STUDIES ON THE INTERACTION BETWEEN PHAGOCYTES AND TUBERCLE BACILLI*

II. The Action of Phagocytes upon C^{14}-Labelled Tubercle Bacilli

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In most investigations on the interaction between phagocytes and bacteria or protozoa the fate of the ingested parasite has been followed by morphological and bacteriological methods. The disappearance (digestion), persistence, or multiplication of the microorganism inside the phagocyte within a given time and under certain conditions has been noted. With tubercle bacilli, however, long term experiments are necessary for observing any changes in the intracellular parasite by such techniques.

The use of radioactively labelled tubercle bacilli made it possible to study some of the influences of phagocytosis on tubercle bacilli in short term experiments. When, in a system of two or more metabolizing components, only one component is radioactively labelled, it is possible to identify radioactive products of this component even in the presence of large amounts of the same but non-radioactive products from the other component. C^{14} is a convenient radioactive label and has the advantage of appearing in all important metabolic end products except water and ammonia. With tubercle bacilli the only catabolite given off in significant amounts is carbon dioxide. Thus, when these organisms are made to incorporate C^{14} into their body structure and fuel reserves the carbon of which is oxidized to CO_2 during respiration, it is possible to obtain an estimate at least of the rate of metabolism of the bacillus in the presence of other respiring cells, such as phagocytes. The total radio-

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activity in the carbon dioxide evolved by the combined system could derive from bacillary respiration and from any conversion of bacterial material to CO_2 by the activity of leucocytic enzymes. Using dead and living bacilli in the presence and absence of leucocytes it is theoretically possible to differentiate between the two sources of radioactive CO_2 in the combined system and to determine the respiratory $C^{14}O_2$ of the bacteria in the presence of leucocytes. The release of radioactive molecules other than CO_2 into the medium by radioactive tubercle bacilli in the presence and absence of phagocytes could also be measured. Such measurements should allow one to draw some conclusions concerning the metabolic activity of tubercle bacilli in the presence of phagocytes and concerning the enzymatic attack by leucocytic enzymes on bacillary components.

In the foregoing paper data on respiration and glycolysis of exudate leucocytes were reported (1). In this paper experiments dealing with the respiratory activity of phagocytized tubercle bacilli and their susceptibility or resistance to enzymatic attack by leucocytes will be presented.

Materials and Methods

Animals.—For the experiments guinea pigs weighing between 350 and 500 gm. were used. The animals, usually males, were received from outside dealers and were fed with pellets, water *ad lib.*, and cabbage.

Leucocyte Suspensions.—The preparation of suspensions rich in polymorphonuclear leucocytes (PMN) and in mononuclear phagocytes (MN) has been described in detail in the preceding paper (1). 16 hours or 4 days after the intraperitoneal injection of the eliciting agent [sodium caseinate for an exudate rich in PMN (85 to 90 per cent) and glycogen for an exudate rich in MN (70 to 95 per cent)] (1), the exudate was collected, washed once, and the cells were suspended in phosphate-free Krebs-Ringer solution at pH 7.4. After a sample for the determination of total cellular phosphorus was removed, phosphate buffer and enough homologous serum was added to give a final concentration of 33 per cent of the latter.

Microorganisms.—The following strains of *M. tuberculosis* were used: a virulent strain Vallée (from Dr. H. Bloch, The Public Health Research Laboratory of the City of New York, New York), an attenuated strain R1Rv (from Dr. R. J. Dubos, The Rockefeller Institute for Medical Research, New York, and a strain of BCG (from Dr. J. D. Aronson, The Henry Phipps Institute, Philadelphia). The strains were maintained by weekly transfers in a liquid medium containing tween 80 and albumin (2). Unless otherwise stated, for all metabolic experiments R1Rv was used. Guinea pigs were vaccinated either with BCG or with R1Rv by injecting intradermally 0.1 ml. of an 8 day old culture. In addition to tubercle bacilli, experiments were done with the following laboratory strains: *M. phlei* (from Dr. C. Gray, Leonard Wood Memorial, Harvard Medical School), *Micrococcus pyogenes* var. *aureus* and *albus*, and *Bacillus subtilis*.

