with methylcholanthrene in the carbowax vehicle. As shown in our table, 14, *i.e.* 24% of the "effective total number" of animals in Group II-A (receiving cortisone) developed tumors during the first 15 weeks of the experiment, as compared with 4 of 45 animals, or 9% in the control group, II-B.

Our results suggest that there was an additional increase in the number of tumor-bearing animals in the groups which received cortisone and in which excision-biopsies were performed at intervals. This was apparent particularly in the experiment with the benzene solution of methylcholanthrene (but was noticeable also in the experiment with the carcinogen in carbowax). Thus, in Group I-A (methylcholanthrene in benzene plus cortisone administration) 9 of 10 mice (90%) which had excisions performed, developed tumors by the end of the 9th week of the experiment—as contrasted with only 28 tumor-bearing animals (57%) among 49 mice ("effective total") which had *not* been subjected to biopsies. On the other hand, 2 (25%) of 8 surviving mice which had been subjected to biopsies in Group I-B (control experiment with methylcholanthrene in benzene, without cortisone administrations) showed tumors by the end of the 9th week, whereas 47% of the animals ("effective total") in this group which were *spared* biopsy excisions, had developed tumors at that time.

Summary. 1. Under the conditions of our experiments, the administration of cortisone to mice given external applications of methylcholanthrene in benzene or in carbowax 1500 was followed by some reduction of the early inflammatory response and by an increase in the incidence of epidermal tumor formation per number of animals, as compared with the results in the control experiments without cortisone injections. 2. Biopsy excisions performed in the methylcholanthrene-exposed skin areas appeared to increase still further the incidence of tumor formation in the mice treated with cortisone.

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Received March 5, 1953. P.S.E.B.M., 1953, v82.

Potassium Metabolism of Liver Mitochondria.* (20216)

S. W. STANBURY† AND GILBERT H. MUDGE.

From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York City.

It has long been postulated that the active transport of potassium (K) or of other alkali metal ions such as sodium (Na) by living cells involves the intermediate formation of ion-carrier complexes(1). More recently, in various cells and tissues, analysis of the kinetics of K exchange by the use of radioactive tracer (K^{42}) has suggested that intracellular K is not homogeneous but that it may consist of separate fractions exchanging at widely differing rates(2,3). Although this finding could be interpreted as indicating the formation of an ion-carrier complex, the evidence is inconclusive and it has become essential to attempt direct observations at the sub-cellular level. Mitochondria were selected for study because aerobic phosphorylation has been implicated in K accumulation by kidney slices(4) and by retina(5); and because the capacity for catalyzing aerobic oxidation and for the aerobic generation of phosphate bond energy has been localized for the most part to this fraction of

* Supported by a grant from the Rockefeller Foundation to Dr. J. V. Taggart. Miss Frances Gillman rendered invaluable technical assistance. Preliminary reports presented at the Potassium Symposium, University of Minnesota, Sept., 1952, and at the 4th Conference on Renal Function, Josiah Macy, Jr., Foundation, Oct., 1952.

† Rockefeller Fellow on leave of absence from the Department of Medicine, University of Manchester, England.

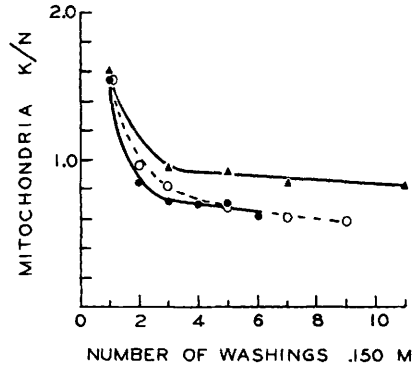

FIG. 1. Changes in mitochondrial K/N ratio following repeated washes in 0.15 M NaCl at 2°C. The 3 curves represent three different experiments. The mean K/N ratios after 3 washings were: 25 experiments with 0.15 M NaCl, 0.79 S.D. ± 0.14; 21 experiments with 0.075 M NaCl, 0.77 S.D. ± 0.06. Under the same conditions the K/N ratio of the nuclear fraction was about one-third that of the mitochondrial, but contamination of the former by the latter fraction cannot be excluded. In 8 determinations the mean K/N ratio of the intact liver was 3.3.

tissue homogenates(6). Relatively high concentrations of K have been previously reported for liver mitochondria(7) and for the protoplasmic granules of Nitella(8), but no systematic studies have been made of the metabolic characteristics of this fraction of cell K. Direct evidence has been obtained in our studies that there is "non-exchangeable"‡ K associated with liver mitochondria and that the turnover of this K in an *in vitro* system is in part dependent on aerobic metabolism.

