Observations on the Labelling of a Strain of *Staphylococcus aureus* with Phosphorus-32

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SUMMARY

The best labelling of *Staphylococcus aureus* (var. *pyogenes*) with ^{32}P was achieved when the organisms were grown in broth containing 33 μg . orthophosphate/ml. and 150-200 μc ./ml. ^{32}P . Cocci labelled in this way were as virulent to mice as control cocci. The phosphorus content of cocci was proportional to the orthophosphate content of the medium over the range 5 μg .-300 μg . PO_4 /ml. ^{32}P was released from cocci when they were incubated with phosphate buffer and other media which did not support cell division. ^{32}P was released from labelled dividing cocci grown in non-radioactive media. About 80% of the ^{32}P was released during division; a maximum of about 55% was released from non-dividing cocci over a similar time period. Most of the ^{32}P released from dividing cocci was present in the form of molecules non-diffusible in dialysis (80%); most of the ^{32}P released into phosphate buffer was diffusible. Cocci deficient or rich in phosphorus were fractionated with trichloroacetic acid (TCA), ether, ethanol and perchloric acid. Cocci containing low amounts of P lacked ^{32}P in the fraction soluble in cold TCA, about 80% of which was orthophosphate. Cocci deficient in phosphorus incorporated ^{32}P from $^{32}\text{PO}_4$ solutions more rapidly than enriched cocci. The distributions of ^{32}P in cocci previously rich or deficient in P were similar, but labelling in deficient cocci was increased in the RNA-containing fraction; and that part of the fraction soluble in hot TCA which had a barium salt insoluble at pH 4. The phosphorus level in the cells had no effect on the amount of phosphorus released into phosphate buffer solution.

INTRODUCTION

Ely (1942) labelled *Staphylococcus aureus* with unstated amounts of ^{32}P and Thorbecke & Benacerraf (1959) labelled *S. pyogenes* with ^{32}P by growing the organisms in the medium containing 20 μc . ^{32}P /ml. The labelled organisms were used for tracer studies in animals and for that purpose the specific activity of the bacterium should be as high as is compatible with normal functioning of the cell. Experiments were made to discover the factors which affected ^{32}P incorporation and those conditions which gave good labelling and viable organisms. Stonier (1956) had previously labelled *Agrobacterium tumefaciens* with ^{32}P and studied the behaviour of the label; the following experiments were made along similar lines with *S. aureus*.

METHODS

Strain. *Staphylococcus aureus*, strain 1531, isolated in Melbourne in 1957 from meninges, was used in these experiments. It has been maintained on nutrient agar under paraffin oil.

Inoculum. Cocci from a 24 hr culture on nutrient agar were suspended in sterile distilled water (about 10^9 cocci/ml.) and 1 drop of this solution from a Pasteur pipette was used as an inoculum.

Media. The cocci were grown in nutrient broth from which orthophosphate had been removed by precipitation with ammonium molybdate. The nutrient broth contained (NaCl, 5.0 g., peptone, 10.0 g., Lab. Lemco, 10.0 g., distilled water, 1000 ml.). The orthophosphate was removed by boiling the broth with 2 ml. concentrated HNO_3 for 15 min. and adding a hot, saturated solution of ammonium molybdate drop by drop until no further precipitate was obtained in the filtered broth. The medium was filtered and adjusted to pH 7.8 with N-NaOH. The phosphate content of the broth, determined by Briggs's (1922) method, was less than $5 \mu\text{g. PO}_4/\text{ml.}$ Untreated nutrient broth contained $700 \mu\text{g. PO}_4/\text{ml.}$ The excess ammonium molybdate was not removed from the broth and the molybdenum present, determined by the method of Sandell (1953), was $320 \mu\text{g./ml.}$ ^{32}P -labelled orthophosphate (from the Commonwealth X-Ray and Radium Laboratories, Melbourne, who obtained it from Radiochemical Centre, Amersham) and PO_4 as Na_2HPO_4 were added to this broth as required. The solution was dispensed in 2-3 ml. volumes into 50 ml. conical flasks and sterilized at 121° for 15 min.

Treatment of cocci. Cocci were separated from the supernatant fluid by centrifuging at 11,000 rev./min. for 30 min. at 4° . Cocci were washed three times either with a physiological saline solution (0.85 g. NaCl/100 ml.) containing 0.025% human serum albumin or with distilled water. Washing with distilled water was used when the specific activity of cocci was determined. Solutions in which cocci were suspended for ^{32}P release studies were placed in 40 ml. centrifuge tubes, previously sterilized with alcohol, and left in a 37° water bath for appropriate times.

