161. *Losses of (Radioactive) Sulphur and Phosphorus from Resting Bacterial Cells.*

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By the use of the radio-isotopes ^{35}S and ^{32}P it has been shown that there is a gradual loss of sulphur and phosphorus from cells of *Bact. lactis aerogenes* which are suspended in a synthetic medium containing all growth requisites except a source of carbon. Such losses occur predominantly while the cells are still alive, and are slowed down abruptly if the cells are killed. Subsequent lysis is (at pH 7) a very much slower process.

No simultaneous uptake of sulphate or phosphate from the medium has been detected under these conditions. The implications of these findings are considered : a lively exchange within the still living cell is suggested, whereby internal concentrations are maintained and feed the slow irreversible outflow.

Considerable interest attaches to the question of the chemical activities of non-growing cells, which are much greater than is implied by the term resting. In particular, it was desired to study material exchanges occurring during the slow decline of a population of *Bact. lactis aerogenes*, since information on this matter is of importance in connexion with the law governing the death-rate (cf. *Nature*, 1951, **167**, 666).

The application of radio-isotopes according to the well-known principles (cf. *Cold Spring Harbor Symp. Quant. Biol.*, 1948, **13**; especially Hevesy, p. 129) was the obvious method of obtaining the required information.

This work has been carried out on suspensions of *Bact. lactis aerogenes* in a synthetic medium containing ammonium and magnesium chlorides, ammonium sulphate, potassium phosphate, and a buffer of either phosphate or phthalate.

Owing to the absence of a source of carbon no growth can take place in such a medium and the cells are said to be in the " non-proliferating " state. According to the temperature, the life of such cells may range from a few minutes to several months, and the object is to discover what changes occur in the sulphur and phosphorus contents during the gradual decline of the viable population.

Loss of Sulphur from " Non-proliferating " Cells.—Bacteria grown on a medium containing sulphate labelled with ^{35}S were suspended in a medium buffered with phosphate (pH 7), and placed in a thermostat. Samples were removed at intervals and the bacteria separated in a centrifuge so that the activity of the clear supernatant medium could be determined with a Geiger counter. The viable count of the suspension was estimated at suitable times by the usual plating technique.

Experiments at both 40° and 25° were conducted. At the latter temperature two parallel runs were made, one with a high concentration of inactive sulphate in the medium, the other with only about the same total amount as in the bacteria themselves.

The results are shown in Fig. 1, where the activity of the supernatant medium represents sulphur lost from the cells.

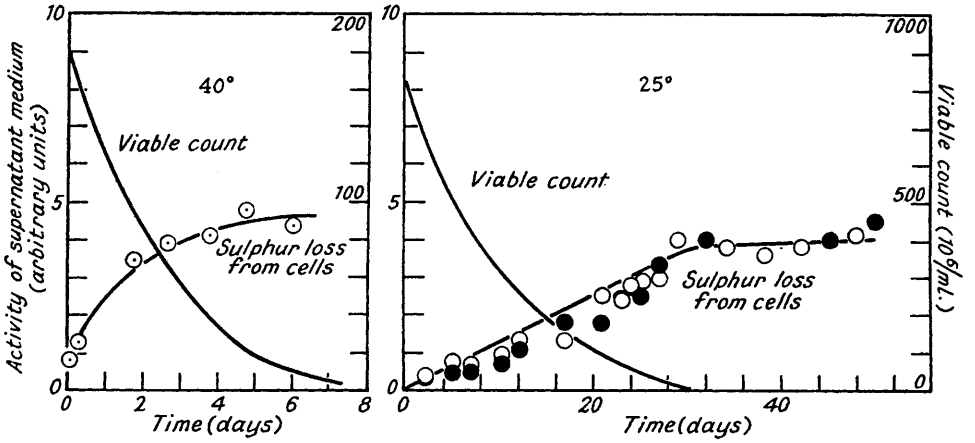
Loss of Phosphorus from " Non-proliferating " Cells.—In order that the phosphate concentration might be varied as desired without effect on the buffering capacity of the medium, a phthalate buffer (pH 6·5) was employed for these experiments, which were made on suspensions of bacteria grown in phosphate labelled with ^{32}P. The difference in pH between a phthalate and a phosphate buffer is in fact insufficient to produce a significant difference in the death rate of *Bact. lactis aerogenes*. At a given temperature, therefore, the results of the respective experiments with sulphur and phosphorus are quite comparable. This was confirmed by the results of an experiment with a culture that had been grown in a medium containing active sulphate and active phosphate together.

