Intracellular Phosphorus Turnover in the Rat Liver Cell.* (20487).

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With the advent of the technic of differential centrifugation for the preparation of large quantities of the sub-cellular particulates in isolated form, it has become possible to study quantitative aspects of the metabolic turnover of biochemical constituents at the level of the cell rather than at the tissue level. The present work represents an attempt to make a broad preliminary survey of the metabolic fate of radiophosphorus labeled phosphate in the rat liver cell following intravenous injection of the labeled phosphate.

Four intracellular fractions from rat liver have been investigated: mitochondria, microsomes, supernatant, and a crude nuclear fraction. The distribution of P32 in these fractions at different times after its injection into the animal as phosphate has been determined. As a preliminary step in the investigation of the individual substances in which P32 is turned over, the phosphorus compounds of each cell fraction have been chemically separated into the 4 following groups: the acid-soluble compounds, the phospholipids, the nucleic acids, and the protein residue. Thus it has been possible to construct time-distribution curves for labeled phosphorus in the various liver cell fractions, and within these, among the various chemical phosphorus fractions. Additional data has also been obtained on the dry weight, nitrogen content and total phosphorus content of the cell fractions from whole livers.

Experimental. Non-fasted male rats of the Curtis-Dunning strain weighing 250 to 300 g were used. For the preparation of the cellular fractions, livers previously perfused with cold Tyrode's solution were forced through a stainless steel sieve which retained the connective tissue, and the pulp was homogenized for 5 minutes in 0.88 molar sucrose in a glass homogenizer fitted with a water-cooled jacket. In general, 5 to 6 g of liver pulp were made up to a volume of 50 ml with sucrose.

Preparation of the cellular fractions. Essentially the method of Hogeboom *et al.*(1) was used for the differential centrifugation of the homogenate. The nuclear fraction was sedimented by centrifuging the homogenate twice for 15 and 10 minutes respectively at 600 x g in an "International" clinical centrifuge fitted with an angle-head rotor. The sediments from these 2 low-speed centrifugations were combined and washed twice with sucrose and constituted the washed crude nuclear fraction. The mitochondrial fraction was obtained by centrifuging the original supernatant removed from the nuclear fraction combined with the 2 washes, at 10,000 x g for 30 minutes in a "Servall" vacuum-type angle centrifuge. The sediment was washed once. The supernatant removed from the mitochondrial fraction, and from the subsequent washing of the mitochondria, was centrifuged for 30 minutes at 100,000 x g in a "Spinco" preparative centrifuge in order to sediment the microsomes. The microsomal fraction was washed once and resedimented. The entire operation of perfusion, homogenization and centrifugation was carried out in the cold at 2°C. Histological examination showed that the fractions obtained corresponded in appearance and staining properties with those described by Claude (2) and by Hogeboom and co-workers(1).

Dry weights of the cellular fractions. Modification of the above procedure was necessary in order to obtain the dry weights of the fractions free from sucrose. For each fraction, the initial centrifugation was carried out in sucrose, to ensure that the same fraction was being sedimented, but all washings were with 0.9% sodium chloride brought to a pH of 7.6 to minimize agglutination of granules. Sodium chloride was used rather than water because of the tendency for granules to disintegrate in water(2). No effort was made to allow for the small amount of sodium chloride con-

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TABLE I. Mean Values for Dry Weight, Nitrogen and Phosphorus Content of Cellular Fractions from 6 Rat Livers.

		Homogenate	Nuclei	Mitochondria	Microsomes	Supernatant	Recovery
Dry wt	Total g	2.37	.44	.75	.41	.77*	—
	% of total	100	18.7	31.0	17.4	32.5 *	
Nitrogen	Total mg	251.8	35.8	78.0	29.5	102.3	97.4
	% of total	100	14.3	31.0	11.7	40.4	
Phosphorus	Total mg	26.9	4.2	7.6	5.0	9.6	97.5
	% of total	100	15.6	28.3	18.7	34.9	
P/N ratio		.11	.12	.10	.17	.09	

* By difference, because of the presence of sucrose.

taminating the granules, which in no case amounted to more than 1 or 2% of the total dry weight of the fraction. The washed nuclei, mitochondria and microsomes were transferred to tared flasks and were dried to a constant weight at 90°C to 100°C. The dry weight of the supernatant was obtained by difference, because of the presence of the bulk of the sucrose in this fraction.