Methods. Mitochondrial suspensions were prepared from rabbit liver by minor modifications of the technic of Lehninger(9). After homogenization in ice-cold 8.5% sucrose, the nuclear fraction was spun down in a refrigerated centrifuge at 600 x G for 5 minutes and was discarded; the supernatant was adjusted to 0.15 M NaCl by the addition of 1 M NaCl, and the mitochondrial fraction deposited by centrifugation at 1,600 x G. The mitochondria were then washed by resuspension in 10 volumes of chilled 0.075 M NaCl; similar results were obtained using 0.15 M NaCl (Fig. 1). The final mitochondrial deposit was suspended in a small amount of saline and incubated in Warburg vessels according to standard technics. Samples of fresh mitochondrial suspension and of mitochondria harvested after incubation were taken for electrolyte determination (internal standard flame photometry) and for nitrogen analysis (micro-Kjeldahl); results are expressed as mEq of electrolyte/g of mitochondrial N. The sample for electrolyte analysis was digested in conc. HNO_3 and the volume made up to 10 ml; when radioactive isotopes were used 2 ml of this dilution was taken for counting with an end-window Geiger-Muller tube. In each experiment the electrolyte content and radioactivity of the separated incubation medium were determined directly. The extent of equilibration between mitochondrial and medium electrolyte was expressed as the specific activity-ratio; this was calculated as specific activity of electrolyte in the mitochondria divided by specific activity of electrolyte in the medium. Oxygen consumption (qO_2) was calculated as μl oxygen consumed per hour per mg mitochondrial N.

Results. It was not possible to obtain a K free suspension when mitochondria were washed repeatedly with cold NaCl solution during their preparation; the mitochondrial K/N ratio of approximately 0.80 remained virtually constant between the third and tenth wash (Fig. 1). This "non-exchangeable" K of the cold mitochondria constituted at least 1.8% of the total K of rabbit liver; this is a minimal estimate based on an assumed complete recovery of the mitochondria during their preparation.

Suspensions of thrice washed *mitochondria* (M_3L) were incubated in the Warburg apparatus at 25°C. In the standard procedure each vessel contained 1 ml M_3L (5-7 mg N), 30 μM Na α-ketoglutarate, 60 μM tris (hydroxymethyl) aminomethane buffer (pH 7.4), 5 μM $MgCl_2$, 75 μM KCl, trace amounts of K^{42} and water to a final volume of 3 ml; oxygen in the gas phase. Although cytochrome-C was added in some experiments no requirement for it could be established and it was generally

‡ The expression "bound K" is avoided since it tends to have a specific physico-chemical connotation. "Non-exchangeable" throughout the present text may be taken to imply "indiffusible" or "not participating in simply physical exchange with ambient cations". This definition does not preclude exchange mediated by the expenditure of energy derived from metabolic processes.

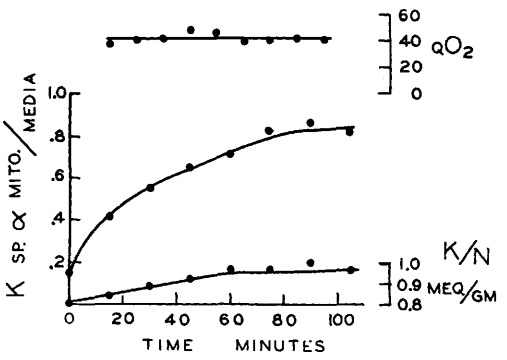

FIG. 2. Equilibration of mitochondrial K with medium K during aerobic incubation. Cup components as in standard procedure, see text.

omitted. Following incubation, flask contents were decanted into 100 ml plastic tubes, flasks rinsed with chilled 0.15 M NaCl and the mitochondria deposited by centrifugation at 2°C. Harvested material was washed 3 times with 75 ml of cold saline and then analysed for radioactivity, total K and N. *Equilibration* between mitochondrial K and the labelled K of the medium was about two-thirds complete with this system after 60 minutes incubation (Fig. 2). In 22 experiments the average K/N ratio after 60 minutes incubation was 109% of the pre-incubation value. Thus a biological steady state can reasonably be assumed even though a precise kinetic analysis cannot be made because of the time required to "harvest" the mitochondria by this technic.