The results are shown in Fig. 2.

Effect of killing the Bacteria on the Sulphur and Phosphorus Losses.—A suspension of radioactive cells was prepared as in earlier experiments. After a few hours at 40° two large

portions were removed. In one the cells were killed by the addition of formaldehyde and in the other by heating them to 70° for five minutes. Each sample was then replaced in the 40° thermostat, and samples were removed at intervals for determination of the activity of the medium, which, as before, would indicate the loss of sulphur or phosphorus from the cells.

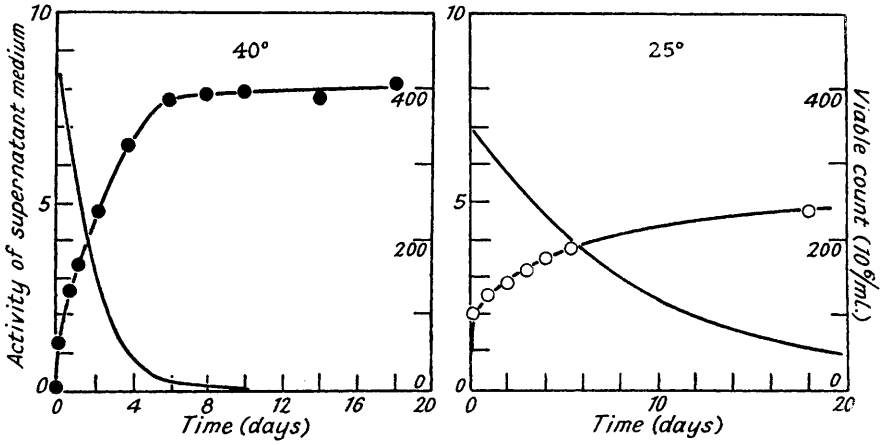
FIG. 1. *Loss of* ^{35}S *from resting cells.*



Rising curves give activity of supernatant liquid in arbitrary units so chosen that total activity of whole suspension = 10 (left-hand scales).

Falling curves give viable counts (right-hand scales). In right-hand diagram, ● 1·82 g. of SO_4'' per l. of medium; ○ $2{\cdot}6 \times 10^{-3}$ g./l.

FIG. 2. *Loss of* ^{32}P *from resting cells.*



Falling curves (right-hand scales), viable count.

Rising curves (left-hand scales), activity of supernatant liquid, *i.e.*, loss of phosphorus from cells.

The results are shown in Fig. 3 and Fig. 4.

Uptake of Sulphur and Phosphorus from the Medium.—For these experiments the bacteria were grown on inactive sulphate and phosphate.

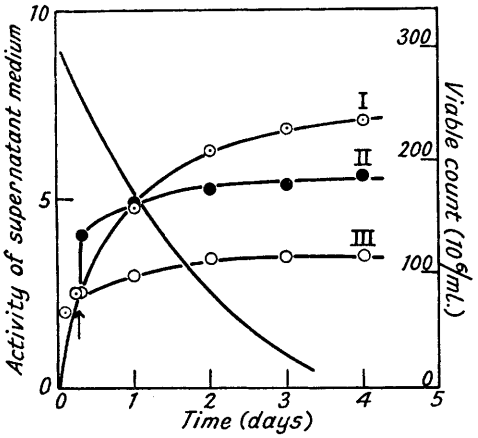
For the study of phosphorus uptake the suspending medium was buffered with phthalate and contained a known amount of labelled phosphate, while for the study of sulphur uptake the medium contained a known amount of labelled sulphate and was buffered with phosphate.

In order that a small uptake by the cells might be detected, the labelled sulphate or phosphate added to the medium was of very small amount but of high specific activity.