Nitrogen determinations. Nitrogen was determined by a micro-Kjeldahl method essentially as described by Umbreit(3). Steam distillation was carried out into a boric acid solution, using an indicator containing bromcresol green and methyl red, which gives a sharp end point.

Phosphorus determinations. The method of Sumner(4) was used for all phosphorus determinations. During digestion of the material with sulfuric acid and hydrogen peroxide, foaming due to large amounts of sugar present in some fractions was prevented or controlled by use of caprylic alcohol.

Analysis of the phosphorus compounds. The method of fractionation of phosphorus compounds into 4 main groups, proposed by Schneider was employed(5). Acid-soluble compounds were extracted with cold 10% trichloroacetic acid, followed by extraction of phospholipids with alcohol and ether, followed by extraction of nucleic acids with hot 5% trichloroacetic acid. The residue was dissolved in 1% sodium hydroxide and will be referred to as the protein residue. The phosphorus of each chemical fraction was determined as previously described. All samples were analyzed in duplicate.

P32 determinations. Approximately 20 μc (10^7 counts per minute) of high specific activity radioactive phosphorus in the form of NaH_2PO_4 were injected into the tail veins of the rats from 30 minutes to 48 hours before sacrificing. The 4 chemical fractions made from each cellular fraction were neutralized where necessary and placed on lens paper in the center of aluminum plates. All counting was done with a Geiger-Müller counter tube with a thin end window. Because this was a preliminary study designed only to indicate the overall picture of phosphorus exchange within the cell, no attempt was made to separate further the chemical fractions before determining the P32 content.

Results. Aliquots of the homogenate prepared from each of 6 rat livers were used for the dry weight, phosphorus and nitrogen determinations shown in Table I. The dry weight of the crude nuclear fraction varied from 14.9% to 22.1% of the dry weight of the homogenate, with a mean value of 18.7%. The mitochondrial fraction accounted for 27.4% to 35.0%, with a mean value of 31.5%, while the microsomes averaged 17.4% with a range of 14.7% to 21.0%. By difference, the supernatant was found to constitute a mean of 32.5% of the dry weight of the homogenate.

The mean phosphorus and nitrogen contents of the cellular fractions are also shown in Table I, both in terms of total milligrams present, and as percentages of the total phosphorus or nitrogen present in the liver. The mean phosphorus content of the whole liver, for this series of rats, was found to be 26.9 mg, 15.6% of which was in the crude nuclear fraction, 28.3% in the mitochondria, 18.7% in the

TABLE II. Phosphorus Distribution in Cellular Fractions of Rat Liver.*

	Homogenate	Nuclei	Mitochondria	Microsomes	Supernatant
Total P	30.11 (24.23-36.75)	4.60 (3.90-5.91)	6.89 (6.17-9.30)	6.13 (4.57-8.07)	12.01 (8.62-14.73)
Acid-soluble P	7.56 (5.96- 9.45)	.35 (.26- .50)	1.30 (1.08-1.62)	.11 (.07- .16)	5.82 (3.40- 7.63)
Phospholipid P	13.04 (10.40-16.50)	1.43 (1.14-1.85)	3.74 (3.20-5.25)	3.46 (2.50-4.32)	3.85 (2.94- 4.98)
Nucleic acid P	7.81 (6.38- 9.71)	2.41 (2.05-3.15)	1.28 (1.04-1.95)	2.26 (1.28-3.44)	1.88 (1.23- 2.66)
Protein residue P	1.23 (.91- 1.53)	.25 (.16- .33)	.51 (.41- .60)	.13 (.06- .23)	.24 (.11- .33)

* The values are means from 10 rats and are expressed as mg P per whole liver. The range is given in parenthesis below each mean.

