INACTIVATION AND MUTATIONS IN MYCOBACTERIUM AVIUM BY DECAY OF INCORPORATED RADIOACTIVE PHOSPHORUS

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INACTIVATION of bacteria and bacteriophages by decay of incorporated radioactive phosphorus (P^{32}) has been reported by STENT and FUERST (1955), FUERST and STENT (1956), STRAUSS, VAHARU, FRICKEY and MATHESON (1956), TESSMAN, TESSMAN and STENT (1957), McFALL, PARDEE and STENT (1958), and KAUDEWITZ, VIELMETTER and FRIEDRICH-FREKSA (1958). These authors have suggested that the P^{32} disintegrations which occur in the bacterial deoxyribonucleic acid (DNA) may be mainly responsible for the inactivation of the cell. Working with an avirulent strain of *Mycobacterium avium*, the present author (TSUKAMURA 1960a) has stated that the mutagenic effect of radioactive isotopes is not always parallel with the radioactivity and that radioactive phosphorus, which can incorporate into the DNA, can exhibit a significant mutagenic effect, while radioactive sulfur, which cannot incorporate into the DNA, can exhibit no significant mutagenic effect. These results have indicated that the mutagenic effect is affected by the intracellular localization of the isotope. The present paper concerns relationship between the intracellular distribution of P^{32} and the inactivation and mutations induced by the presence of P^{32} . The results have revealed that inactivation and mutations in this organisms are produced rather by the decay of incorporated radioactive phosphorus (change of P^{32} into S^{32}) rather than by the beta-ray irradiation.

MATERIALS AND METHODS

Strain: *Mycobacterium avium*, an avirulent strain Jucho (S type), was used throughout. This strain was subcultured in modified Sauton medium at 37°C for seven days and then washed three times in physiological saline (0.9 percent NaCl solution). This culture age corresponded to the stationary growth phase and contained about 5×10^7 visible cells or about 5×10^6 viable cells per mg wet weight (TSUKAMURA 1960c). The modified Sauton medium consists of 4.0 gm of sodium glutamate, 0.5 gm of KH_2PO_4 , 0.5 gm of $MgSO_4 \cdot 7H_2O$, 0.05 gm of ferrous ammonium citrate, 2.0 gm of sodium citrate, 40 ml of glycerol, and 960 ml of distilled water. The pH of the medium was adjusted to 7.0 by sodium hydroxide. The medium was dispensed in 50 ml amounts into 200 ml Erlenmeyer flasks and sterilized by autoclaving at 115°C for 20 minutes.

Labeling of cells by P^{32} : Cells were homogenized by shaking with glass beads for ten minutes and were incubated in solutions containing P^{32} -orthophosphate.

Two major procedures were used for labeling of cells by P^{32} . (1) In order to increase incorporation of P^{32} into cellular fractions other than DNA and, at the same time, to decrease incorporation into the DNA, the cells were incubated at 37°C in saline containing relatively high concentrations of P^{32} -orthophosphate. (2) On the other hand, in order to increase incorporation of P^{32} into the DNA, the cells were incubated at 37°C in phosphorus-deficient medium containing relatively low concentrations of P^{32} -orthophosphate as a sole phosphorus source. The phosphorus-deficient medium consists of the modified Sauton medium from which phosphate has been omitted.

P^{32} -orthophosphate (specific activity: C.F.; radiochemical purity: 99 percent) was supplied by the Radiochemical Center, Amersham, England.

Fractionation of phosphorus compounds and determination of radioactivity: The cells labeled with P^{32} were washed repeatedly in 20 volumes of physiological saline until a 0.2 ml sample of the washing water had no significant radioactivity. Usually, five washings were satisfactory for this purpose.

One portion of the washed cells were once again washed in distilled water, weighed and fractionated according to the procedure of SCHMIDT and THANNHAUSER (1945) (Table 1). The remaining portion of the washed cell mass was stored in a refrigerator and used for experiments.

