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UTILIZATION OF CHOLESTEROL BY ORGANISMS OF THE GENERA *MYCOBACTERIUM* AND *NOCARDIA*[1]

ANNE O. MUELLER, HERMAN P. SCHMITT, AND GEORGE P. MUELLER

Department of Bacteriology and Department of Chemistry
The University of Tennessee, Knoxville, Tennessee

INTRODUCTION

Of the numerous studies dealing with microbial transformations of steroids, those concerned with cholesterol were of particular interest to us. For example, Tak (1942), using enrichment culture techniques, isolated several cholesterol-decomposing organisms belonging to the genus *Mycobacterium*. Turfitt (1944*a*, 1944*b*, 1947, 1948) found no organisms except *Nocardia* spp. and *Mycobacterium lacticola* to be capable of utilizing cholesterol, with *N. erythropolis* chiefly responsible for the breakdown of this sterol in soils. Gram negative organisms were of considerable importance in the disposal of steroids lacking the C-17 side chain. However, using Turfitt's two media, Schatz, Savard and Pintner (1949) were less successful in isolating cholesterol oxidizers from the soil, but with their own medium they obtained better results, although the organisms proved to be Gram negative Eubacteria. Horvath and Kramli (1947, 1948*a*, 1948*b*, 1949) discarded the genus *Nocardia* as a good oxidizer of cholesterol in favor of their new *Azotobacter oxydius*. Their experiments indicate the facultative anaerobic bacteria to be more efficient in the oxidation of cholesterol than species of the aerobic genus *Nocardia*. The assimilation of cholesterol by *Mycobacterium smegmatis* was found (Sobel and Plaut, 1949) to be slow in the absence of supplements but rapid if beef heart infusion broth was added to the medium. Possibly due to its slower growth, *Mycobacterium tuberculosis* was less vigorous in consuming cholesterol.

MATERIALS AND METHODS

In further exploration of this field we desired to limit the source of available carbon in the fermentation medium to cholesterol. Several organisms have been found which are vigorous decomposers, but since most of them were shown to belong to the genus *Mycobacterium* and the closely related genus *Nocardia* the study was principally limited to these. Organisms of other genera were used for comparison under given test conditions. We had planned to examine the fermentation mixture for degradation products of cholesterol and so to extend the study of Turfitt (1948) to the identification of fission products resulting from the action of other bacteria. However, the program was interrupted in the preliminary stages of the analytical work.

[1]This investigation was supported by a grant from the Research Corporation, New York, N. Y., for which the authors express their thanks.

The preparation of satisfactory suspensions of cholesterol in water was realized by using Tak's method of boiling the solvent away from aqueous-acetone solutions. This procedure does not always give uniform suspensions due to a certain amount of agglomeration occurring on sterilization in the autoclave, but in spite of this deficiency most of our suspensions were prepared in this manner. Later, as the apparatus became available, heavy suspensions of cholesterol in a little water were irradiated at 400 kilocycles in an ultrasonic field of about 500-watt intensity. The creamy suspension resulting was then poured into the sterile salt solution and no further heating was necessary. Of moderate success was the use of a Waring Blendor, but here the particles were considerably larger, although several of the resulting media were successfully incubated with marked consumption of the steroid.

Bacteria were obtained from soil samples collected from garden, farm, forest, and lake-bottom lands in East Tennessee, and in addition from areas of rich garden soil in which cholesterol and hog brains and spinal cords had earlier been buried. Tables, floors, and waste-disposal areas of several abbatoirs were sampled as well as various portions of the Knoxville sewage system and many septic tanks.[2]

Earlier in this study all the raw samples were inoculated separately into Tak's and Turfitt's cholesterol medium and both groups of cultures were incubated at 30° C. and 47° C. Incubation at the higher temperature prevented the overgrowth with molds and actinomycetes which frequently occurred at 30° C. In order to isolate those mycobacteria which grow only at low temperatures, incubation at 30° C. was continued. Tak's medium was found superior for isolating cholesterol oxidizers from soils. The preliminary screening test was the organism's ability to survive and multiply using cholesterol as the sole carbon source in the medium. Actinomycetes and fungi as well as acid-fast and nonacid-fast bacteria were collected. It was observed that the native ability of the various organisms to utilize the medium varied from negligible to very pronounced. In addition, some of those of lesser ability could, through suitable culturing, acquire a high degree of activity.