TABLE III. Phosphorus of Chemical Fractions Expressed as % of Phosphorus of a Given Cellular Fraction.*

	Homogenate	Nuclei	Mitochondria	Microsomes	Supernatant
Total P, %	100	100	100	100	100
Acid-soluble P, %	25.1	7.6	18.9	1.8	48.5
Phospholipid P, %	43.3	31.1	54.3	56.4	32.1
Nucleic acid P, %	25.9	52.4	18.6	36.9	15.7
Protein residue P, %	4.1	5.4	7.4	2.1	2.0

* Mean values from 10 rats.

microsomes, and 34.9% in the supernatant. The same livers contained, on an average, 251.8 mg of nitrogen, 14.3% of which was in the nuclei, 31.0% in the mitochondria, 11.7% in the microsomes, and 40.4% in the supernatant.

Tables II and III show the intracellular distribution of phosphorus in the livers of a second series of rats. In Table II, the total phosphorus content of the cellular fractions is given in mg and this is broken down between the different chemical fractions. The mean and range for 10 rats are given. Although the wet weight of the livers of the 10 rats varied not very greatly, the phosphorus content ranged from 24.2 mg to 36.7 mg per liver. Where the phosphorus content was low the fraction most affected was invariably the supernatant. Comparison of Tables I and II shows that the mean values for the phosphorus contents of the cellular fractions were not exactly the same for the two series of rats; however, the differences between the means were not statistically significant by the *t* test.

In Table III, the chemical form in which the phosphorus is present in each cellular fraction is stressed by expressing the phosphorus content of a given chemical fraction as a percentage of the total phosphorus of the cellular fraction from which it was obtained. This brings out more clearly several points. Each cellular fraction, including the supernatant, contains a high percentage of phospholipids, which in the case of the mitochondria and microsomes amounts to more than 50% of the total phosphorus present. The supernatant contains a very high percentage of acid-soluble phosphorus, which might be expected, but the microsomes contain extremely little (1.8% of their total phosphorus), as compared with the mitochondria in which 18.9% of the total phosphorus is acid-soluble. The reverse is true of the nucleic acid content, where nucleic acid phosphorus accounts for 36.9% of the phosphorus of the microsomes but only 18.6% of the phosphorus of the mitochondria. Protein residue is low in all fractions. It averages 7.4% of the total phosphorus in the mitochondria but only 2.1% and 2.0% in the microsomes and supernatant respectively.†

Uptake of P32 by the cellular fractions.

† The amount of protein residue phosphorus obtained depends upon the method of extraction used. The Schneider method used here gives values 10 times greater than those obtained by the Schmidt-Thannhauser method(6).

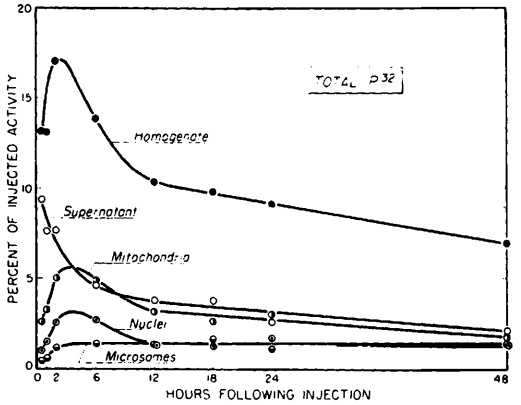

FIG. 1. Distribution of total P32 activity in various fractions of rat liver cells as a function of time following injection.