Radioactive Substrates.—Three different radioactive substrates were used to label tubercle bacilli. They were α-C^{14}-glycerol, $CH_3C^{14}OONa$ and $C^{14}O_2$. For other bacteria uniformly C^{14}-labeled glucose was used in some cases. The sources of these substrates were as follows: α-C^{14}-glycerol was synthesized in these laboratories as described previously (3), carboxyl-labeled acetate was synthesized by a standard method (4), and $C^{14}O_2$ was generated from $BaC^{14}O_3$ with 40 per cent perchloric acid (5). The $BaC^{14}O_3$ was obtained from the Oak Ridge Laboratory, Oak Ridge, Tennessee. Uniformly labeled glucose was purchased from the Nuclear Corporation, Chicago.

Cultivation and Preparation of Labeled Organisms.—The medium used with glycerol as labeling substrate was the liquid medium containing tween 80 and albumin (2) for submerged and dispersed growth with the following alterations: the casein digest was omitted, asparagine was replaced by an equimolar amount of ammonium sulfate, 1 per cent glycerol, containing α-C^{14}-glycerol, was added. These alterations were adopted to increase the ratio of radioactive to non-radioactive carbon sources. The same medium (including 1 per cent non-radioactive glycerol) was used with acetate as a labeling substrate, and 0.1 per cent of C^{14}-acetate was added. Higher concentrations of acetate were found to be growth inhibiting.

With $C^{14}O_2$ as labeling agent, the complete Dubos-Middlebrook (2) medium was used. The bacilli were first grown for 3 to 4 days, then the system was closed and $C^{14}O_2$ was generated by addition of perchloric acid to $BaC^{14}O_3$ in a double sidearm of the flask as shown in Fig. 1. Between 50 and 150 ml. of culture were set up in one container. Before harvesting the bacilli, the CO_2 was first removed from the atmosphere of the culture flask by aspiration and absorption in alkali.

The cultures were harvested by centrifugation in 50 ml. celluloid centrifuge tubes for 20 minutes at 10,000 *g*, removing the supernatant and washing the bacilli twice in water containing 0.01 per cent tween 80 and 0.1 per cent bovine albumin fraction V. After the second washing the bacilli were resuspended in water containing 0.01 per cent tween 80. If dead bacilli were to be used, this suspension was kept for 15 minutes in a boiling water bath, while the suspension with the living bacilli was kept at room temperature. Both suspensions were then centrifuged a fourth time and the organisms were resuspended in the complete medium for use in the experiments in the Warburg respirometer.

Sonic Disruption.—Disruption of over 95 per cent of the bacilli was achieved by using a Raytheon 10 kc. oscillator (magnetostrictive), model DF 101. The washed tubercle bacilli were suspended in 15 to 20 ml. of complete medium and exposed to supersonic vibration for 10 minutes at 2°C. The oscillator was equipped with a power stabilizer and special circuit to permit accurate tuning and reproducibility.

Respirometry.—Measurements were made at 37°C. After closing the system in the Warburg respirometer readings were taken over a half-hour period, and then the content of the side arm was tipped in. Readings were taken at regular intervals over a period of 4 hours. At the end of the experiment each manometer was taken out, the chimney was removed from the flask and 0.5 ml. 1 N HCl was quickly added through the side arm of the flask. After closing it, the acid was tipped into the main compartment and all flasks were shaken for 15 minutes.

Recovery of CO_2 and Soluble Molecules.—After addition of the acid and after the final incubation period the total CO_2 absorbed in the center well and on the filter paper was collected quantitatively in a conical centrifuge tube by rinsing the center well three times with distilled water. The filter paper was then washed with distilled water on the edge of the centrifuge tube. 0.03 ml. of a solution of 1 N $NaHCO_3$ was added as carrier. The carbonate was then precipitated by adding 2 ml. of a solution containing 1.4 per cent BaCl and 2.7 per cent NH_4Cl in water (6). The tubes were left at room temperature for at least 2 hours or in the refrigerator over night. The precipitates were washed by repeated centrifugation and resuspension in 50 per cent ethanol. The total precipitate from each center well was then plated on one planchet, washing the tube carefully with small amounts of 50 per cent ethanol.

If small organic molecules were to be recovered after the experiment, the cell suspension was removed from the flasks and centrifuged. The supernatant fluid was filtered through a syringe type of Seitz filter. Aliquots of these filtrates were then plated on planchets.

