Res., 35:1826.

135. Muirhead, KA, Tometsko, A, and Meisler, AI, 1978, Biochim. Biophys. Acta, submitted for publication.

136. Muirhead, KA, 1977, unpublished data (personal communication)

137. Nachbar, MS, Oppenheimer, JD, and Aull, F, 1974, Am. J. Med. Sci., 268:122.

138. Neal, JL, 1972, J. Theor. Biol., 35:113.

139. Nicolson, GL, 1976, Biochim. Biophys. Acta, 458:1.

140. Nilsen-Hamilton, M, and Hamilton, RT, 1976, J. Cell Physiol., 89:795.

141. Oshiro, Y, and DiPaolo, JA, 1974, J. Cell Physiol., 83:193.

142. Otsuka, H, and Moskowitz, M, 1975, J. Cell Biol., 67:A319.

143. Otsuka, H, and Moskowitz, M, 1975, J. Cell Physiol., 85:665.

144. Otsuka, H, and Moskowitz, M, 1975, J. Cell Physiol., 86:379.

145. Oxender, DL, Lee, M, Moore, PA, and Cecchini, G, 1977, J. Biol. Chem., 252:2675.

146. Oxender, DL, Lee, M, Moore, PA, and Cecchini, G, 1977, J. Biol. Chem., 252:2680.

147. Pardee, AB, 1968, Science, 162:632.

148. Pardee, AB, 1969, J. Gen. Physiol., 54:S79.

149. Pardee, AB, 1971, In Vitro, 7:95.

150. Pardee, AB, 1974, Proc. Nat. Acad. Sci., 71:1286.

151. Pariser, RJ, and Cunningham, DD, 1971, J. Cell Biol., 49:525.

152. Pariza, MW, Butcher, FR, Becker, JE, and Potter, VR, 1977, Proc. Nat. Acad. Sci., 74:234.

153. Parnes, JR, Garvey, TQ, and Isselbacher, KJ, 1976, J. Cell Physiol., 89:789.

154. Pasternak, CA, 1974, Biochem. J., 144:593.

155. Patterson, MK, 1974, J. Nat. Cancer Inst., 53:1493.

156. Paul, D, 1973, Biochem. Biophys. Res. Commun., 53:745.

157. Pearlstein, E, and Waterfield, MD, 1974, Biochim. Biophys. Acta, 362:1.

158. Perdue, JF, 1976, J. Cell Physiol., 89:729.

159. Plagemann, PG, and Richey, DP, 1974, Biochim. Biophys. Acta, 344:263.

160. Plagemann, PGW, Richey, DP, Zylka, JM, and Erbe, J, 1975, J. Cell. Biol., 64:29.

161. Plagemann, PGW, and Sheppard, JR, 1975, J. Cell Physiol., 85:163.

162. Quinlan, DC, and Hochstadt, J, 1974, Proc. Nat. Acad. Sci., 71:5000.

163. Quinlan, DC, Parnes, JR, Shalom, R, Garvey, TQ, Isselbacher, KJ, and Hochstadt, J, 1976, Proc. Nat. Acad. Sci., 73:1631.