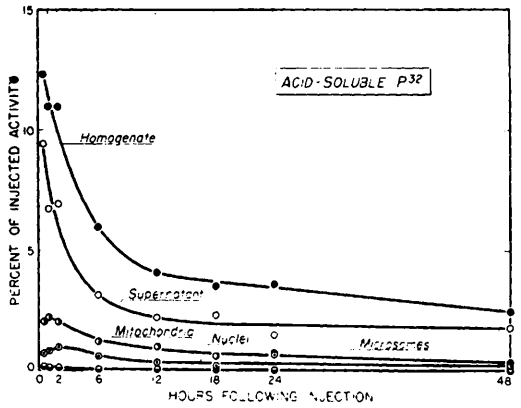

FIG. 2. Distribution of acid-soluble P32 activity in various fractions of rat liver cells as a function of time following injection.

Fig. 1 to 4 show the % distribution of the total injected dose of P32 in the cellular fractions of the rat liver one-half hour to 48 hours after intravenous injection of P32. Each point represents the mean value of 2 rats. From Fig. 1 it appears that the P32 reaches a maximum in the liver as a whole about 2 hours after intravenous injection. In the first few hours there is a similarity in the curves of the mitochondrial and crude nuclear fractions, which both take up P32 rapidly and reach a maximum at about 4 hours after injection. The microsomes on the other hand take up relatively little P32, very slowly. The P32 uptake of the supernatant follows an entirely different course, reaching a maximum some time before one-half hour after injection, and dropping off very rapidly at a time when the P32 is being incorporated most rapidly into the particles.

The incorporation of P32 into the acid-soluble compounds of the cellular fractions is shown in Fig. 2. This includes both inorganic and esterified phosphate compounds. Quantitatively, the supernatant is clearly the most important component at all times, while the nuclei and particularly the microsomes are relatively unimportant.

Fig. 3 gives the distribution of phospholipid P32 among the cellular components. The most noticeable features are the similarity of the curves for all fractions including the supernatant, the maximum occurring in all cases about 6 hours after injection.

The distribution of nucleic acid P32 is

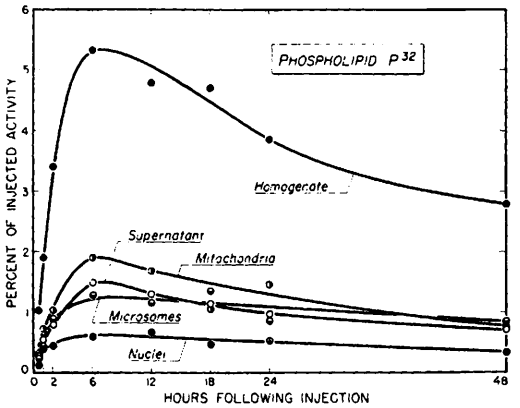

FIG. 3. Distribution of phospholipid P32 activity in various fractions of rat liver cells as a function of time following injection.

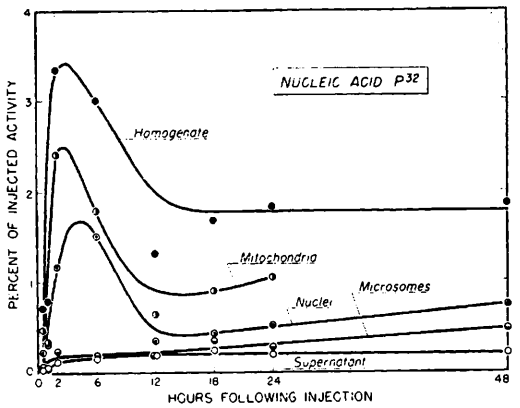

FIG. 4. Distribution of nucleic acid P32 activity in various fractions of rat liver cells as a function of time following injection.

given in Fig. 4. This fraction sometimes showed considerable variation between 2 rats sacrificed at the same time, and in general seems to be a less constant component than the phospholipids or acid-soluble compounds. Initially, only a very small amount of P32 is found in the nucleic acids of the microsomes and supernatant compared with the nuclei and mitochondria. Incorporation into the mitochondrial fraction is the most rapid, a maximum being reached 2 to 3 hours after injection, as compared with 5 to 6 hours for the nuclei, and considerably longer for the microsomes and supernatant.