The contributions made by each of the components in this test system are indicated in Table I. The highest K/N ratios and most complete K exchange were obtained under conditions which permitted aerobic oxidation of added substrate. Comparable results were obtained using α-ketoglutarate, citrate or succinate. In several experiments with α-ketoglutarate, anaerobic incubation yielded K/N ratios approximately 60% of the values obtained with the complete system, and the specific activity ratio was correspondingly reduced. Anaerobic incubation with succinate produced similar but less regular results. No systematic attempt has yet been made to study the effects of anaerobic incubation in the presence of various other substrates. It is conceivable that the K turnover found in the absence of added substrate (Table I) was associated with the oxidation of endogenous substrate but the significance of this has not been explored. Although the addition of adenosine-5-phosphate (AMP) or adenosinetriphosphate (ATP) greatly increased respiration, these compounds had no apparent effect on K metabolism and were therefore not routinely employed.

When mitochondrial K had been actively labelled with K^{42} during the period of incubation, it was found that the final radioactivity was the same whether the harvested mitochondria were washed at 2°C with 0.15 M NaCl or with 0.15 M KCl (Table II). This experiment demonstrates the exchangeability of mitochondrial K in a metabolizing system and its failure to exchange with either Na or K during the washing procedure. However, certain inconsistencies are noted when attempting to correlate K turnover with the degree of oxidative activity. During incubation, the procedures which reduced aerobic metabolism

TABLE I. Component Study. For additions see *standard procedure* in text. Mitochondrial K/N ratio was 0.86 before incubation. Incubated 60 minutes at 25°C. With the complete system the average specific activity ratio in 20 experiments was 0.65 after 60 minutes incubation.

	Values after incubation		
	qO_2	K/N	Specific activity ratio
Complete system	43	.90	.64
No α-ketoglutarate	15	.47	.70
" $MgCl_2$	40	.73	.50
" Tris buffer	42	1.05	.53
" cytochrome	39	.86	.71
" K^{39} added	41	.50	.09
" oxygen	2	.59	.46

TABLE II. Incorporation of K^{42} into Mitochondria. After paired aliquots of mitochondria had been incubated with α-ketoglutarate for 1 hour, one sample washed 3 times with 75 ml of isotonic NaCl (2°C); the other sample washed with an equal volume of KCl, which represents a thousand-fold excess of K for each wash. For discussion see text.

	Treatment of mitochondria after incubation			
	Washed in NaCl			Washed in KCl
Exp.	K/N, mEq/g	Sp. activity ratio, mito/media	K^{42}, cts/mg N	K^{42}, cts/mg N
1	1.02	.82	136	104
2	.81	.71	152	154

only partially inhibited K exchange (Table I), while virtually complete inhibition of exchange is indicated for the cold, non-metabolizing mitochondria by the type of experiment shown in Table II. An explanation for these inconsistencies is not immediately at hand.

A variety of experimental variables was examined using the incubation procedure described above. K metabolism was not detectably influenced by a change in pH between 7.2 and 7.6, but below pH 7.0 and above 8.0 the K/N ratio was reduced with a parallel depression in respiration.

Variations in osmotic pressure between 165 and 300 mOsM/1 had no significant effect on respiration or mitochondrial K. With higher osmotic pressures (produced by the addition of either NaCl or sucrose) there was a progressive fall in the K/N ratio and in the oxygen uptake, so that at 400 mOsM/1 these values were about 30% of the controls.

In the standard experiment the final K concentration in the medium was 25 mEq/1; this was selected because of the constancy of the K/N ratios and the magnitude of K exchange. When lower concentrations of K were used there was a slight reduction in the K/N ratio but a more marked effect on the exchange reaction (Table I). The effects of higher K concentrations have not yet been systematically examined.

In a system to which no $MgCl_2$ had been added, the presence of $CaCl_2$ (final concentration 3 x 10^{-4} M) reduced markedly both the rate of oxygen consumption and the level of K maintained by the mitochondria. The addition of an excess of $MgCl_2$ (final concentration 1.5 x 10^{-3} M) prevented the inhibitory effects of Ca on oxygen consumption; in some experiments depression of the K/N ratio was prevented, but this was not constant.