TABLE 1
Fractionation of cells according to the procedure of SCHMIDT and THANNHAUSER

Each 0.2 ml of the fractions obtained was placed in a stainless steel planchet and heated to dryness. The radioactivity was counted by a Geiger-Mueller counter (Phillips, Holland, GM 481D). The amounts of P³²-isotope were expressed as "counts per minute" (cpm) per mg, wet weight, of cells or per μ g of DNA. The dry weight was 20 ± 1 percent of the wet weight in the test organism. The amount of DNA was determined by the diphenylamine reaction (DISCHE 1930) in the DNA fraction. Appropriate corrections were made for natural decay and for background.

The radioactivity of whole cells also was determined prior to the fractionation. Each 0.2 ml of the appropriate cell suspension was placed in a stainless planchet and heated to dryness, and its radioactivity was measured. The radioactivity of whole cells was expressed as cpm per mg, wet weight, of cells.

Determination of the number of surviving cells and of the numbers of streptomycin-resistant mutants and isoniazid-resistant mutants in the surviving fraction: The radioactive, washed cell mass was stored at -7°C ($\pm 2^\circ\text{C}$) after washing as described above. This refrigeration of the cells considerably decreased the number of surviving (colony-developing) cells during the first 24 hours of refrigeration. Subsequently, experiments were begun taking the second day of refrigeration as the zero time. As shown later, inactivation of bacteria occurred slowly also on further refrigeration (Figure 1). Therefore, the amount of survival fraction in radioactive cells was determined by subtracting the decrease in viable number in nonradioactive cells (control, Figure 1) from the amount of the survival fraction estimated in the labeled cells.

FIGURE 1.—Percent survival and mutation frequencies to isoniazid resistance and to streptomycin resistance in nonradioactive cells (control) which were stored at -7°C in a refrigerator. (Cells were washed in saline three times and then suspended in phosphorus-deficient medium (without P³²) at 37°C for 24 hours. The cells, thereafter, were washed in saline three times and refrigerated. Open and closed marks indicate independent determination respectively. Lines indicate averages of two experiments.

Mutations to isoniazid resistance per 10^5 surviving organisms ∇ $\blacktriangledown$.

Mutations to streptomycin resistance per 10^5 surviving organisms Δ $\blacktriangle$.

At appropriate time intervals, a portion of the refrigerated cell mass was homogenized by shaking with glass beads for ten minutes and was suspended in saline. The optical density of an appropriate dilution of the cell suspension was measured by a Leitz's Rouy-Photometer; the number of milligrams, wet weight, of cells per ml of suspension was determined by comparing readings with a previously prepared standard curve.

Ratios of streptomycin-resistant mutants and isoniazid-resistant mutants, arising spontaneously or induced by the presence of P^{32} , occurring in the total survival population were used as markers of the mutation frequencies. Counts were performed in the conventional manner by utilizing Sauton agar plates. Sauton agar medium consisted of modified Sauton medium added to three percent agar. One tenth ml of each of the different dilutions of cell suspension was inoculated onto each of five plates, nine cm in diameter; containing no drug, containing 1 μg of streptomycin per ml, and containing 10 μg of isoniazid per ml. Plates were incubated at 37°C for five days, and the number of colonies was counted in the usual manner. Sterile dihydrostreptomycin sulfate (manufactured by Meiji Co.) and isoniazid (manufactured by Tanabe Co.) were added to Sauton agar media immediately before use.

Amounts of the survival fraction were determined as numbers of surviving organisms per mg, wet weight, and then survival ratios were calculated taking the survival fraction at the zero time as the unit.

Figures shown in the present study were plotted without correcting decays of P^{32} occurring during the first 24 hours of refrigeration and during the incorporation process. However, errors derived from those are believed to be minor ones because of a relatively short time of those periods when compared with the total time of observation.

RESULTS

Conditions for labeling of cells by P^{32} are shown in Table 2.

Distribution of radioactivity of P^{32} between the bacterial cells and the supernatant and washing waters, ratio of uptake of P^{32} by bacterial cells, distribution of radioactivity in cellular fractions fractionated according to the procedure of SCHMIDT and THANNHAUSER, and radioactivity of the DNA fraction per μg of DNA are shown in Table 3.