Fractionation of cocci. Cocci were frozen and thawed twice in about 1 ml. water. Phosphorus-containing compounds were fractionated according to the method of Mudd, Yoshida & Koike (1958). Each fraction was not analysed chemically but the probable content of each was as follows:

Phosphorus compounds that were soluble in 5% trichloroacetic acid (TCA) after 1 hr at 4° . Hancock (1958) showed that inorganic phosphorus and amino acids were present in a similar extract prepared from *Staphylococcus aureus*. Phosphorus in this solution was precipitated with barium acetate at pH 4 and the remaining soluble phosphorus was precipitated at pH 7.4 with barium acetate. Metaphosphates are normally precipitated from TCA solutions at pH 4. Metaphosphate has not been identified from *S. aureus* and the precipitate obtained in these experiments has not been definitely characterized. The phosphorus precipitated at acid pH with barium acetate (2-20% total) has been tentatively called 'metaphosphate' since others have found metaphosphate in similar extracts of Mycobacteria (Mudd *et al.* 1958); 80-85% of the phosphorus remaining in acid solution was precipitated with barium acetate at pH 7.4. The remaining phosphorus compounds were not characterized.

P compounds that were soluble in alcohol and 3+1 alcohol+ether mixtures. Park & Hancock (1960) showed that lipid and polypeptides were present in this extract and the phosphorus extracted was probably combined in these compounds.

P compounds that were soluble in 5% TCA after 5 min. at 95°. Park & Hancock (1960) showed that nucleic acids were present in this extract with virtually all the teichoic acid from the cell wall. This fraction was treated at pH 4 with barium acetate and the precipitate obtained tentatively called 'polyphosphate', although polyphosphate was not characterized. The supernatant fluid from acid barium acetate precipitation was treated with barium acetate at pH 7.4. 50–80% of the phosphorus present was precipitated with this reagent. Some PO_4 was probably present with organically bound phosphorus since Mudd *et al.* (1958) found that hydrolysis of polyphosphate occurred during extraction.

P compounds that were soluble in cold N-perchloric acid after 24 hr. Ribonucleic acids were extracted by Mudd *et al.* using this technique and the phosphorus present in this extract probably represented mostly RNA phosphorus.

P compounds that were soluble in 5% TCA after 15 min. at 95°. Deoxyribonucleic acid was extracted from mycobacteria by Mudd *et al.* (1958) using this technique. It seems likely from the studies of Park & Hancock (1960) that some nucleic acid-P would also have been extracted with TCA at 95° for 5 min. The P extracted would then be less than the total P present in DNA.

Phosphorus which remained in the cocci after the extraction procedure described was extracted after 2 hr hydrolysis with $\text{N-H}_2\text{SO}_4$ at 100°.

Radioactivity measurements. ^{32}P -containing solutions were counted in a Geiger-Müller counter for liquids attached to a high-tension power supply and scaler. Appropriate corrections were applied to measurements for paralysis time, radioactive decay and counter efficiency. The specific activity of cocci was determined by drying the cocci to constant weight on a watch glass and dissolving them in 10 ml. concentrated HNO_3 .

Counts of viable cocci and measurements of turbidity. Viable bacterial counts were measured after the method of Miles & Misra (1938). Optical extinctions of the bacterial suspensions were read on a Unicam SP 600 spectrophotometer at 470 $\text{m}\mu$ and standardized against direct haemocytometer counts.

RESULTS

Phosphate requirements and uptake

The number of cocci harvested from broth was a maximum when 6 $\mu\text{g. PO}_4/\text{ml.}$ was present. When less than this amount was added per flask the yield decreased slightly but the harvest of about 10^8 cocci/ml. was not increased when the phosphate was increased in 6 $\mu\text{g./ml.}$ increments to 36 $\mu\text{g./ml.}$ Viability of the cocci grown in broth containing less than 6 $\mu\text{g./ml.}$ was poorer, 7.6% total cocci compared with 9.3% when cocci were grown in broth containing PO_4 in excess of that amount. An aqueous suspension of cocci from a nutrient agar slope required only small amounts of PO_4 to increase to about 10^8 cocci/ml.

Experiments were made to discover whether the cocci would accumulate more phosphorus than the minimum amount required for growth and viability. The incorporation of PO_4 into the cocci was studied using tracer amounts of $^{32}\text{PO}_4$ added

with orthophosphate. Cocci were grown in media which contained 70–450 $\mu\text{g. } ^{32}\text{PO}_4/\text{ml.}$ (Fig. 1). The cocci appeared to be saturated with phosphorus after growing in a medium containing 300 $\mu\text{g./ml.}$ The percentage of ^{32}P in the cocci remained at 4–5 % of that present in the broth when 70–300 $\mu\text{g. PO}_4/\text{ml.}$ were present, but decreased to about 3 % at higher concentrations. The cocci were able to accumulate more phosphorus than was necessary for normal growth and viability. The nutrient agar used to grow cocci for inoculation contained 700 $\mu\text{g. PO}_4/\text{ml.}$ and the inoculum cocci probably contained reserve phosphorus. This may have enabled them to grow in broth containing less than 6 $\mu\text{g. PO}_4/\text{ml.}$

Fig. 1. The relationship between cell ^{32}P and concentration of $^{32}\text{PO}_4$ in the medium.