Determination of Radioactivity.—The main radioactive measurements were (*a*) determination of the radioactivity of the bacterial suspension by combustion or by direct plat-

ing, (*b*) determination of the radioactivity in the center well, and (*c*) determination of the radioactivity of a filtrate of the medium at the conclusion of the experiment by direct plat-

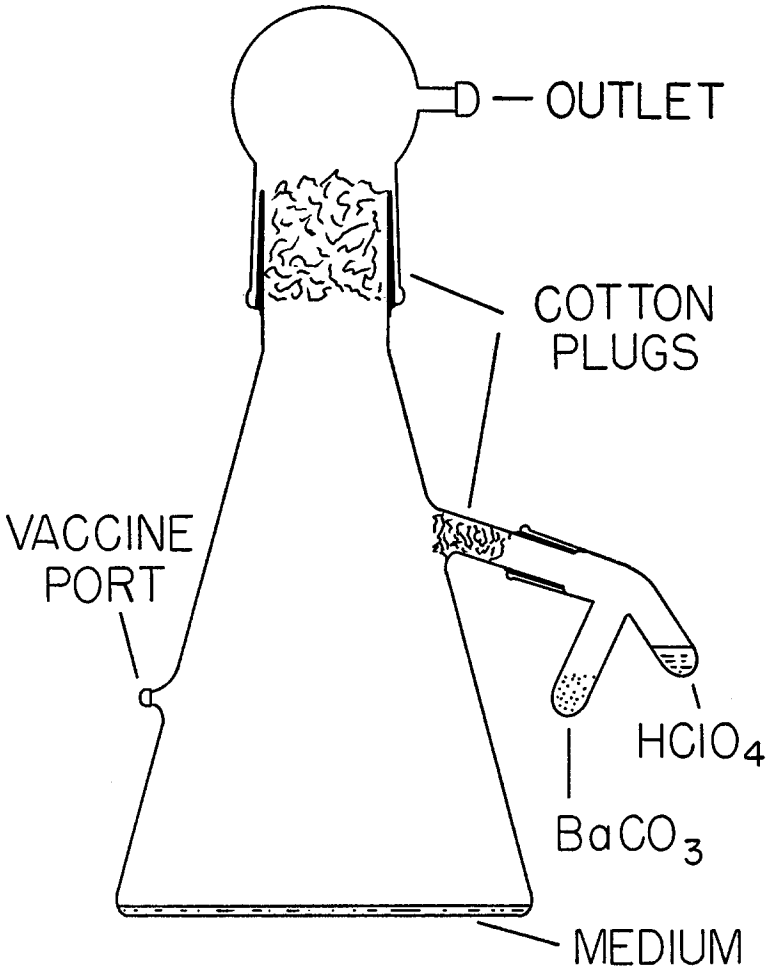

FIG. 1. Diagrammatic representation of the flask used for the growth of tubercle bacilli in an atmosphere of $C^{14}O_2$. The $C^{14}O_2$ was generated from the V-shaped side arm by tipping the $HClO_4$ into the $BaC^{14}O_3$. Before the bacteria were harvested CO_2 was removed from the atmosphere in the flask by aspiration through the vaccine port and the opening in the spherical cap. CO_2 was absorbed in sodium hydroxide solution.

ing. Radioactive measurements were made using a gas flow counter (7). Respiratory CO_2 was counted as $BaCO_3$ (8). Organic compounds were counted directly as described previously (9).

Calculations.—Cellular activity is expressed on the basis of 100 μg. of leucocyte phosphorus (1) and the radioactivity is expressed as C.P.M. evolved per hour of incubation and per 10,000 C.P.M. put into the flask.

RESULTS

I. *Breakdown of Bacillary Material by Leucocytes*

A. Complete Oxidation to CO_2.—

The extent of attack by leucocytic enzymes on tubercle bacilli was measured by exposing various preparations of dead bacilli to leucocytes (PMN). For control and comparison, studies were also carried out with several organisms other than tubercle bacilli.

1. Heat-Killed, Dried Tubercle Bacilli (Strain H37Rv) (12).—When these organisms were incubated for 4 hours in the presence or in the absence of leucocytes, the amounts of radioactivity recovered from the center well were

TABLE 1

$C^{14}O_2$-Production from Heat-killed (100°C.) Labelled Bacteria by Leucocytes

Bacterium	Label introduced by growth on:	$C^{14}O_2$ liberated:		No. of experiments
		Counts from bacteria alone	Increase in counts due to leucocytes	
M. tuberculosis R_1R_v	C^{14} glycerol	0.2 ± 0.1	0.2 ± 0.1	3
"	C^{14} acetate	2.8 ± 1.9	0.02 ± 0.02	2
"	$C^{14}O_2$	0.4 ± 0.1	1.0 ± 0.2	28
M. phlei	C^{14} glucose	1.0 ± 0.1	7.2 ± 1.3	2
M. pyogenes	C^{14} glucose	0.00	10.4 ± 0.7	4
B. subtilis	C^{14} glycerol	0.8 ± 0.7	14.2 ± 0.3	4

The results are expressed as counts recovered from the center well per hour of experiment, per 10^4 counts used, and per 10^8 phagocytes. (The mean and standard deviation of the mean are given.)

not significantly above background. These organisms were thus metabolically inert, and there was also insignificant conversion of bacillary material to CO_2 by leucocytes.