Data for the protein residue are not included because the amounts of radioactivity and phosphorus were in some cases so small that accurate determinations were not feasible. However, the high specific activity that has frequently been reported(7) when the Schmidt-Thannhauser method of separation is used was not found in this investigation where the Schneider extraction method was employed. The role of this class of compounds in the metabolism of the cell will remain obscure until their chemical nature is more rigorously defined.

Discussion. There is considerable variation in the dry weights of all cellular fractions. This is particularly noticeable in the crude nuclear fraction, where it can be ascribed in part to deficiencies in the separation procedure, since this fraction is not pure and may well contain varying numbers of unbroken cells. Nuclear volumes from 10 to 18% of the liver cell volume have been reported(8) so the dry weight figure of 18.7% of the dry weight of the liver is probably not much too high. Although the nuclear fraction is admittedly impure, nevertheless its properties are probably largely those of pure nuclei. The results with this fraction are included for general comparative purposes, and to complete the quantitative recovery figures.

The situation is somewhat different with regard to the mitochondria. Losses were kept to a minimum by reclaiming mitochondria from the nuclear washes, and although Jackson *et al.*(9) have found that repeated washing is necessary to rid the mitochondria of all sub-microscopic particles, the degree of contamination involved here is not sufficiently great to account for the observed variations in dry weights. Many conditions such as nutritional status(10), or glycogen-glucose balance(11), have been shown to influence the state or number of the mitochondria of various cells, and this would clearly be reflected in the dry weight.

Greater variation was found in the dry weights of the microsomes than of the mitochondria. The rats used in this experiment were non-fasted as this was considered preferable for the subsequent experiment where the animals were sacrificed from one-half hour to 48 hours after the injection of P32. Changes in liver metabolism, for example the utilization of glucose, have been shown to occur with prolonged fasting(12). However the disadvantage of using non-fasted rats is that glycogen granules are present in the liver and these appeared largely in the microsome fraction. Variation in the amount of glycogen present greatly affected the dry weight of this fraction. Another experiment in which fasted rats were used gave much more consistent and considerably lower values for the dry weight of the microsome fraction.

In the crude nuclear fraction the nitrogen content was 14.3% of the total nitrogen of the homogenate, which is in quite close agreement with Schneider's finding of 13.3%(13). Many figures are available for the nitrogen content of the mitochondria and they range between 23% and 26% of the total nitrogen. However, after correcting for losses, Schneider and Hogeboom(14) have estimated that 30 to 35% of the total nitrogen is present in this fraction. Their estimate agrees well with the value of 31% found in this investigation, where losses were kept to a minimum.

The nitrogen and phosphorus values for the microsomes reported here are considerably lower than those found in some investigations where centrifugation was more prolonged and smaller particles were sedimented(1). The dry weight of this fraction can clearly be increased by increasing the time or speed of centrifugation. This acceleration of 100,000 x g for 30 minutes used in the present experiments was somewhat arbitrarily chosen, in order to reduce the time of separation to a

reasonable minimum. On the other hand, the high phosphorus to nitrogen ratio, which is characteristic of microsomes, was obtained, being 0.17 for the microsomes as compared with 0.10 for the mitochondria and 0.09 for the supernatant.

The data presented in Tables II and III reveal a number of points concerning the partition of phosphorus compounds between the cellular components. It is not surprising that there is a large amount of acid-soluble phosphorus present in the supernatant, as it would be expected that a wide variety of relatively small, phosphorus-containing molecules is present in the non-granular part of the cytoplasm. But the very large amount of these substances present in the mitochondria is striking, especially when compared with the extremely small amount associated with the microsomes. It may be that the presence of semi-permeable membranes around the mitochondria permits these granules to retain acid-soluble substances where no such mechanism exists in the smaller granules. It was noticed that the acid-soluble extract of the mitochondria was quite green compared with other fractions; possibly they are responsible for the elaboration of some of the constituents of bile.