A method was later evolved for the selection of species of the genera *Mycobacterium* and *Nocardia*. This was based on the observation that organisms of these genera, after being purified through repeated culturing on cholesterol media, were always able to reduce malachite green when it was added to the medium. Thus this dye gave us a crude selectivity test. When a 1:100,000 dilution of malachite green was added to the glycerol broth, glycerol agar, or Tak's cholesterol medium, in which these organisms were inoculated, reduction took place in three to six days. The rate of reduction de-

[2]We wish to thank Messrs. E. Huey, Herbert Madden, and L. E. Bailey for sampling privileges and to acknowledge in particular the efforts of Mr. John M. MacDonald in supplying us with various samples.

pended on the rate of growth of the organisms at hand. After decolorization was complete, smears were made from the cultures and treated with Ziehl-Neelsen stain to verify the presence of acid-fast organisms. All species of *Mycobacterium*, both stock strains and the unknowns isolated in this work, and all the species of *Nocardia* tested decolorized malachite green quickly. To purify cultures, dilutions were plated on malachite green-glycerol agar and bacteria from those colonies which were surrounded by a clear colorless area were transplanted. Again the Ziehl-Neelsen stain was used to verify the presence of acid-fast bacteria. The purified cultures were stored on glycerol-agar slants.

Incubation at various temperatures was carried out in Erlenmeyer flasks ranging in size from 250 to 4000 cc. Some of the cultures were stationary, but most were agitated gently on a rotary shaker. Aeration was achieved in some cases by passing compressed air through a Berkefeld filter.

The total amount of soluble organic materials remaining after fermentation was determined by carefully duplicated solvent extraction procedures. Large volumes of media were extracted with chloroform, the two-phase mixture being filtered through macerated filter paper in a sintered-glass funnel to remove inert material at the interface. The chloroform layer was separated, the extraction repeated three times and the combined organic layers evaporated. The residue was dried *in vacuo* at room temperature and weighed. A simpler procedure for small volumes of media was to evaporate in a stream of air at room temperature and extract the dry residue three times with ether and once with acetone, treating the combined solutions as usual.

We realize that the gross weight of the organic fermentation products, when subtracted from the weight of the original cholesterol, gives a difference which does not necessarily represent the true amount of the sterol consumed. However, it would be the true amount when the residue was itself pure cholesterol or other compounds having the same molecular weight. This is hardly to be expected. Similar objections may be made to the seemingly more accurate method of determining the amount of cholesterol in the fermentation products by precipitating the digitonide. Thus it is possible for the precipitation method to be completely in error since it is not specific for cholesterol but also determines other digitonin-precipitable compounds, such as 7-dehydrocholesterol, and possibly others yet to be identified from fermentation. However, since Tak (1942) and Turfitt (1948) had used the method, we too used it with cholesterol, and determined optimum amounts of sample, solvent, reagents, and conditions for the precipitation. We were able nearly to duplicate the percent of cholesterol in the digitonide, given by Osato and Mutsuo (1930), which was 0.2431, with the factor 0.2465. This factor was the average of twelve determinations, using fixed conditions of precipitation and varying the concentration from about

2 mg. to 25 mg. of cholesterol in 2 ml. of anhydrous ether. However, when applied to the crude fermentation products, the method gave such erratic results that, considered together with its questionable value, we declined to pursue it further.

Turfitt reported that the degradation of cholesterol by species of *Nocardia* was influenced by variations in the hydrogen ion concentration in the medium and he recommended the use of buffered media to prevent acid accumulation. Our observations are in accord with his.

Results

Table 1 shows that in unbuffered fermentations which were adjusted weekly to pH 7.0 by the addition of aqueous sodium hydroxide, the consumption of cholesterol by *Mycobacterium berolinense* was greater than in those which were untreated and allowed to remain acidic.

TABLE 2. *Comparison of nitrogen utilization by the mycobacteria*[1] *tested*

Nitrogen Source[2]	Reaction of Growth	N used [3]	N not used [4]
$(NH_4)_2SO_4$	acid	34	5
NH_4Cl	acid	29	5
$NaNO_3$	alkaline	33	8
NH_4NO_3	acid	20	5
Asparagine	alkaline	10	4

[1]All acid-fast organisms collected, both stock strains and unidentified strains, were tested.