2. Fresh Heat-Killed Tubercle Bacilli (Strain R1Rv).—The results obtained with these organisms (Table I) were similar to those mentioned above, although the counts observed in these experiments were usually significantly above the background, with or without leucocytes (Table I, columns 3 and 4). This was probably due to the higher specific activity of the carbon of these organisms than of those used in the dried state.

3. Other Heat-Killed Organisms.—In Table I are also presented results obtained when three organisms other than the tubercle bacillus were heat-killed and exposed to leucocytes. There was significant conversion of bacterial carbon to $C^{14}O_2$ by the leucocytes (Table I, columns 3 and 4).

4. Sonically Disrupted Tubercle Bacilli.—Table II contains results obtained when sonically disrupted, live tubercle bacilli (R1Rv) were exposed to PMN.

Conversion of bacillary material to CO_2 may be observed to be considerable (column 3, Table II). When heat-killed tubercle bacilli were treated in the sonic oscillator and exposed to leucocytes, 17.2 ± 3.0 C.P.M. were produced per hour of the experiment, 10^4 counts used and 10^8 leucocytes. Comparing this result with the figure obtained with sonicated live tubercle bacilli (8.7 ± 1.2; Table II), it is evident that heated bacillary material is more easily attacked and oxidized by leucocytic enzymes than is the unheated material.

B. Conversion of Bacillary Material to Filterable Fragments.—

The data cited above represent only that part of the bacillary material which is converted to CO_2. It is probable that not all of the carbon compounds that might have been split off the bacteria by leucocytes would have been completely oxidized. In order to obtain some idea of the amount of material split off the bacteria, filtrates of the system were prepared after incubation, as described under Materials and Methods.

TABLE II

$C^{14}O_2$-Production from Sonically Disrupted Labelled Live Tubercle Bacilli by Leucocytes

Label introduced by growth on:	$C^{14}O_2$ liberated:	
	Counts from disrupted bacteria alone	Increase in counts due to leucocytes
C^{14}-glycerol	3.5 ± 2.5	7.1 ± 5.0
$C^{14}O_2$	9.4 ± 2.5	8.7 ± 1.2
C^{14}-acetate	2.5 ± 1.8	23.5 ± 0.4

Results expressed as C.P.M./hour and 10^4 counts used and 10^8 leucocytes.

When dead labelled bacilli corresponding to 10^4 C.P.M. were incubated without PMN an average of 52 C.P.M. per hour of experiment appeared in the bacterium-free filtrate. In three out of the four cases tested in which PMN were also present, the filtrate contained less than this number of counts. Only in one case was there a detectable increase in soluble bacillary material due to leucocytic action.

II. *Respiration of Phagocytized Tubercle Bacilli*

A. CO_2 Production.—

After demonstrating that, using intact heat-killed tubercle bacilli, only negligible amounts of bacillary material are converted to CO_2, it was assumed that the $C^{14}O_2$ evolved by a mixture of live tubercle bacilli and leucocytes represents the respiratory CO_2 of the tubercle bacilli.

Table III summarizes the results of experiments in which the $C^{14}O_2$ given off by living tubercle bacilli alone, or in the presence of leucocytes, was measured. It may be seen that tubercle bacilli give off, in general, less CO_2 after they have been phagocytized than when they are merely suspended in the medium (Table III, column 4).

In order to imitate more closely conditions *in vivo*, an experiment was carried out in which the atmosphere in the flask contained CO_2. For this purpose, the gas phase was air with 5 per cent CO_2, and absorption of the CO_2 was not carried out until the conclusion of the incubation. Dixon-Keilin flasks were used (10) instead of the conventional flasks, and the stop-cock containing the absorbent was connected to the center well after 4 hours incubation. Experiments in conventional flasks, using air and with the center well in contact with the atmosphere in the flask throughout the experiment were conducted simultaneously.