In each suspension initially the total amount of labelled element (active plus inactive) in the medium was approximately equal to that in the cells themselves (inactive). In order to fulfil this condition one litre of a suspension containing, for example, 200 million/ml. would require only 2·5 mg. of sulphate ion or 20 mg. of phosphate ion in the medium.

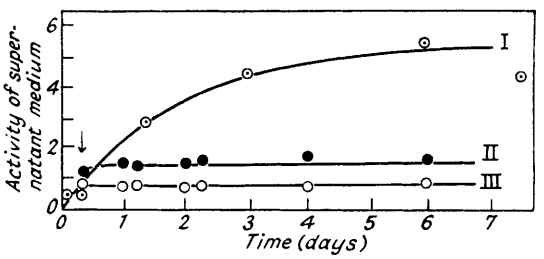
In the conditions of the experiments no significant uptake of the active elements could be detected.

FIG. 3. *Loss of* ^{32}P *(at 40°) from cells killed by heat or formaldehyde.*



I, Control—cells not killed.
II, Killed by heat after 6·5 hours (indicated by arrow).
III, Killed by formaldehyde after 6·5 hours (indicated by arrow).
Activity of whole suspension = 10 units.

FIG. 4. *Loss of* ^{35}S *(at 40°) from cells killed by heat or formaldehyde.*



I, Control—cells not killed.
II, Killed by heat after 8 hours (indicated by arrow).
III, Killed by formaldehyde after 8 hours (indicated by arrow).
Activity of whole suspension = 10 units.

Discussion of Results

When cells of *Bact. lactis aerogenes* are suspended in a synthetic medium containing all growth requirements except a source of carbon the number of viable cells per ml. (viable count) decreases with time, the death rate depending on the temperature of the suspension. At 40° in the conditions of these experiments it is such that after 7 days the viable count falls to less than one-tenth of its initial value, while at 25° a corresponding fall occurs in about 30 days.

At both these temperatures cells labelled with ^{35}S are found to lose their activity to the surrounding medium at a rate which decreases in much the same way as the viable count itself (Fig. 1). The rate at which the ^{35}S appears in the medium as a consequence of this loss is independent of the concentration of inactive sulphate outside the cell.

As can be seen from Fig. 2 the behaviour of phosphorus is very similar in that the rate of loss of ^{32}P from the cells to the medium falls off approximately in parallel with the viable count itself. The main difference is that the cell can lose phosphorus much more

extensively than sulphur, so that by the time the viable count becomes negligible at least 80% of the total phosphorus has been lost, but only 40% of the total sulphur. This result is perhaps hardly surprising since a considerable part of the cell phosphorus may be present as phosphate esters while the sulphur is probably present mainly as protein.

The experiments in which the cells were deliberately killed were designed to answer the question whether the loss of sulphur and phosphorus from bacteria is due to an activity of the viable cell, or rather to a rapid autolysis of the cell after death. In either case the rate of loss would run parallel with the viable count as is actually found. If, however, the cells are killed soon after suspension, then a clear-cut answer can be obtained.

The experiments made with ^{35}S and with ^{32}P respectively (Figs. 3 and 4) show conclusively that it is the viable cells which are principally responsible for the loss of sulphur and phosphorus to the medium. In each case there is an appreciable loss from the cells during the actual process of killing by heat, and this is almost certainly due to the increase in metabolic activity of the cells as the temperature is raised. After this, however, the rate of loss falls to quite small values. It is interesting to note in the case of phosphorus that even after the killing the loss continues though at a much diminished rate for approximately 36 hours, whence it appears that the partially degraded phosphate esters presumably present in the cell at the moment of death can continue to escape by a purely non-biological process. The absence of such a phenomenon with the sulphur suggests that under these conditions the protein breakdown can be accomplished readily only by the living cell.

The next question is whether or not these losses from the viable cell are accompanied by a simultaneous uptake of sulphate and phosphate from the medium. This is answered by the experiments on the suspension of non-radioactive bacteria in the synthetic medium containing radioactive sulphate or phosphate, the conditions being so arranged that initially there is approximately the same amount of either element in the cells as in the medium.