P^{32} decay-survival curves and P^{32} decay-mutation curves are shown in Figures 2 to 7. Since, as has been stated, some part of the frozen cells lost their viability during the refrigeration, decay-survival curves were plotted by subtracting decrease of survival due to the refrigeration alone (Figure 1) from data of the survival fraction in P^{32} -labeled cells. On the other hand, the mutation frequencies, ratios of mutants in total surviving population, were not affected by the refrigeration (Figure 1). Therefore, data of the mutation frequencies in the labeled cells were plotted without any correction.

In the present study, experiments A and B, C and D, and E and F were performed in pairs, respectively.

TABLE 2
Labeling condition of bacterial cells

Experiment	Condition of labeling of cells with P ³²
A	Cells (113 mg/ml) were incubated in saline containing 1.5 microcuries P ³² -orthophosphate/ml for three hours
B	Cells (134 mg/ml) were incubated in phosphorus-deficient medium containing 0.3 microcuries P ³² -orthophosphate/ml for three days (before labeling, the cells were preincubated in phosphorus-deficient medium for one day to exhaust their phosphorus deposit)*
C	Cells (94 mg/ml) were incubated in saline containing 3.0 microcuries P ³² -orthophosphate/ml for three hours
D	Cells (132 mg/ml) were incubated in phosphorus-deficient medium containing 1.0 microcuries P ³² -orthophosphate/ml for 24 hours*
E	Cells (80 mg/ml) were incubated in saline containing 3.0 microcuries P ³² -orthophosphate/ml for 24 hours
F	Cells (88 mg/ml) were incubated in phosphorus-deficient medium containing 0.5 microcuries P ³² -orthophosphate/ml for 24 hours (before labeling, the cells were preincubated in phosphorus-deficient medium for three days to exhaust phosphorus deposit)*

* P³²-orthophosphate was added as a sole phosphorus source.

Ratio of uptake of P³² by bacterial cells, radioactivity of whole bacterial cells and distribution of radioactivity in cellular fractions: These varied according to the labeling condition (Tables 2 and 3). As had been expected, incubation of cells in phosphorus-deficient media containing P³² as a sole phosphorus source represented an increased ratio of uptake of P³² by cells and an increased amount of P³² incorporated into the DNA fraction, accompanying frequently a relative decrease of radioactivity in the acid-soluble fraction (Experiments B, D and F). On the other hand, generally reverse relations were found in cells incubated in saline containing P³² (Experiments A, C and E).

2. Decay-survival curves and decay-mutation curves: Percent survival and mutation frequencies were expressed as a function of P³² decay. These are shown in Figures 2 to 7 and partially summarized in Table 4.

(1) Decay-survival curves. Different types of survival curves were found (Figures 2 to 7). It was remarkable that inactivation of cells (loss of ability to develop colonies) was much more significantly affected by the amount of P³² found in the DNA fraction than done by the amount of total radioactivity of cells which must parallel the amount of beta-ray irradiation.

As shown in figures, inactivation occurred most rapidly in experiment B. The order in respect to the rapidity of inactivation was as follows: Experiments B, F, D, C, E, A. On the other side, the amount of total radioactivity was the highest in experiment E. The order in respect to the amount of total radioactivity, total amount of P³² contained in cells, was as follows: E, C, F, D, B, A. This order is apparently incompatible with the former order concerning inactivation.

The amount of radioactivity (P³²) in the DNA fraction was the highest in experiment B, and the order in respect to the amount is as follows: B, E, F, D, C,

TABLE 3

Distribution of radioactivity in culture, uptake ratio of P₃₂ by bacterial cells, radioactivity of whole bacterial cells, and distribution of radioactivity in cellular fractions