It was found that the presence of 5 per cent CO_2 in the atmosphere in the flasks reduced $C^{14}O_2$ production by the bacilli in the absence of leucocytes to a level 65 per cent of that in air. This effect was even more marked in the case of phagocytized bacilli, *i.e.*, the ratio "counts in presence of leucocytes/counts in absence of leucocytes" (as calculated in Table III) was somewhat lower

TABLE III

$C^{14}O_2$-Production by Labelled Living Tubercle Bacilli outside and inside Phagocytes in an Atmosphere of Air

Phagocytes derived from	Label introduced by growth on:	$C^{14}O_2$ as counts from bacilli alone	Ratio: Counts $\frac{+\text{ leucocytes}}{-\text{ leucocytes}}$	No. of experiments
Normal guinea pig	C^{14}-glycerol	75.3 ± 10.2	0.81 ± 0.06	13
" " "	C^{14}-acetate	52.5 ± 1.8	0.85 ± 0.01	2
" " "	$C^{14}O_2$	48.5 ± 3.4	1.02 ± 0.05	16
Infected " "	$C^{14}O_2$	48.5 ± 3.4	0.92 ± 0.04	6
Normal rabbit	$C^{14}O_2$	48.5 ± 3.4	0.79 ± 0.03	2
Infected "	$C^{14}O_2$	48.5 ± 3.4	0.85 ± 0.03	2

when the experiment was performed in an atmosphere containing 5 per cent CO_2 than when it was performed in an atmosphere devoid of CO_2.

B. Oxygen Uptake.—

Although the rate of CO_2 production by tubercle bacilli gives some indication of the rate of oxidative processes, it does not parallel the rate of oxygen uptake, since the respiratory quotient of tubercle bacilli can vary considerably, depending mainly on the available substrates and the oxygen concentration (11). To investigate the metabolism of tubercle bacilli inside phagocytes it was thus desirable to measure the oxygen uptake of intracellular bacilli. For this purpose it was assumed that the oxygen uptake rate of PMN is altered to the same extent whether they phagocytize living or dead tubercle bacilli. If the O_2 consumption in two Warburg flasks, each containing the same amount of PMN but one containing in addition living and the other dead tubercle bacilli, is measured, any difference in oxygen uptake between the two flasks should be due to the respiration of the living, phagocytized bacilli. This oxygen uptake can then be compared to that of the same amount of bacilli when they respire in the absence of leucocytes.

Five experiments of this type were done with the strain R1Rv. The oxygen uptake of intracellular bacilli was found to be 73.4 ± 11.5 per cent of that of extracellular bacilli suspended in the complete medium. In similar experiments with the saprophyte *M. phlei*, the oxygen uptake of the ingested bacteria was reduced to less than 20 per cent of the value in the absence of leucocytes.

C. Effect of Oxygen Concentration.—

All experiments so far reported were done in an atmosphere having the oxygen concentration of air. Since tubercle bacilli depend on the availability of oxygen and since the oxygen tension in tissue fluids is lower than that of air, a number of experiments was conducted in which the atmosphere consisted of 1 per cent oxygen, 99 per cent nitrogen. In the medium used in our experiments, the oxygen uptake of tubercle bacilli was reduced by 37.0 ± 3.0 per cent in an atmosphere with 1 per cent O_2, as compared with air.

TABLE IV

$C^{14}O_2$-Production by Living Labelled Tubercle Bacilli with and without Leucocytes in an Atmosphere with 1 per Cent Oxygen

Phagocytes derived from	Label introduced by growth on:	$C^{14}O_2$ as counts from bacilli alone	Ratio: counts $\frac{+ \text{ leucocytes}}{- \text{ leucocytes}}$	No. of experiments
Normal guinea pig	C^{14}-glycerol	73.6 ± 9.2	0.8 ± 0.1	3
" " "	$C^{14}O_2$	62.7 ± 9.5	1.0 ± 0.1	5

The figures for the counts obtained from radioactive tubercle bacilli, when they respired in the presence and absence of leucocytes in 1 per cent oxygen, are given in Table IV. They are not significantly different from those obtained in an atmosphere of air. That means that tubercle bacilli, although they take up less oxygen in 1 per cent oxygen than in air, give off the same amount of CO_2 in both atmospheres. If the $C^{14}O_2$ evolved is representative of the total respiratory carbon dioxide of tubercle bacilli, then the respiratory quotient of these microorganisms increases by about 40 to 60 per cent when the oxygen concentration is changed from 21 per cent to 1 per cent. The ratio for $C^{14}O_2$ production with leucocytes/without leucocytes is approximately the same with both oxygen concentrations (column 4 in Tables III and IV).