[2]Merrill's synthetic medium was used throughout with the addition of 0.5% of the nitrogen source.

[3]Number of different cultures which used the nitrogen successfully.

[4]Number of cultures which did not successfully use this nitrogen.

The nitrogen requirement of several of the cholesterol utilizers was studied with the hope of finding more favorable conditions for growth and consumption of the substrate. The synthetic medium of Merrill (1930, 1931) was used with variations made in the nitrogen source. Inoculations scraped from eight-day growths on glycerol-agar slants were floated on the surface of the medium, and the tubes incubated at 30°C. for ten days. Determinations of pH were made simultaneously on these and on uninoculated controls. Turbidity, pellicle formation and changes in the medium towards increased acidity or alkalinity were used as indications of the availability of the nitrogen source tested. With the ammonium salts (chloride, sulfate, and nitrate), the medium became acid. When sodium nitrate or asparagine was the nitrogen source it tended to become alkaline. However, the desire to make cholesterol the sole carbon source precluded the use of the amino acid to furnish nitrogen. Even so, the nitrogen

TABLE 1. *Comparison of cholesterol consumption by M. berolinense after six weeks incubation of shaken cultures*

Unbuffered Medium				Buffered Medium			
Medium No.	Cholesterol Incubated in mg.	Cholesterol Remaining in mg.	Cholesterol Consumed %	Medium No.	Cholesterol Incubated in mg.	Cholesterol Remaining in mg.	Cholesterol Consumed %
109	750	486	36	15	750	375	50
24	250	142	43	25	250	14	94
110	750	304	60	22	750	82	89

TABLE 3. *Consumption of cholesterol suspended by various methods*

Method[1]	M. phlei[2]; three months' incubation with shaking				M. phlei[3]; two months' stationary incubation			
	Medium No.	Cholesterol			Medium No.	Cholesterol		
		Amount at beginning mg.	Residue mg.	Amount Consumed %		Amount at beginning mg.	Residue mg.	Amount consumed %
Crystalline.....	76	400	78	81	31	500	390	23
Acetone.....	143	12,000	1,422	89	....	.....	.....	..
Irradiated.....	144	7,000	38	99	146	6,000	2,005	65

[1]Methods of subdivision used were crystallization, suspension by Tak's acetone procedure, and irradiation in an ultransonic field, respectively.

[2]Isolated from sewage, strain M.

[3]Obtained from the American Biological Company, Chicago, Illinois.

requirement of all the cholesterol decomposers was easily satisfied with ammonium salts, as is evident from table 2. So ammonium sulfate was used throughout.

Significant differences in the multiplication of bacteria were observed on suspensions of cholesterol prepared by the various methods described (Table 3). Finely suspended material was more quickly attacked as indicated by the more rapid rise in bacterial count and increasing acidity of the medium.

Since the mycobacterial oxidation of cholesterol is an aerobic process, the effects of shaking and aeration were examined, and bacterial counts, pH changes, and cholesterol utilization under these conditions compared with similar data from stationary cultures. It