	Experiment A	Experiment B	Experiment C	Experiment D	Experiment E	Experiment F
Amount of bacterial cells used:	3,390 mg	4,000 mg	3,780 mg	5,300 mg	3,200 mg	4,420 mg
Distribution of radioactivity in culture:						
(1) Bacterial cells	157,000 cpm	884,000 cpm	1,150,000 cpm	1,324,000 cpm	6,720,000 cpm	1,569,000 cpm
(2) Supernatant and washing waters	2,990,000 cpm	133,000 cpm	10,568,000 cpm	4,140,000 cpm	4,075,000 cpm	399,000 cpm
(3) Total	3,147,000 cpm	1,017,000 cpm	11,718,000 cpm	5,464,000 cpm	10,795,000 cpm	1,968,000 cpm
Uptake ratio of P ₃₂ by bacterial cells:	5.0 percent	87.0 percent	9.8 percent	24.2 percent	62.2 percent	80.0 percent
Radioactivity of whole cells:	46.3 cpm/mg	221. cpm/mg	314. cpm/mg	250. cpm/mg	2,096. cpm/mg	355. cpm/mg
Distribution of radioactivity in cellular fractions: *	cpm/mg	cpm/mg	cpm/mg	cpm/mg	cpm/mg	cpm/mg
(1) Acid-soluble fraction	18.1 (38.8%)	43.2 (26.9%)	80.0 (28.2%)	48.6 (22.7%)	410. (27.0%)	63.5 (17.8%)
(2) Phosphate fraction	3.15 (6.7%)	2.64 (1.6%)	4.74 (1.7%)	13.5 (6.3%)	81.0 (5.3%)	11.7 (3.3%)
(3) DNA fraction	0.086 (0.18%)	14.4 (8.9%)	3.89 (1.4%)	6.3 (2.9%)	13.9 (0.91%)	10.2 (2.9%)
(4) RNA fraction	21.3 (45.7%)	85.0 (52.6%)	173. (61.0%)	111.3 (51.9%)	825. (54.2%)	233. (65.5%)
(5) Protein fraction	4.00 (8.6%)	16.2 (10.0%)	22.5 (7.9%)	34.6 (16.2%)	190. (12.6%)	37.6 (10.6%)
(6) Total	46.7 (100%)	161.4 (100%)	284.1 (100%)	214.3 (100%)	1,520. (100%)	356.0 (100%)
Radioactivity of the DNA fraction per one μ g of DNA:	0.029 cpm/ μ g DNA	4.76 cpm/ μ g DNA	1.30 cpm/ μ g DNA	2.10 cpm/ μ g DNA	4.62 cpm/ μ g DNA	3.21 cpm/ μ g DNA

mg = mg in wet weight.

* Fractionated according to the procedure of SCHMIDT and THANNHAUSER.

FIGURE 2.—Decay-survival curve and decay-mutation curves in experiment A (see Tables 2 and 3).

Mutations to isoniazid resistance per 10⁵ surviving organisms ●—●.

Mutations to streptomycin resistance per 10⁵ surviving organisms ▲—▲.

FIGURE 3.—Decay-survival curve and decay-mutation curves in experiment B (see Tables 2 and 3).

Mutations to isoniazid resistance per 10⁵ surviving organisms ●—●.

Mutations to streptomycin resistance per 10⁵ surviving organisms ▲—▲.

TABLE 4

Correlations among total radioactivity of cells, radioactivity of DNA fraction, inactivation of cells, and induced mutation in *Mycobacterium avium* (Jucho)

Exp.*	Radioactivity of whole cells in cpm/mg, wet weight	Radioactivity of DNA fraction in cpm/mg, wet weight	DNA cpm Total cpm†	Percent survival		Increase of mutation to isoniazid resistance
				(after 14 days)	(after 28 days)	
{A	46.3 cpm/mg	0.086 cpm/mg	0.18%	59.0%	14.0%	1.4 times
{B	221.	14.4	8.9	5.6	0.5	12.6
{C	314.	3.89	1.4	21.0	4.7	8.8
{D	250.	6.30	2.9	10.0	2.8	13.0
{E	2,096.	13.9‡	0.91	6.4	3.4	10.7
{F	255.	10.2	2.9	6.9	0.6	10.8

* Experiments A and B, experiments C and D, and experiments E and F were performed as a pair experiments, respectively.