It can be seen from Table I that phospholipids constitute the major group of phosphorus-containing compounds in the liver cell, accounting for 43% of the total phosphorus. They are more concentrated in the granules than in the nuclei or cytoplasm, but, unlike the acid-soluble compounds they occur in quantity in every cellular component.

There has been some question as to whether all the nucleic acid of the cell is bound to particles, or whether some is free in the supernatant. Table I shows that a considerable amount of nucleic acid, 24%, is not sedimented by centrifugation at 100,000 x g for 30 minutes, and several investigators have confirmed the presence of nucleic acid which is nonsedimentable at even higher speeds(14, 15). However, Szafarz(16) has shown that this nucleic acid does not exist in the supernatant in the form of relatively small molecules, but is bound to proteins by stable bonds analogous to those existing in mitochondria and microsomes.

A large number of somewhat conflicting estimates are given in the literature for the nucleic acid content of cell particles(14). The data presented in Table II support the general view that the highest concentration of nucleic acid occurs in the microsomes, with considerably lower values for the mitochondria and supernatant.

Considering the more dynamic aspect of phosphorus distribution, Fig. 1 to 4, showing the incorporation of P32 into the cellular components, reveal several interesting points. Because the purpose of this investigation was to make a broad survey of the outlines of phosphorus metabolism within the cell, rather than an attempt to establish in accurate detail the turnover of any one class of compounds, only the general nature of the curves obtained should be considered. Furthermore, no attempt was made, at this time, to separate inorganic phosphate from the remainder of the acid-soluble fraction. Therefore the activity of this fraction represents not only the incorporation of P32 into esterified compounds, but also the penetration into the cell fractions of the original injected phosphate. However, it appears that after the first 2 or 3 hours, the bulk of the acid-soluble P32 is in a bound form, as the specific activity of the supernatant acid-soluble phosphorus is, by that time, lower than that of any other fraction.

Labeled phosphorus enters the liver cell very soon after injection and the activity of the phosphorus of the non-particulate cytoplasm rises immediately. Incorporation of P32 into the mitochondria and nuclei follows rapidly, but proceeds more slowly in the microsomes. The specific activity of the phosphorus of the microsomes was at all times lower than that of the other fractions. This rapid incorporation of P32 into the mitochondria as compared with the microsomes, is in keeping with the many metabolic activities ascribed to them. There is a great deal of evidence that much of the production of energy takes place at the level of the mitochondria. Here the various reactions of the Krebs cycle occur, with the production of many phosphorylated intermediates(17,18); and it is probably also in

the mitochondria that this energy is made available to the rest of the cell by the production of high energy phosphates(14). It is therefore not surprising to find that the incorporation of P32 into acid-soluble compounds is particularly extensive in the mitochondria.

In contrast to the behavior of the mitochondria is the relatively slight incorporation of P32 by the microsomes: particles known to have very incomplete enzyme systems and to function to only a minor extent in the oxidative processes of the cell. This difference in activity of the 2 particles is yet another indication that the 2 types of particles exist in the cell, and that the microsomes obtained by differential centrifugation are not merely broken up mitochondria, or the mitochondria simply aggregates of microsomes.

The acid-soluble phosphorus of the crude nuclei presents a problem requiring further investigation. Preliminary study of the specific activities of the phosphorus of the acid-soluble fractions has shown that the specific activity of the acid-soluble phosphorus of the crude nuclei appears to be higher than that of any other cellular fraction, in some cases being as much as 5 times greater, and therefore cannot represent merely contamination by other cellular constituents. The acid soluble phosphorus compounds of the nuclei are apparently metabolically very active, even though small in amount. Perhaps they contain an exceptionally high proportion of substances such as adenosine triphosphate, which is known to have a rapid turnover in the liver (19). It will be necessary to use pure nuclear preparations and to separate out the various known components of the acid-soluble fraction in order to determine the compound or compounds involved.