Neither with sulphur nor with phosphorus is any uptake indicated, though the counting techniques employed would detect an uptake of 5—10% of the labelled component. Any uptake, therefore, is insufficient to offset more than a small fraction of the simultaneous loss from the cell. Once the great majority of cells are dead, survivors may well use lysed cell material as a source of carbon and maintain a steady turnover of essential elements with little overall loss, and though such systems might yield results of interest they are outside the scope of the present discussion.

As to the general implication of these various results, it might perhaps have been expected that one of two types of behaviour would be observed: (*a*) an irreversible loss of material from dead and lysing cells, or (*b*) a more or less lively exchange between living cells and the surrounding medium. In fact, while there is predominantly a loss, it is one which occurs from still living cells.

The simplest interpretation of this finding is as follows. There is an active internal exchange of material between various parts of the living cell. The bacterial mechanism is still largely organised for syntheses so that breakdown products from one part are to some considerable extent salved and used for re-synthesis of other parts. The salvage is, however, imperfect and is accompanied by a steady leakage from the cell. There is little or no corresponding uptake from the medium since the internal concentrations maintained by the exchange within a cell exceed the relevant external concentrations.

The significance of processes of this kind in connexion with the law governing the death rate of bacteria has recently been discussed (*Nature*, 1951, **167**, 666) and the theory put forward that internal exhanges of material play an important part in determining the form of this law. The present results appear to provide further evidence that the slow process of cell decline is indeed superposed on a vigorous internal activity.

Experimental

Materials.—Organism. A strain of *Bact. lactis aerogenes* was used and was maintained by daily serial subculture in a standard synthetic medium.

Synthetic media. (1) *Standard synthetic medium.* This was prepared by mixing under aseptic conditions sterile solutions of glucose (50 g./l.), phosphate buffer (2·96 g. of KH_2PO_4 +

16·0 g. of $Na_2HPO_4,12H_2O$/l.), ammonium sulphate (5 g./l.), and magnesium sulphate (1 g./l.) in the ratios 10 : 10 : 5 : 1.

(2) *Sulphur-free medium.* The ammonium and magnesium sulphates in the standard synthetic medium were replaced by equivalent amounts of ammonium and magnesium chlorides.

(3) *Phosphorus-free medium.* The phosphate buffer of pH 7·1 in the standard synthetic medium was replaced by an M/15-potassium hydrogen phthalate buffer of pH 6·5.

Preparation of Suspensions.—Bacteria containing ^{35}S. The strain of *Bact. lactis aerogenes* in the standard synthetic medium was inoculated into the sulphur-free medium and was serially subcultured in it several times until the bacterial count was limited by the sulphur present as impurity in the medium. 0·1 Ml. of the latter culture was then inoculated into 100 ml. of the sulphur-free medium and, when growth had ceased after the assimilation of all the sulphur present as impurity (a bacterial count of about 20×10^6 per ml. was usually reached), 1·0 mc of carrier-free $^{35}SO_4''$ in phosphate buffer at pH 7·12 was added. When growth had again ceased sufficient inactive sulphate was added to bring the bacterial count to the desired value. In these experiments the specific activity of the $^{35}SO_4''$ was such that 1·0 mc corresponded to the support of a population of about 8×10^9 bacteria, which was usually about one-half of the total bacterial mass required. The bacteria from the fully grown culture were then separated in a centrifuge, and the cells were washed three times with phosphate buffer and then re-suspended in a medium containing phosphate buffer, ammonium chloride, and magnesium chloride in the ratios 10 : 5 : 1 together with the requisite amount of inactive sulphate.

Bacteria containing ^{32}P. A suspension of bacteria in the standard synthetic medium was inoculated into 26 ml. of phosphorus-free medium to which had been added enough inactive phosphate to give a bacterial count of 10^9 per ml. After several subcultures in this medium a small inoculum (0·02 ml.) was transferred to 260 ml. of phosphorus-free medium containing 0·5 mc of $^{32}PO_4'''$ and enough inactive phosphate to support a bacterial count of 230×10^6 per ml. The cells were separated in the usual manner, washed three times with phthalate buffer, and re-suspended in a medium containing phthalate buffer, ammonium sulphate, and magnesium sulphate in the ratio 10 : 5 : 1 and the desired amount of inactive phosphate.