† Percentage of radioactivity of the DNA fraction per total radioactivity.

‡ This amount was suggested to be derived from contamination of P³² (see text).

FIGURE 4.—Decay-survival curve and decay-mutation curves in experiment C (see Tables 2 and 3).

Mutations to isoniazid resistance per 10^5 surviving organisms ●—●.

Mutations to streptomycin resistance per 10^5 surviving organisms ▲—▲.

FIGURE 5.—Decay-survival curve and decay-mutation curves in experiment D (see Tables 2 and 3).

Mutations to isoniazid resistance per 10^5 surviving organisms ●—●.

Mutations to streptomycin resistance per 10^5 surviving organisms ▲—▲.

A. This order is apparently compatible with the order in respect to the rapidity of inactivation of cells, although experiment E only was an exceptional case.

In experiments E and F (Tables 2 and 3 and Figures 6 and 7), which were performed as a pair at the same time, the cells in experiment E showed a somewhat higher P^{32} amount in the DNA fraction than those in experiment F. Inactivation of cells occurred, in contrary, much more rapidly in the cells of experiment F. This phenomenon would be interpreted as follows. In these experiments, the DNA fraction contained usually a relatively low amount of radioactivity. Subsequently, it appears quite possible that the DNA fraction is contaminated by the other fractions containing relatively high amounts of P^{32} . Since the cells of experiment E contained the highest amount of radioactivity as whole cells, radioactivity in the DNA fraction in experiment E is thought to be increased by such contamination: Thus, the DNA fraction of experiment E might represent a higher amount of P^{32} than is actually present.

FIGURE 6.—Decay-survival curve and decay-mutation curves in experiment E (see Tables 2 and 3).

Mutations to isoniazid resistance per 10⁵ surviving organisms ●—●.

Mutations to streptomycin resistance per 10⁵ surviving organisms ▲—▲.

FIGURE 7.—Decay-survival curve and decay-mutation curves in experiment F (see Tables 2 and 3).

Mutations to isoniazid resistance per 10⁵ surviving organisms ●—●.

Mutations to streptomycin resistance per 10⁵ surviving organisms ▲—▲.

It is apparent that the amount of total radioactivity, which parallels the amount of beta-ray irradiation, has no close correlation with the incidence of inactivation. The ratio of uptake of P³² may have any positive correlation with the inactivation, although a definite conclusion was not made from the present data. The amounts of radioactivity in other cellular fraction did not appear to have any close correlation with the occurrence of inactivation.

In addition, the data on decay-survival curves appear to indicate a tendency that the higher the amount of P³² in the DNA fraction, the more exponential becomes the decay-survival curves (Figures 2 to 7 and Table 3 and 4). This finding appears to indicate an important role of P³² decay in the DNA fraction for the induction of cell inactivation. For example, the decay-survival curves in experiments B, E and F (Figures 3, 5 and 7), of which the DNA fractions contained relatively high amounts of P³², resemble the exponential curve. On the other side, there appears to be also the tendency that the lower the amount of P³² in the

DNA fraction, the curve resembles a multihit curve. The decay-survival curves of experiments A and C (Figures 2 and 4) more or less resemble the multihit curves.

(2) Decay-mutation curves. In the decay-mutation curves (Figures 2 to 7), the most striking fact is a discrepancy between mutations to isoniazid resistance and to streptomycin resistance. The mutation frequency to isoniazid resistance was rapidly increased as the P^{32} decay progressed, while the mutation frequency to streptomycin resistance was not increased or only slightly increased.

There appeared to exist the tendency that the higher the amount of P^{32} in the DNA fraction, the more rapid becomes the decay-mutation (to isoniazid-resistance) curve, and, in most cases, the decay-mutation curve tended to be as if exponential to the decay of P^{32} . However, further experiments are needed to determine whether or not this correlation indeed exists since this correlation was not as clear as that found in the decay-inactivation curves. Determination of the mutation frequency involved a larger number of errors than did the determination of the survival, for the former contained errors involved in both determinations of the number of surviving cells and of the number of mutants.