III. *Effect of Phagocytosis on Tubercle Bacilli Heated at 56°C.*

When a suspension of tubercle bacilli is kept in a boiling water bath considerable alteration of the physico-chemical state of the constituents of the bacilli probably occurs. To reduce this alteration to a minimum, some experiments were performed in which the microorganisms were killed by keeping them suspended in the complete medium, for 1 hour in a water bath of 56°C. Tubercle bacilli treated in this way did not give rise to any visible

growth on the oleic acid agar medium. Such "mildly" killed radioactive bacilli were then used for the same type of experiment as reported for the live bacilli (Table V).

Although tubercle bacilli heated for 1 hour to 56°C. have to be regarded as dead from the point of view of multiplication, they still had a small residual oxygen uptake when suspended in the complete medium. They also produced some CO_2, as can be seen from the counts indicated in Table V, column 2. When they were phagocytized, however, they gave off 5.2 times more $C^{14}O_2$ than in the absence of leucocytes (column 3). It has been shown above that leucocytes did not convert significant amounts of the carbon of intact tubercle bacilli to CO_2. It follows then that the bacilli which have been treated at 56°C. are apparently stimulated with respect to respiratory CO_2 production when they are surrounded by leucocyte protoplasm. A similar effect can be brought about by adding such bacilli to a suspension of sonically disrupted PMN; the stimulation is 2.9 fold (Table V) and of a lower degree than with

TABLE V

$C^{14}O_2$-Production by Heat-treated (56°C.) Labelled Tubercle Bacilli in Presence and Absence of Phagocytes, in an Atmosphere of Air

Tubercle bacilli exposed to	$C^{14}O_2$ as counts from bacilli alone	Ratio: counts $\frac{+\text{ phagocytes}}{-\text{ phagocytes}}$	No. of experiments
Intact guinea pig leucocytes.....	5.5 ± 2.3	5.2 ± 1.3	11
Sonicated guinea pig leucocytes.....	5.5 ± 2.3	2.9 ± 0.1	2

whole leucocytes. The decrease of stimulation with sonicated leucocytes is probably due to the fact that the leucocytic protoplasm surrounding the bacilli is diluted compared to the conditions inside the intact phagocyte.

In one experiment, an attempt was made to measure the oxygen uptake of intracellular, heat-treated (56°C.) tubercle bacilli. This was done in the same way as with the living microorganisms. It was noted that the oxygen uptake of the heated bacilli was also stimulated by phagocytosis, although to a somewhat lesser degree than CO_2 production.

DISCUSSION

The results presented in this paper indicate that with the technique employed a distinction could be made in mixtures of leucocytes and tubercle bacilli between the metabolic activity of leucocytes and that of the bacilli. Theoretically, phagocytosis of microorganisms can result in an alteration of their metabolic activity, in breakdown of material of the parasite, or in both. Leucocytic attack on bacteria has been followed by measuring the conversion of bacillary carbon to $C^{14}O_2$ or the liberation from the bacilli of labelled substances capable of passing a bacterial filter. In addition, changes in leucocytic

metabolism occur during phagocytosis. These have been discussed in the preceding paper.

$C^{14}O_2$-Fixation by Leucocytes.—To eliminate the possibility that significant amounts of $C^{14}O_2$ might escape detection through fixation by the leucocytes, an experiment was carried out in which leucocytes were incubated in the complete medium in an atmosphere containing $C^{14}O_2$ generated from $BaC^{14}O_3$ of very high specific activity. After 4 hours of incubation the leucocytes were centrifuged, washed, combusted and the resulting $BaCO_3$ was counted. The medium was also examined. Insignificant amounts of radioactivity were detected in the leucocytic material, and low, though significant, amounts in the medium. It was concluded that the fixation of $C^{14}O_2$ into the leucocytes (0.1 per cent) or the medium (1.0 per cent) in the phagocytosis experiments was too low to affect the results. It should be noted that the presence of a CO_2 absorber in the center well further reduced the possibility of such interference.

Leucocytic Attack on Bacilli.—The results clearly showed that leucocytes from normal guinea pigs were unable to convert significant amounts of carbon of dead but structurally intact tubercle bacilli to CO_2 within 4 hours of incubation. This was also the case with leucocytes obtained from animals previously infected with tubercle bacilli (BCG, R1Rv, or Vallée strains). The fact that protoplasmic components of other microorganisms such as *B. subtilis* and *M. phlei* were converted to CO_2 in significantly measurable amounts indicated that the technique used allowed the determination of bacillary breakdown by leucocytic enzymes. It must thus be concluded that the tubercle bacillus is resistant to such enzymatic activity. This resistance of the tubercle bacillus is not due to a lack of enzymes on the part of the phagocyte, since the carbon of sonically disrupted tubercle bacilli was converted to CO_2 to a degree comparable to that observed with whole bacteria other than tubercle bacilli.