Uptake of ^{35}S.—A culture of bacteria in the standard synthetic medium was centrifuged. The cells were washed three times with phosphate buffer and re-suspended in a medium containing phosphate buffer, ammonium chloride, and magnesium chloride in the ratios 10 : 5 : 1 together with carrier-free $^{35}SO_4''$.

Uptake of ^{32}P.—The method was similar to that for the experiments on the uptake of ^{35}S except that the cells were washed with phthalate buffer and were re-suspended in a medium containing this same buffer, ammonium sulphate, and magnesium sulphate in the ratios 10 : 5 : 1, and the necessary $^{32}PO_4'''$.

Measurement of Radioactivity.—A separate appendix on counting techniques is given.

Appendix

The determination of radioactive sulphur in experiments with bacterial suspensions

In tracer experiments the measurements of activity are usually made either on solid or on liquid samples by the use of the appropriate type of Geiger counter. Liquid samples are particularly convenient and usually require little preparation, but as the minimum wall thickness of conventional counter-tubes for liquids corresponds to about 25 mg./cm.^{2} these cannot be used with tracers which emit only low-energy β-radiation. ^{35}S has consequently been determined almost exclusively in solid samples, with the use of thin end-window counters or of counters in which the sample is placed inside the tube itself (Hendricks, Bryner, Thomas, and Ivie, *J. Phys. Chem.*, 1943, **47**, 469). With low-energy β-emitters large corrections for self-absorption of the radiation become necessary and considerable errors can arise from small irregularities in the solid samples. This is particularly evident in work with small specific activities which necessitates relatively thick samples. Comparison of different solid samples is inaccurate unless the self-absorption correction is the same for all, *i.e.*, unless they are in the same chemical form. With sulphur, quantitative conversion into sulphate is usually necessary—a process which is frequently difficult.

In the present work it has been found possible to measure ^{35}S in liquid samples by the use of a conventional thin-window counter placed very close to the upper surface of an extended area of the liquid. Errors due to uncertainties in the self-absorption corrections are eliminated as all samples in the same solvent are of substantially the same density and of virtually

infinite thickness for the radiation from ^{35}S (greater than 0·3 mm. of water). The chemical nature of the dissolved substance is not significant (unless adsorptive phenomena take place, as discussed below).

Experimental

The liquid samples were mounted on glass plates on which they were confined by a wax ring about 6 cm. in diameter, deposited from a solvent. By a screw mechanism and an optical system the samples were raised till their upper surfaces were a standard small distance from the window of the counter (mica, 2 mg./cm.^{2}; counter, type EHM 2). The electronic counting equipment was of the conventional kind, and the corrections applied to the observed rates of count were as described previously (Carroll, Danby, Eddy, and Hinshelwood, *J.*, 1950, 946).

Sources of Error, especially Those due to Adsorption.—Preliminary experiments with solutions of inorganic ^{35}S compounds showed that the liquid-counting technique was satisfactory provided

Fig. 5.

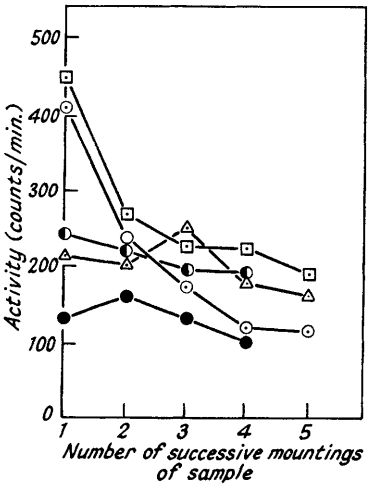


Fig. 6.