DISCUSSION

Experimental conditions: Experiments in the present study were performed under the conditions excluding any selective process. As seen in control experiment (Figure 1), the refrigeration does not increase the mutation frequency. Therefore, the procedure of refrigeration is not selective for the marker mutants. Both procedures of incubation of cells in physiological saline as well as in phosphorus-deficient medium did not show any significant change of turbidity of cells. Therefore, these are believed to be unselective. It is also believed that any change of intracellular distribution of P^{32} did not occur after the determination on distribution of P^{32} , since the cells were stored at a freezing state during the progress of P^{32} decay.

In addition, it appears better to describe some additional properties of the test organism. The strain Jucho of *Mycobacterium avium* is inactivated by ultraviolet irradiation showing survival curves of a single-hit type, like other mycobacteria (TSUKAMURA 1960b). Furthermore, this strain is a member of the group showing the lowest DNA content per mg, wet or dry weight, in the genus *Mycobacterium* (unpublished data).

Mode of inactivation of cells: As stated above, it is remarkable that inactivation is affected by the amount of radioactivity in the DNA fraction rather than by the amount of total radioactivity that parallels the amount of beta-ray irradiation. If inactivation were caused by beta-ray irradiation, the rapidity of inactivation would parallel the amount of total radioactivity. This is not the case, as described above. Inactivation occurs showing a close correlation with the amount of P^{32} in the DNA fraction. The decay-survival curves tended to be exponential, as the amount of P^{32} in the DNA fraction increased. These findings strongly suggest that inactivation of cells is caused by decay of P^{32} —change of P^{32} into

S³²—in the DNA fraction. This change must cause a change of the DNA structure. However, it is unlikely that the decay of P³² in the DNA fraction is the sole mechanism responsible for cell inactivation although it is believed to be the most important factor.

As shown in experiments A and C, especially in the former (Figure 2), cells having a slight labeling of the DNA fraction by P³² represented decay-survival curves of a multihit type. The existence of decay-survival curves of the multihit type indicate that inactivation may occur by decay of incorporated P³² in multiple loci. These multiloci seem to be outside of the DNA on the basis of the following points: (1) The existence of decay-survival curves of the single-hit type, or resembling it, in cells showing heavy labeling of the DNA fraction would suggest the existence of only one nucleus according to the target theory. (2) It is not practical to suppose the existence of such an excess of nuclei as considered from the shape of the decay-survival curve in experiment A (Figure 2). Subsequently, these multiloci appear to be in the cytoplasm, and it is possible that they may be ribosomes.

There are two conflicting claims as to the number of nuclei of mycobacteria by cytochemical observations. KNAYSI (1952, 1957, 1959) and KNAYSI, HILLIER and FABRICANT (1950) claimed that mycobacteria possess several nuclei. On the other hand, MUDD and others (MUDD, WINTERSCHIED, DELAMATER and HENDERSON 1951; WINTERSCHIED and MUDD 1953; TODA, KOIKE, HIRAKI and TAKEYA 1957) claimed that mycobacteria have only one (or two) nucleus. The results of the present study would support the one-nucleus theory.

The test organism contained about 5×10^7 cells per mg (wet weight). However, the number of viable cells per mg was determined to be about 5×10^6 per mg, wet weight, under the culture age used for experiments. If one supposed that only viable cells could incorporate P³² into their DNA, it would be possible that, under the conditions shown in the experiment B (Figure 3), only a few disintegrations of DNA-P³² atoms per cell may be sufficient to cause cell inactivation, although this supposition remains yet impractical because of considerable experimental errors.

Mutations induced by P³² decay: As stated above, there is no close correlation between the induced mutation and the amount of total radioactivity of cells, which parallels the amount of beta-ray irradiation. Consequently the induced mutation is not thought to be mainly derived from the beta-ray irradiation. It appears noteworthy that the decay-mutation curves were roughly exponential to the P³² decay (Figures 3 to 7). It appears also that the induced mutation frequency may be roughly in accordance with the amount of P³² incorporated into the DNA fraction (Table 4), although this could not be accurately demonstrated, possibly, because of the inaccuracy of determinations of the mutation frequency. In view of the findings, it appears apparent that the induced mutation is in some manner derived from the P³² decay.