When tubercle bacilli are labelled by means of different radioactive substrates such as C^{14}-glycerol, C^{14}-acetate, or $C^{14}O_2$, the distribution of activity among the chemical fractions of the bacilli varies for each substrate (12). Some of the differences observed in the experiments reported above might be explained by this fact. Further, the actual composition of the growth medium may affect the chemical constitution of the bacillus; *e.g.*, the presence of glycerol or acetate in the medium has been shown to increase the lipide content of the tubercle bacillus (13). The results obtained in the present work do not yet allow an interpretation in terms of the nature of the bacillary components most readily attacked by leucocytes and converted to CO_2.

There was no indication that radioactive substances capable of passing through bacterial filters were liberated from tubercle bacilli in large amounts by leucocytes. In fact less activity was detected in the filtrate of the medium when phagocytes were present than when the bacilli were incubated alone.

This was also the case with sonically disrupted bacilli. This fact indicated that leucocytes take up or bind some of the bacillary material. No attempt has been made to analyze the leucocytic protoplasm for radioactivity after incubation with tubercle bacilli—intact or sonicated.

Respiration of Phagocytized Bacilli.—In the present study only gross overall changes of metabolism have been studied; *viz.*, changes in oxygen consumption and CO_2 production. Since heat-killed intact tubercle bacilli are not significantly converted to CO_2 by leucocytes, all the CO_2 produced by phagocytized living tubercle bacilli can be considered to be due to bacillary respiration. This assumption was strengthened by the finding that in the case of the sonicated tubercle bacilli, heat-treated sonicated material was more extensively attacked than sonicated living bacilli.

The O_2 consumption of living intact bacilli was reduced by 27 per cent and the CO_2 production by 20 per cent on phagocytosis. These figures might be interpreted as demonstrating a slight change in the R. Q. of the bacilli, but they could also indicate that the ratio of $C^{14}O_2/C^{12}O_2$ produced depended on the environment of the bacilli; *i.e.*, the bacilli could have access to different substrates within the leucocyte from those available outside.

One of the most vulnerable points in the metabolism of tubercle bacilli is the need for oxygen. When the atmosphere contains 21 per cent oxygen, this need is probably satisfied even inside phagocytes. However, when the oxygen concentration is lowered to a value which corresponds to the oxygen tension of the extracellular tissue fluids, the situation becomes more critical. Although tubercle bacilli are able to multiply in an atmosphere with 1 per cent oxygen, they grow more slowly and the growth obtained per unit of oxygen used is smaller than with higher oxygen concentrations (14). Judging from the $C^{14}O_2$-production in the present experiments it seems that as much oxidation occurs in the tubercle bacillus in an atmosphere with 1 per cent oxygen as in 21 per cent oxygen (Tables III and IV); oxygen uptake measurements, however, show that this is not the case, since the oxygen uptake rate in 1 per cent oxygen is 37 per cent lower than that in 21 per cent oxygen. This situation seems to be even more accentuated when the bacilli are phagocytized; there is still the same $C^{14}O_2$-production in 1 per cent oxygen as in an atmosphere of air (Tables III and IV), but preliminary experiments suggest that the oxygen uptake of intracellular tubercle bacilli is only 33 per cent of that of extracellular organisms in an atmosphere with 1 per cent O_2. Since the energy production of tubercle bacilli depends almost completely on oxidative processes, it can be calculated that these microorganisms produce about five times less energy inside a phagocyte with an atmosphere of 1 per cent oxygen than they do in a favorable environment with plenty of oxygen available, *i.e.*, extraleucocytically in air. Both reduction of oxygen concentration and phagocytosis appear to increase the respiratory quotient of tubercle bacilli and the

combination of these two environmental changes seems to have a potentiated effect, since our data indicate that the R. Q. of intracellular tubercle bacilli in an atmosphere with 1 per cent oxygen is almost five times higher than that of free bacilli respiring in the complete medium in an atmosphere of air. What the mechanism of this increase in R. Q. is remains to be explored. The possibility exists that lactic acid plays some part, because the concentration of this compound is higher in the presence of leucocytes and becomes still higher when the oxygen concentration is lowered. Heplar (11) has shown that lactic acid favors an increase in R. Q. which is brought about by a decrease in oxygen concentration. However, if the tubercle bacilli utilize C^{12}-lactate produced by the leucocytes, rather than their own endogenous C^{14}-labelled energy stores, the R. Q. would tend to appear low since $C^{14}O_2$ is the measure of bacillary CO_2 production in our experiments.