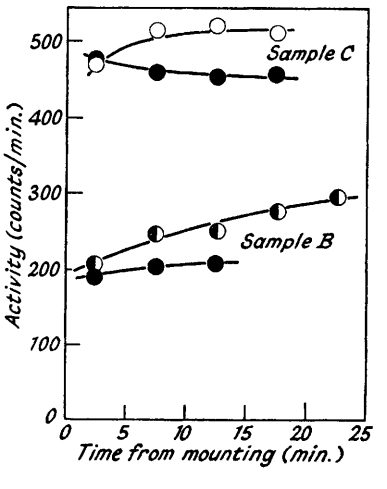


Fig. 5. *Effect of successive remounting of sample on observed activity.*

Sample *A*, treated with: ⊙ methionine, cysteine, and sulphite; ⊡ with sulphite; △ with bacterial lysate.

Sample *B*, treated with: ◐ alkaline sulphide; ● bromine water.

Fig. 6. *Variation in observed activity with time after mounting.*

○ Sample untreated.
◐ Treated with alkaline sulphide.
● Treated with bromine water and sulphate.

that sufficient inactive carrier was present to eliminate the effects of adsorption. In the bacterial work, however, the concentrations of sulphur compounds were very small (about 10^{-8} g./10^6 cells). This necessitated the use of ^{35}S of high specific activity, and adsorption on glassware, particularly on the plates on which the samples were mounted, became important. Adsorption at the air–liquid interface can also take place and lead to a higher concentration of the active material at the surface; with a weak β-emitter this leads to too high an apparent activity, as determined by a counter placed above the surface (Salley, Weith, Argyle, and Dixon, *Proc. Roy. Soc.*, 1950, *A*, **203**, 42). With a bacterial suspension containing ^{35}S the sedimentation of cells away from the surface can lead to a progressive decrease in the rate of count recorded.

Experiments on these effects are described below.

(1) *Adsorption on glass.* In Fig. 5 is shown the progressive decrease in activity observed when a typical sample of the solutions used in the work already described is successively remounted on a series of clean glass plates. This fall is probably due largely to adsorption of the sulphur-containing substance on the glass. The influence of a number of added sulphur compounds in reducing the loss was therefore studied. Methionine, cysteine, and sulphate ions show

little effect. Sulphite, sulphide, and a solution containing lysed bacteria reduce the loss in activity. A water-repellent silicone film coating all glassware greatly reduces adsorption and leads to more reproducible results, but when this is used on the mounting plates a more effective barrier than a wax ring is required to contain the samples.

(2) *Adsorption at the air–liquid interface.* Sulphur which leaves bacterial cells seems to be in a reduced state and may be partly in combination with various long-chain protein residues as surface-active compounds. Their concentration at the surface would explain the high initial rates of count observed with several of the samples illustrated in Fig. 5. Added sulphide and bacterial lysate both appear to reduce the effect of this adsorption, probably by undergoing exchange with the active sulphur in the surface film and so distributing it more uniformly through the liquid. Oxidation of sulphur to sulphate with bromine water and addition of inactive sulphate to counteract the effect of adsorption on the glass proved to be the most effective treatment. Fig. 6 shows the variation in the observed rate of count during the first 15—20 minutes after the placing of the samples on the mounting plates. Where the concentration of active material in the surface layers is not prevented there is a progressive slow increase in activity. In contrast, the samples treated with bromine show little change in observed rate of count. A drop of wetting agent added to one of the latter samples reduced the counting rate to half its previous value: here the surface layer contains no active sulphur and acts as a barrier to the escape of radiation from the liquid below (Judson, Argyle, Salley, and Dixon, *J. Chem. Phys.*, 1950, **18**, 1302).

(3) *Sedimentation of bacterial cells.* The activity contained in the cells of a culture grown in the presence of radioactive tracer can be determined in two ways: either from the difference in the activity of the whole suspension and that of the clear medium from which the cells have been removed by centrifuge, or by direct measurement on the sludge of bacteria obtained by centrifugation, allowance being made for the activity of the liquid remaining in it (Carroll *et al.*, *loc. cit.*). With ^{35}S the activity of the whole suspension cannot be determined accurately owing to the sedimentation of the cells away from the surface which leads to a progressive decrease in the rate of count. This difficulty is avoided by destruction of the cells with a little concentrated sulphuric acid, followed by oxidation of the bacterial sulphur to sulphate by bromine. By a similar treatment it is possible to measure the activity of the centrifuged bacterial sludge.

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