164. Regan, DH, Lavietes, BB, Regan, MG, Demopoulis, HB, and Morris, HP, 1973, J. Nat. Cancer Inst., 51:1013.

165. Renner, ED, Plagemann, PG, and Bernlohr, RW, 1972, J. Biol. Chem., 247:5765.

166. Richelson, E, 1973, Anal. Biochem., 52:563.

167. Richelson, E, 1974, J. Biol. Chem., 249:6218.

168. Rieber, M, Bacalda, J, and Alonso, G, 1975, Cancer Res., 35:3009.

169. Ring, K, 1970, Angew. Chem., 9:345.

170. Robinson, JH, 1976, J. Cell Physiol., 89:101.

171. Roblin, R, Albert, SO, Gelb, NA, and Black, PH, 1975, Biochem., 14:347.

172. Romano, AH, and Colby, C, 1973, Science, 179:1238.

173. Romano, AH, 1976, J. Cell Physiol., 89:737.

174. Ronquist, G, Ågren, G, Ponten, J, and Westermark, B, 1976, J. Cell Physiol., 89:433.

175. Roth, JD, Amster, RC, and Meisler, AI, 1974, Exptl. Cell Res., 85:57.

176. Rozengurt, E, and Pardee, AB, 1972, J. Cell Physiol., 80:273.

177. Rozengurt, E, and Jiminez de Asua, L, 1973, Proc. Nat. Acad. Sci., 70:3609.

178. Rubin, H, 1971, J. Cell Biol., 51:686.

179. Salter, DW, and Cook, JS, 1976, J. Cell Physiol., 89:143.

180. Salzman, NP, 1959, Biochim. Biophys. Acta, 31:158.

181. Sander, G, and Pardee, AB, 1972, J. Cell Physiol., 80:267.

182. Sanford, KK, 1974, J. Nat. Cancer Inst., 53:1481.

183. Schor, S, and Rozengurt, E, 1973, J. Cell Physiol., 81:339.

184. Schulman, JD, Wrathall, JR, Sigali, S, and Doores, L, 1974, J. Nat. Cancer Inst., 52:275.

185. Schultz, AR and Culp, LA, 1973, Exptl. Cell Res., 81:95.

186. Sefton, BM and Rubin, H, 1971, Proc. Nat. Acad. Sci., 68:3154.

187. Shodell, M, and Isselbacher, KJ, 1973, Nature New Biology, 243:83.

188. Snedecor, GW and Cochran, WG, 1967, Statistical Methods, 6th ed., Iowa State Univ. Press, Iowa, Chapter 6.

189. Spaggiare, S, Wallach, MJ, and Tupper, JT, 1976, J. Cell Physiol., 89:403.

190. Stambrook, PJ and Sisken, JE, 1972, J. Cell Biol., 52:514.

191. States, B, Harris, D, and Segal, S, 1974, J. Clin. Invest., 53:1003.

192. Stoien, JD and Wang, RJ, 1974, Proc. Nat. Acad. Sci., 71:3961.

193. Swaffield, MN, 1960, Arch. Biochem. Biophys., 86:219.

194. Tupper, JT, Mills, B, and Zorgniotti, F, 1976, J. Cell Physiol., 88:77.

195. Ullrey, D, Gammon, MT, and Kalckar, HM, 1975, Arch. Biochem. Biophys., 167:410.

196. Vaheri, A, Ruoslahti, E, and Nordling, S,1972, Nature New Biology, 238:211.

197. Venuta, S,and Rubin, H, 1973, Proc. Nat. Acad. Sci., 70:653.

198. Waller, JM and Kirsten, WH, 1971, Virchows Arch. B Cell Path., 6:183.

199. Wang, RJ, 1975, Photochem. and Photobiol., 21:373.

200. Waymouth, C, 1972, in Growth, Nutrition, and Metabolism of Cells in Culture, vol. I, ed. Rothblat, GH and Cristofalo, VJ, Academic Press, N.Y., p. 28.

201. Weber, MJ, and Edlin, G, 1971, J. Biol. Chem., 246:1828.

202. Weber, MJ and Rubin, H, 1971, J. Cell Physiol., 77:157.

203. Weber, MJ, 1973, J. Biol. Chem., 248:2978.

204. Weber, MJ, Hale, AH, Yau, TM, Buckman, T, Johnson, M, Brady, TM, and Larossa, DD, 1976, J. Cell Physiol., 89:711.

205. Wiebel, F, and Baserga, R, 1969, J. Cell Physiol., 74:191.

206. Wilkinson, GN, 1962, Biochem. J., 80:324.

207. Wohlhueter, RM, Marz, R, Graff, JC, and Plagemann, PGW, 1976, J. Cell Physiol., 89:605.

208. Yamada, KM, and Pastan, I, 1976, J. Cell Physiol., 89:827.