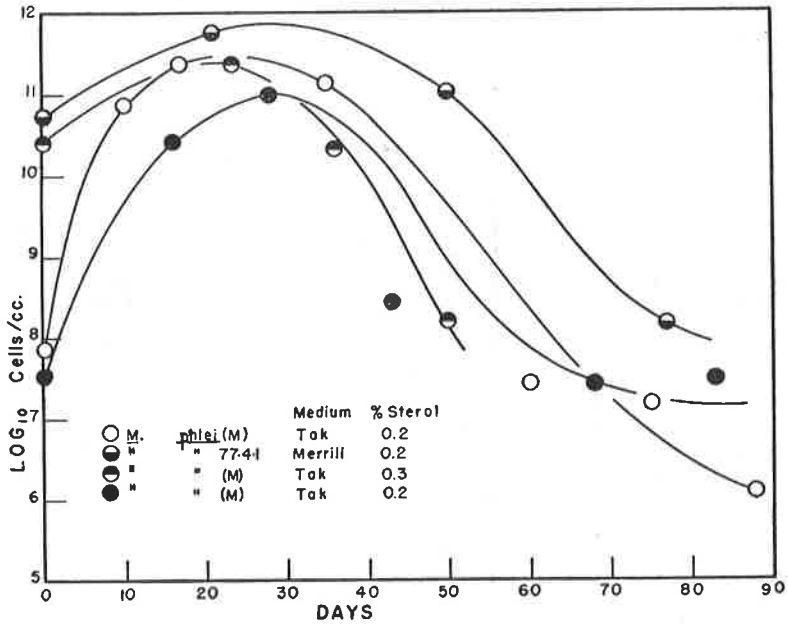

Fig. 1. Growth curves of *Mycobacterium phlei* in shaken media prepared by precipitating cholesterol from aqueous-acetone solution. Each point represents an average bacterial count of at least eight cultures incubated simultaneously. The lower curves show the reproducibility of two experiments which were identical except that one was initiated three weeks after the other.

is apparent from table 5 that while the complete oxidation process is more than twice as efficient in the shaken than in the stationary culture, the most striking effect, with one exception, was achieved by aeration. The pH values ranged from 6.2 to 6.9 in all three methods except in the case of *M. berolinense,* where the average value was 5.0 in stationary culture and 6.8 and 6.7 respectively in shaken and aerated media. While this effect may be regarded as resulting from direct facilitation of the aerobic process, the suggestion by Sobel and

TABLE 4. *Improvement of cholesterol-consuming ability of M. berolinense*

Lot No.	Cholesterol Incubated mg.	Cholesterol Remaining at End of Incubation mg.	Treatment of culture used for inoculum
4[1]	750	362.9	3 month glyc. agar transfer at 2 week intervals
22	750	82.0	1 month cholesterol medium transfer at 2 week intervals
41	500	23.0	1 month cholesterol medium transfer at 2 week intervals
86	385	5.3	10 weeks in cholesterol medium, 10 weeks on glyc. agar transfers at 2 week intervals
93	750	57.7	10 weeks in cholesterol medium, 10 weeks on glyc. agar, 4 weeks in cholesterol medium transfers at 2 week intervals

[1]All samples were treated the same way after being inoculated: Tak's medium with 1 month incubation in shaken culture, except No. 4 which was incubated 10 weeks.

TABLE 5a. *Cholesterol consumption by the bacteria studied*

Bacteria Studied	Strain	Stationary Cultures			Shaken Cultures		
		No.	Length of Incubation	Cholesterol Consumed	No.	Length of Incubation	Cholesterol Consumed
				ave. %			ave. %
Mycobacterium							
Group I:							
M. lacticola	Tak	1	5½ mo.	34	2	1 mo.	13
M. lacticola	1472	1	2 mo.	19	3	1 mo.	50
M. lacticola	1472	1	5½ mo.	51	1	2 mo.	48
M. berolinense	Tak	3	1 mo.	38	7	1 mo.	67
M. berolinense	Tak	2	5½ mo.	50	8	6 wks.	70
Group II:							
M. cholesterolicum	Tak	1	1 mo.	22	6	1 mo.	71
M. cholesterolicum	Tak	1	5 mo.	92	1	6 wks.	57
M. sp.	77–4–1	2	3½ mo.	59	1	6 wks.	87
Group III:							
M. phlei	ABC	1	2 mo.	65	1	1 mo.	23
M. phlei	Tak	1	1 mo.	16	1	6 wks.	82
M. phlei	Tak	1	5 mo.	17	1	2 mo.	75
M. phlei	M	1	3½ mo.	81	1	1 mo.	48
M. phlei	M	.	.....	..	1	2 mo.	99
M. phlei	M	.	.....	..	1	2½ mo.	95
M. phlei	M	.	.....	..	2	3 mo.	94
M. phlei	10	2	2 mo.	78	.	.....	..
M. phlei	13–47	2	2 mo.	65	3	1 mo.	76
M. phlei	13–47	1	5½ mo.	60	.	.....	..
M. phlei	X–11	1	2 mo.	72	2	3 wks.	61
M. phlei	X–11	1	5½ mo.	48	1	1 mo.	36
M. phlei	X–11	.	.....	..	1	7 wks.	44
M. phlei	2T32	.	.....	..	1	6 wks.	54
Nocardia erythropolis	2569	2	1 mo.	59	7	1 mo.	66
N. erythropolis	2569	1	2 mo.	81	1	10 wks.	72

TABLE 5a (*Continued*)