The large amount of phospholipid present in each cellular fraction and the extreme similarity with regard to the incorporation of P32 into phospholipids in all parts of the cell is very striking, and would tend to favor a theory of the structural function of phospholipids in the cell. This is in marked contrast to the nucleic acids which vary greatly in their turnover, depending upon their location within the cell.

Summary. 1. A broad preliminary survey has been made of the metabolic fate of P32 labeled phosphate within the rat liver cell, following intravenous injection of the labeled phosphate. 2. By means of differential centrifugation of rat liver homogenates, 4 intracellular components have been separated out: mitochondria, microsomes, the remaining supernatant, and a crude nuclear fraction. 3. Time-distribution curves have been constructed for the P32 in the 4 cell fractions, and further resolution has been obtained by chemically fractionating the P32 activity in each cell fraction into 4 groups of phosphorus compounds: the acid-soluble compounds, phospholipids, nucleic acids, and protein residue. 4. The mitochondria and nuclei took up P32 rapidly, whereas the microsomes incorporated relatively little P32, and much more slowly. Characteristically different patterns of P32 uptake were also noted for the various chemical fractions. A brief interpretation of the findings is given. 5. Additional data are given for the dry weight, phosphorus and nitrogen content of the cellular fractions from whole rat livers.

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Virulence Enhancement of *Candida albicans* by Antibiotics and Cortisone. (20488)

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It was previously reported that the intraperitoneal injection of a non-pathogenic strain of *Candida albicans* produces a rapidly fatal disseminated infection if aureomycin is administered simultaneously by the same route (1). That this effect was not due to this special strain of Candida is evident from the fact that similar results were obtained with 9 other strains of Candida, 2 isolated from the oral cavity, 4 from urine, and 3 from stool of patients. With the exception of minor quantitative differences, the results with these strains were similar to those reported in the original communication. The death ratio varied from 60-100%; with an average of 76.6% (83 deaths among 107 mice). None of these strains alone was capable of causing death of the mice without concomitant intraperitoneal administration of aureomycin.

Routes of administration of aureomycin and fungus. When the drug was administered orally (2 mg aureomycin in 0.2 cc saline), and the fungus was given *per os,* subcutaneously or intraperitoneally, no death occurred; after the subcutaneous injection of aureomycin and oral or intraperitoneal infection, no deaths occurred; when the drug and fungus were mixed and given subcutaneously 2 out of 10 animals succumbed; after the intraperitoneal injection of aureomycin, oral and subcutaneous infection proved harmless. However, a mixture of drug and fungus administered intraperitoneally invariably proved fatal (Table I).

TABLE I. Various Routes of Drug and Fungus Administration.

Application of drug	Infection with Candida	Results with Aureomycin	Results with Terramycin
Per os	*Per os*	0/10	0/10
	Subcut.	0/10	0/10
	Intraper.	0/9	0/10
Subcut.	*Per os*	0/10	1/10
	Subcut.	2/10	4/10
	Intraper.	1/10	4/10
Intraper.	*Per os*	0/10	1/10
	Subcut.	1/10	2/10
	Intraper.	10/10	10/10

Legend: Doses of aureomycin, 2 mg in 0.2 ml; of terramycin, 3.3 mg in 0.2 ml. Doses of *Candida albicans,* 0.2 ml of an emulsion of packed cells in 2.5 ml saline (320 million living cells).
Numerator = Mice dead within 14 days of observation. Denominator = Total No. of mice.

All of 6 mice survived the intravenous administration of 1 mg aureomycin in 0.1 ml saline; 21 of 30 mice succumbed after the intravenous administration of 30, 40, or 80 million Candida cells in 0.1 ml saline, but only 5 of 21 mice succumbed when the same number of Candida cells were suspended in 0.1 ml (1.0 mg) of aureomycin. It is evident that intravenously introduced *Candida albicans* is pathogenic for mice and that the pathogenic effect of the fungus is remarkably reduced when combined with aureomycin. These results contrast with all other combinations of routes of administration.

Tests with other antibiotics. In another series of experiments other antibiotics were