A surprising observation was the profound effect of inorganic orthophosphate on mitochondrial K (Fig. 3). In confirmation of the work of many others, orthophosphate was found to stimulate respiration but at the same time there was a marked lowering of the K content of the harvested mitochondria. No explanation for the latter effect can be given at present but it may be pointed out 1) that the K/N ratios fell to levels much lower than

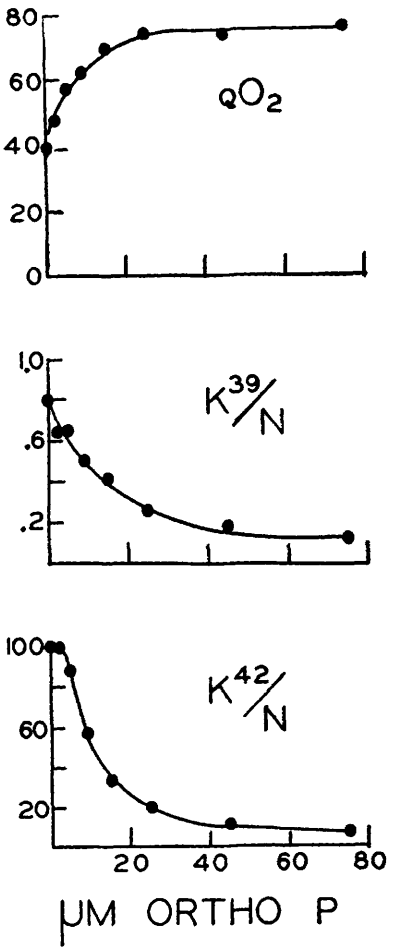

FIG. 3. Effect of orthophosphate on mitochondrial K. Standard procedure except for additions of orthophosphate, indicated as μM added per cup (final volume 3.0 ml). Osmotic pressure kept constant at 240 mOsM/1 by variations in NaCl.

those obtained during anaerobic incubation and 2) that a maintained high level of mitochondrial K is apparently not essential for aerobic metabolism under these circumstances.

Various observations, including the effects of the addition of orthophosphate described above, suggested the possibility that changes in phosphorus metabolism were specifically concerned in the turnover of mitochondrial K. Previous work had also implicated aerobic phosphorylation as a determinant in the maintenance of normal levels of tissue K. A study was made, therefore, of the effect of 2,4-dinitrophenol (DNP), measuring simultaneously the changes in both K and phosphate metabol-

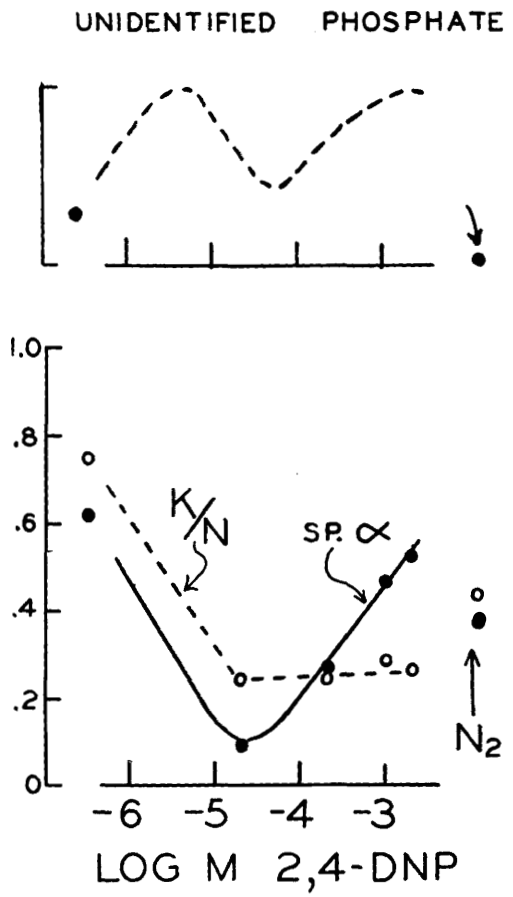

FIG. 4. Effect of DNP on mitochondrial K. The relative amount of orthophosphate esterified under these conditions is shown schematically at top. Comparison with anaerobic incubation (without DNP) is indicated by N_2.