Nocardia erythropolis	2569	.	.....	..	2	1 mo.	98
Nocardia restrictus	6846	1	2 mo.	9	1	10 wks.	97
Nocardia asteroides	Duggar	.	.....	..	1	6 wks.	1
Azotobacter chroococcum	stock	1	5½ mo.	39	.	.....	..
Azotobacter chroococcum	37–3	1	5½ mo.	12	.	.....	..
Azotobacter vinelandii	stock	1	5½ mo.	28	.	.....	..
Flavobacterium dehydrogenans	Arnaudi	1	2 mo.	40	.	.....	..
Flavobacterium sp.	Pollard	1	2½ mo.	21	.	.....	..
Bacterium sp. 303	Schatz	.	.....	..	1	3 wks.	13
Escherichia coli	Tenn.	.	.....	..	1	6 wks.	34
Escherichia coli[1]	Tenn.	.	.....	..	1	6 wks.	47
Escherichia coli	1st Crk.	1	5½ mo.	31	3	1 mo.	88

[1]Cholesterol-adapted strain.

TABLE 5b. *Cholesterol consumption by the bacteria studied*

Bacteria Studied	Strain	Aerated Cultures		
		No.	Length of Incubation	Cholesterol Consumed
				ave. %
Mycobacterium berolinense.....	Tak	2	1 mo.	73
Mycobacterium sp.....	77–4–1	1	6 wks.	53
Mycobacterium phlei.....	10	1	2 mo.	92
Mycobacterium phlei.....	13–47	1	2 mo.	98

Plaut (1949) that the direct attack of oxygen cannot be disregarded admits the possibility that the reaction between cholesterol and oxygen may have resulted in a more readily assimilable sterol. The overall reaction would still be for the most part microbial since the nearly complete disappearance of cholesterol cannot be attributed to the presence of air alone.

From the appearance of the media, the rate of disappearance of cholesterol was at first slow but became pronounced simultaneously with the phase of rapid multiplication of organisms and decreased with the stationary phase of the growth curve. In stationary cultures the phase of logarithmic multiplication reached a maximum in six to seven weeks and in shaken culture in less than a month. Figure 1 illustrates the rate of growth with time and shows that the bacterial population after 20 days is of the same order of magnitude regardless of the initial value.

The incubation temperature further influenced the rate of sterol assimilation depending on the group of acid-fast organisms tested. Growth at 47° C. is used as a basis for the classification of acid-fast organisms. Group I will grow at 47° C. but fails to survive at 60° C. for one hour. Group II will not grow at 47° C. nor will it survive at 60° C. for one hour. Group III will grow at 47° C. and will survive at 60° C. for one hour. It is evident that Group III will withstand greater variations in incubation temperatures while Group II (acid-fast organisms which infect cold-blooded animals) will be more sensitive to incubation temperatures. On this basis, *M.* 77-4-1, isolated from garden soil in which hog brains and spinal cords were buried, is classified in Group II. It was found to grow more abundantly at 30° C. than at 25° C. *M. phlei* (strain M) is classified in Group III and was found to give equally good results in cholesterol consumption at 40° C. and 47° C.

No organisms which originally had the ability to use cholesterol lost that ability during the course of this study. Some few species of *Mycobacterium* with slight cholesterol-consuming ability improved upon continuous transfer in liquid cholesterol medium. An unknown saprophytic species of *Mycobacterium*, labeled 2T32, was received from the 3rd Army Medical Laboratory. At first it grew slowly and

scantily on cholesterol medium, but improved on continuous transfer until after three months an experimental lot showed but 2809 mg. of cholesterol left as residue from the 6000 mg. incubated. This culture was identified as *M. phlei. M. berolinense* kept on glycerol agar and then used as inoculum into cholesterol medium was fair in its cholesterol consuming ability but this ability was also improved by transfers in cholesterol medium.

Malachite green inhibits bacteria in general, but despite this action, it was used in selecting active cholesterol utilizers. Its function in aiding this selection is not known. Most striking affirmation of this test was obtained in the decolorization of the dye by conditioned *utilizers* such as 2T32, just described, which prior to conditioning had been unable either to effect decolorization or cholesterol assimilation. Also a cholesterol-adapted variant of a stock strain of *Escherichia coli* (Tennessee strain) was produced after four months of exposure to cholesterol medium. After this treatment the variant reduced malachite green and had acquired an increased ability to consume cholesterol. Although the dilutions of this dye used here have been found to be inhibitory for Gram positive organisms, all such organisms that we tested grew well in the dye-containing nutrient broth, even though no decolorization was shown.