It is of interest to note that the ratio of $C^{14}O_2$ production by free and phagocytized tubercle bacilli was different for bacilli grown on C^{14}-glycerol from the ratio obtained with bacilli originally labelled by growth in an atmosphere of $C^{14}O_2$. This difference is significant ($p < 0.02$). This again might be due to differences of distribution of the various radioactive labels among bacillary fractions. When leucocytes from infected animals were used for these experiments the ratio of $C^{14}O_2$ production by free and phagocytized tubercle bacilli was not significantly different ($p < 0.1$) from the ratio obtained with leucocytes from normal guinea pigs. Observations on the effect of phagocytosis on O_2 consumption by living organisms other than tubercle bacilli are in sharp contrast to those with the tubercle bacillus and indicate that alteration of metabolic activity is accompanied by disruption of the organisms in the leucocytes.

Leucocytic Stimulation of $C^{14}O_2$ Production by Bacilli Killed at 56°C.—The observation that tubercle bacilli heated for 1 hour at 56°C. showed a significant increase of $C^{14}O_2$ production when phagocytized is unexplained. The gaps in the metabolic pathways of the tubercle bacillus produced by heating may be filled partially by intermediates, enzymes, or coenzymes provided by the leucocytes. This reactivation seems comparable to the observation that bacteria which have been killed by chemicals or heat and are no longer able to multiply in ordinary bacteriological media can be revived by the addition of tricarboxylic acid cycle intermediates (15).

Role of the Bacillary Surface in Resisting Leucocytic Attack.—These studies demonstrate that the tubercle bacillus, in contrast to saprophytic mycobacteria, is endowed with the power to maintain its respiratory metabolism in an intracellular environment. The intact bacillus is resistant to attack by leucocytic enzymes which are capable of degrading the bacillary material of disrupted tubercle bacilli. It seems possible that this ability of tubercle bacilli to behave as intracellular parasites is due at least partially to the physico-

chemical state of the bacterial surface. Alterations of the surface of the tubercle bacilli can be brought about by various means other than sonic disruption, such as extraction with petroleum ether or simple aging, and frequently result in partial loss of pathogenicity for laboratory animals (16). It is conceivable that such structural changes on the surface of the organisms render them susceptible to enzymatic attack by leucocytic enzymes or cause metabolic changes after phagocytosis similar to those observed with saprophytic organisms. The observation that the non-ionic surface-active agent triton WR 1339 has a suppressive effect on experimental tuberculosis (17) further supports this interpretation. This substance has no inhibitory effect on tubercle bacilli *in vitro* and it accumulates in phagocytes *in vivo*. Furthermore, these antituberculous agents have an effect on the sensitivity of erythrocytes to cold shock similar to the effect due to removal of cholesterol from the surface of the red cells. It has been suggested that these surface-active agents remove some hydrophobic lipide or lipides from the surface of tubercle bacilli (18) thus causing an alteration of the outcome of the interaction between host and parasite on a cellular level (19).

SUMMARY

Tubercle bacilli labelled with C^{14} were prepared by growth on radioactive substrates such as glycerol, CO_2, and acetate. These organisms were exposed *in vitro* to leucocytes (mostly polymorphonuclear leucocytes) from peritoneal exudates of guinea pigs. The respiration of the leucocytes and of the bacilli, alone and together, was followed by determining oxygen uptake and $C^{14}O_2$ production. When heat-killed labelled tubercle bacilli were exposed to leucocytes there was little or no degradation of bacillary material to $C^{14}O_2$ by leucocytic enzymes. On the other hand, conversion of components of sonically disrupted bacilli to $C^{14}O_2$ by leucocytes was significant.

It was possible to determine the oxygen uptake and $C^{14}O_2$ production of phagocytized living tubercle bacilli, and it was found that after phagocytosis the bacilli maintained their rates of oxygen consumption and $C^{14}O_2$ production. This finding was in contrast to observations made with *Mycobacterium phlei*, a saprophytic acid-fast organism, and with *Bacillus subtilis*. In these cases oxygen consumption and $C^{14}O_2$ production declined after phagocytosis, and bacterial components were converted to carbon dioxide to a significant degree by leucocytic enzymes.

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