ism. These experiments revealed a previously undescribed polyphasic effect of DNP on phosphate metabolism and on respiration; they also suggest a relationship between K exchange and the formation of certain phosphate esters. Very low and high concentrations of DNP each increased the esterification of added orthophosphate§ in the absence of added phosphate acceptors and produced a considerable augmentation of oxygen consumption; at intermediate concentrations, esterification and respiratory stimulation were minimal. The studies in phosphate metabolism will be described in detail elsewhere but the observed changes are presented schematically in Fig. 4 to permit comparison with the changes in K. Low concentrations of DNP (10^{-5} M) *increased* the amount of orthophosphate which disappeared during incubation and there was a simultaneous reduction in the concentration of mitochondrial K. Intermediate concentrations (10^{-4} M) of DNP, which are known to inhibit oxidative phosphorylation, were found to diminish further the concentration and exchange of mitochondrial K and to *decrease* orthophosphate esterification. The specific activity ratio of K in the harvested mitochondria at this concentration of DNP (10^{-4} M) was very low, much less than that obtained following incubation without oxygen. When the DNP concentration was increased to 10^{-3} M there was a marked stimulation of respiration and esterification of orthophosphate again *increased,* as did the apparent exchange of mitochondrial K. Further studies have shown that the phosphate ester thus formed is completely hydrolyzed by N HCl in 40 minutes at 100°C. The greater part of the ester formed could be recovered from the incubation medium, but separate analysis of washed mitochondria showed that incubation in the presence of 10^{-3} M DNP produced an increase in the 40-minute hydrolyzable phosphate of the actual mitochondrial particles. Since this concentration of DNP also increased the rate of exchange of mitochondrial K it seems possible that the two phenomena are related.

Further studies are now in progress to clarify the precise relationship of K to the phosphate fractions of the mitochondria. It may be noted that DNP has virtually no effect on the small amount of Na demonstrable on the mitochondria (Fig. 5). The effect on K appears to be specific.

Discussion. It is considered that the observations described warrant the conclusion that there is a fraction of cell K, associated with the mitochondria, which may be "non-exchangeable" under certain circumstances but which is capable of exchange under appropriate metabolic conditions. When metabolism

§ In a system without added orthophosphate DNP produced similar effects on respiration and phosphate esterification: the "apparent orthophosphate" estimated (Fiske and SubbaRow) in trichloracetic acid extracts of harvested mitochondria showed a completely comparable polyphasic variation.

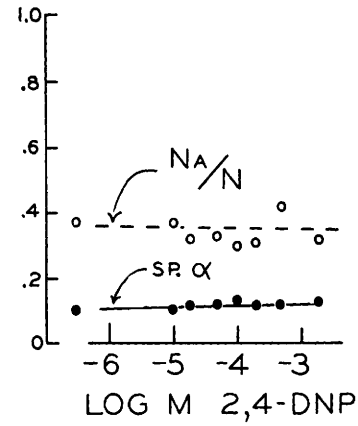

FIG. 5. Effect of DNP on mitochondrial Na. Conditions as in standard procedure except that after incubation the mitochondria were washed three times with chilled 0.15 M KCl, instead of NaCl. In 5 experiments the mean values of the control (*i.e.* without DNP) were: Na/N, 0.34; and specific activity ratio, 0.17. Under these conditions the mitochondria contain much less Na than K, and its rate of exchange is very low.

is suppressed, both by cooling to 0°C and by simultaneous dilution of substrate, this K is apparently indiffusible and unavailable for exchange with either the Na or K of the wash fluid; during active metabolism the same fraction is in a state of dynamic exchange with ambient K. Although the highest K/N ratios and the highest rates of exchange were obtained under conditions which permitted oxidation of added substrate, certain observations indicated that the behavior of mitochondrial K could not be correlated directly or solely with oxygen uptake. Most striking were the extremely low K/N ratios obtained with added orthophosphate or after incubation with DNP concentrations higher than 10^{-4} M. In both instances, although oxygen consumption increased, the K/N ratio in harvested material was lower than that observed after anaerobic incubation. In addition, there was no correlation between K turnover and the respiratory stimulation produced by AMP, nor between K/N values and the increased oxygen consumption and phosphate esterification noted with 10^{-5} M DNP.