Mutations (to isoniazid resistance) could occur even under the conditions in which P³² was scarcely contained in the DNA fraction (Figure 2). These mutations appear to result from the following possibilities: (1) mutations by decay

of P^{32} atoms in the DNA; (2) mutations derived from abnormal protein synthesis caused by decay of P^{32} in the cytoplasm; (3) mutations by beta-ray irradiation. The third possibility appears to be unimportant in the test organism, for the beta-ray irradiation has had no significant mutagenic effect on the organism (TSUKAMURA 1960a). The second possibility may be present, considering the data shown by WITKIN (1956), HAAS and DOUDNEY (1957) and DOUDNEY and HAAS (1958, 1959). Mutations from such origin would be possible in respect to isoniazid resistance but would be impossible in respect to streptomycin resistance (streptomycin inhibits protein synthesis).

The existence of discrepancy between mutations to isoniazid resistance and to streptomycin resistance was a remarkable phenomenon. The same phenomenon had been observed in the induced mutations by ultraviolet irradiation (TSUKAMURA 1960b). At that time, the phenomenon was interpreted by the presence of some phenomic lag for phenotypic expression of mutation to streptomycin resistance, while there is no phenomic lag for expression of isoniazid resistance. Since experiments of the ultraviolet study were performed concerning the zero point mutation, it was suggested that low mutation frequency to streptomycin resistance is derived from delay of phenotypic expression. In experiments of the present study, the cells were inoculated immediately on media containing isoniazid or streptomycin. Therefore, the mutation frequencies observed concern the zero point mutation. The discrepancy may be derived from the absence or presence of phenomic lag in mutations to isoniazid resistance and to streptomycin resistance.

Previously, the author (TSUKAMURA 1960a) found that cells of this organism growing actively in Sauton medium containing 0.5 microcuries of P^{32} -phosphate per ml represented a marked increase of the both mutation frequencies, unlike the results of the present study. That finding is interpreted by assuming that, in growing cultures, induced streptomycin-resistant mutants could accomplish their phenotypic expression.

SUMMARY

Inactivation and mutation occurred in *Mycobacterium Jucho* by decay of incorporated radioactive phosphorus (P^{32}) under the conditions in which cells labeled with P^{32} were stored in freezing state during the progress of P^{32} decay, and the existence of any selective process of marker mutants could be excluded.

Relationships between the inactivation and mutations and the intracellular distribution of P^{32} were studied. Fractionation of radioactive cells was performed according to the procedure of SCHMIDT and THANNHAUSER.

Inactivation of cells was much more markedly affected by the amount of P^{32} in the DNA fraction than by the amount of total radioactivity of cells that parallels the amount of beta-ray irradiation. Inactivation curves, survival curves plotted as a function of the amount of P^{32} decay tended to be exponential, if the DNA fraction was labeled heavily by P^{32} . It is strongly suggested, thus, that beta-ray irradiation is not responsible for inactivation of bacterial cells and decay of

P³² in the DNA fraction, but that change of P³² into S³² in DNA structure is mainly responsible for it.

If cells were labeled by P³² representing a relatively low content of P³² in the DNA fraction, inactivation of cells occurred showing decay-survival curves of a multihit type. Thus, it is also suggested that inactivation of cells may occur also by decay of P³² in multiloci outside of the DNA.

Mutation frequency to isoniazid resistance was rapidly increased with progress of P³² decay, while mutation frequency to streptomycin resistance was not increased or only slightly increased. Thus, a marked discrepancy between these two mutations was observed. Mechanism of this discrepancy was discussed. The incidence of mutation (to isoniazid resistance) did not parallel the amount of total radioactivity of cells that corresponded to the amount of beta-ray irradiation. The induced mutation was increased roughly in accordance with the progress of P³² decay. It is conceivable that the induced mutation also occurs by decay of incorporated P³².

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