Among other differentiations afforded with this dye, *E. coli* and two Gram negative species of *Flavobacterium,* reduced malachite green, but very much more slowly than any of the sterol-using species of *Mycobacterium* or of *Nocardia.* The reported differentiation (Bent and West, 1940) between *M. tuberculosis* and *M. smegmatis* was not confirmed either with malachite green or brilliant green. However, bacterium 303, a Gram negative rod-shaped organism isolated by Schatz, Savard and Pintner (1949) and reported to be a cholesterol-oxidizer, decolorized malachite green rapidly. One variant culture of *M. berolinense,* which failed to decolorize this dye, was run as a control with the normal strain under identical conditions of culture in Tak medium at pH 6.8, containing 750 mg. of cholesterol at 0.3 percent concentration and 1:100,000 of malachite green. After shaking it was found that the normal strain had consumed 70 percent of the sterol while the variant strain had used but 31 percent.

A summary of the extent of complete degradation of cholesterol by the organisms studied, both those isolated in this study and those obtained from other laboratories, is given in table 5. The acid-fast mycobacteria were in general most active. The partially acid-fast species of *Nocardia* in accordance with Turfitt's findings, were also very active, whereas the non-acid-fast bacteria which we tested were inferior in this ability. Of the three groups of saprophytic mycobacteria, those belonging to Group III were most active in stationary cultures though major differences between Groups I, II, and III became less apparent in shaken cultures.

The organic residues appeared to consist largely of cholesterol along with impurities which were difficult to separate by direct chromatography. The residues from *M. lacticola* (Tak strain) and *M. berolinense* were also subjected to chemical separation (Dobriner, Liebermann and Rhoads, 1948); the acid fractions were respectively 5.4 and 6.4; phenolic, 9.9 and 1.3; ketonic 21.6 and 1.9; and non-ketonic 59.2 and 78.3 percent. The ketonic fraction in the former case was largely delta-4-cholestenone which was also frequently noted during the chromatographic separation which was applied to thirty samples. Turfitt (1948) sought but was unable to find small fragments of the steroid molecule in the form of volatile carbonyl compounds. With a similar purpose in mind, we distilled the acidified liquors from several fermentations, but could detect no volatile acids by titration. It is thus apparent that a method of continuous removal of breakdown products, as described by his investigation, is necessary if intermediates are to be isolated. However, it must be mentioned that the continuous method as used may not be very efficient either. Without replenishment of the organisms, the original inoculum, after it reached the death phase, would effect little change in the substrate.

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Summary

Species of *Mycobacterium* and of *Nocardia* were found in general to possess or acquire the ability to consume cholesterol suspended in an aqueous solution of inorganic salts which provided the nitrogen and mineral requirements. The temperature required for most efficient incubation was found to vary but was, within rather broad limits, correlated with the classification of the organisms. Aeration by means of agitation or bubbling greatly accelerated the growth of bacteria and increased the consumption of the sterol.

Of the acid-fast bacteria, the saprophytic mycobacteria, Group III, and species of the partially acid-fast *Nocardia* were most efficient. Non acid-fast bacteria were poor cholesterol consumers. Reduction of malachite green was found to be a criterion of the ability to utilize cholesterol and was used as a screening test in the isolation of active organisms from wastes and soils.

The complete degradation to carbon dioxide and water or other products of low molecular weight must follow the initial attack on cholesterol very rapidly. Were this not so, it should be possible to isolate intermediates in the degradation process. Actually, in spite of the large amounts of cholesterol which disappeared during certain fermentations, exploratory chemical studies of the fermentation residues revealed that these consisted largely of cholesterol together with small amounts of other compounds which were not completely identified.

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NEWS OF TENNESSEE SCIENCE

(Continued from page 248)

The Isotopes Division, U. S. Atomic Energy Commission, Oak Ridge Operations, has added the following to its staff: Mr. F. W. Kittrell, formerly Sanitary Engineer with T.V.A., Knoxville; Dr. Robert P. Geckler, formerly Assistant Professor in Biology at Vanderbilt University; Dr. Robert Bryden, formerly Associate Professor in Biology at the Middle Tennessee State College.

(Continued on page 270)