It must be emphasized that in a study of this kind the precise results obtained may be determined largely by the chosen experimental conditions. It is, therefore, not surprising that others have reached conclusions quite different from our own. Thus, Harmon(10) concluded that the mitochondria were fully permeable to both Na and K and that accumulation did not occur even during aerobic metabolism. It may be noted that his analyses were limited to determination of radioactivity and that the washing procedures were less thorough than ours; moreover, all experiments were carried out in the presence of a concentration of orthophosphate which we have found to depress the level of mitochondrial K. While the present paper was in preparation Bartley and Davies(11) reported that the establishment of concentration gradients for a variety of ions, including K, by kidney mitochondria was dependent on metabolic activity. Both their experimental technics, and the results obtained differ from those of the present investigation. From the standpoint of technic it is noteworthy that they incubated in the presence of orthophosphate (0.01 M) and that after incubation the mitochondrial material was centrifuged down at 20°C and the deposit sampled for analysis without further washing. With this technic the mitochondria were sampled while metabolism continued and the sample must have contained diffusible potassium in the extra-mitochondrial fluid; with our own technic, metabolism was arrested and any diffusible K in extra-mitochondrial fluid was presumably replaced in the course of washing by the Na of the wash fluid. Thus, whereas we were dealing with a very few micro-equivalents of K firmly associated with the mitochondria, the K content of the samples taken by Bartley and Davies may have been largely determined by the K concentration in the original incubation medium. The K concentration used by them is not reported; but if this was high it is conceivable that the "immeasurably high" rates of K exchange which they observed represented equilibration between added K^{42} and diffusible K in the fluid *between the mitochondrial particles*. The slower turnover of the smaller amount of K *on the mitochondria* may have been completely masked. Extension and comparison of the two technics should provide further insight into the problem.

No attempt will be made to integrate all the

described phenomena; in fact, some of our observations as yet elude adequate explanation. Nonetheless, it has been demonstrated that mitochondria have an individual K metabolism and certain apparently specific mechanisms have been elicited. The observations on the effects of DNP at high concentrations suggest that complexes may be formed between mitochondria, phosphorylated derivatives and K; breakdown and resynthesis of such complexes could be responsible for the observed exchange of mitochondrial K. If such a mechanism occurs in the intact cell it could conceivably be utilized for the function of K accumulation; these mitochondrial mechanisms may constitute an "ion-complex carrier" for K.

Summary. Rabbit liver mitochondria contain K which cannot be removed by repeated washings with NaCl solutions at 2°C. This K fraction exchanges with ambient K under appropriate metabolic conditions, indicating a dependence of K metabolism on aerobic oxidation. Orthophosphate depresses the mitochondrial K level. A newly observed polyphasic action of DNP indicates a specific relationship between mitochondrial ester phosphate and K exchange.

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Received March 5, 1953. P.S.E.B.M., 1953, v82.

Determination of Deuterium Exchange Rates Between Maternal Circulation and Amniotic Fluid.* (20217)

ALBERT A. PLENTL AND DONALD L. HUTCHINSON.
From the Department of Obstetrics and Gynecology, Columbia University College of Physicians and Surgeons, New York City.

The function of the amniotic fluid is clearly defined but its origin, rate of formation and its fate represent controversial subjects among investigators in this field. Morphologic studies on cellular structures involved did not supply an adequate body of information to explain the formation of large amounts of amniotic fluid in certain pathologic states nor could it explain the rapid rate of replacement in the normal pregnant organism as demonstrated by Flexner and his associates(1). These authors reached the conclusion that the amniotic fluid is completely replaced once every 2.9 hours. Such a rapid turnover rate is contrary to the prevailing opinion that the amniotic fluid represents a stagnant pool or consists of fetal urine which is swallowed by the baby. Since it would be difficult to present an acceptable technical, mechanical or morphologic explanation for the phenomenon described by Flexner, a critical examination of these experiments is in order. As a working hypothesis, one may regard the pregnant organism as a multi-compartment system which should lend itself to a mathematical approach provided certain reservations are kept in mind. Flexner and associates considered this approach but apparently discarded it in favor of approximations which are not entirely above criticism. The theoretically correct treatment of multicompartment sys-

* This investigation was supported by a Research Grant from the National Institutes of Health, Public Health Service.