The effect of the specific activity of $^{32}\text{PO}_4$ on growth and viability

The following experiments were made to discover the maximum amount of ^{32}P a coccus could contain without detriment to viability and growth and also the amount of $^{32}\text{PO}_4$ necessary in the broth to achieve this labelling. The total number of cocci/ml. and the total number viable were compared in cultures which contained PO_4 only and $^{32}\text{PO}_4$ with various specific activities. Replicate cultures contained $^{32}\text{PO}_4$ with specific activities of 3.7 $\mu\text{c./}\mu\text{g.}$; 5.5 $\mu\text{c./}\mu\text{g.}$; 13.8 $\mu\text{c./}\mu\text{g.}$, 1 mc./ $\mu\text{g.}$ and 67 $\mu\text{g.}$, 33 $\mu\text{g.}$, 18 $\mu\text{g.}$, 0.25 $\mu\text{g. PO}_4/\text{ml.}$ respectively. Control cultures contained the same amount of PO_4 . No growth occurred in the culture containing 250 $\mu\text{c. } ^{32}\text{P}$ and 0.25 $\mu\text{g. PO}_4/\text{ml.}$; the control culture contained 2.1×10^8 cocci about 8 % of which were viable. Growth of cocci was 60% that of the control culture when the specific activity of the $^{32}\text{PO}_4$ was 13.8 $\mu\text{c./}\mu\text{g.}$ and the viability was decreased from 0.3 % (control) of total cocci to 0.0003%. Growth was about 25% of the control when the specific activity was 5.5 $\mu\text{c./}\mu\text{g.}$ but viability was unaffected; 0.23 % of total cocci were viable compared with 0.1 % (controls). Growth and viability were not affected when the specific activity of the $^{32}\text{PO}_4$ was 3.7 $\mu\text{c./}\mu\text{g.}$ The total number of cocci was 8×10^9 in control and experimental flasks and the number viable was

0.5% total (control), 0.6% total (^{32}P -containing broth). The greatest $^{32}\text{PO}_4$ uptake, 44% of that present in the broth, occurred when the specific activity of the $^{32}\text{PO}_4$ was 13.8 $\mu\text{c.}/\mu\text{g.}$ and 18 $\mu\text{g. PO}_4/\text{ml.}$ were present. However, these cells had a low viability (0.0003% of total cells) and were thus unsatisfactory for tracer experiments in animals. When the specific activity of the $^{32}\text{PO}_4$ was 5.5 $\mu\text{c.}/\mu\text{g.}$ with 33 $\mu\text{g. PO}_4/\text{ml.}$ present in the medium, 14% of the $^{32}\text{PO}_4$ present in the broth was incorporated into the cocci. Cocci grown under these conditions were as viable as control cocci and were as virulent in mice as non-radioactive cocci. They had a specific activity of 3.3×10^{-8} $\mu\text{c. }^{32}\text{P}/\text{coccus}$, which was similar to the specific activity of the cocci with reduced viability from cultures containing 13.8 $\mu\text{c.}/\mu\text{g. }^{32}\text{PO}_4$. The specific activity of cocci from cultures containing 3.7 $\mu\text{c.}/\mu\text{g. PO}_4$ and 67 $\mu\text{g. PO}_4/\text{ml.}$ was 2.4×10^{-9} $\mu\text{c.}/\text{coccus}$ and only 4% of the $^{32}\text{PO}_4$ in the broth was incorporated in the cocci. Cocci labelled under these conditions were not used for tracer experiments in animals.

Distribution of ^{32}P in coccal fractions after uptake during growth

Cocci were labelled with ^{32}P by growing them in broth containing 18–500 $\mu\text{g. PO}_4/\text{ml.}$ and 150–200 $\mu\text{c. }^{32}\text{P}/\text{ml.}$ They were washed three times with a solution of 0.025% albumin in 0.85% NaCl. Cocci washed in this way lost about 0.2–0.3% of their ^{32}P if they were suspended in the albumin-saline solution for 1 hr. They lost about 50% of their ^{32}P content when they were suspended in PO_4 buffer for 1 hr. Therefore, albumin saline was assumed to be a safe washing medium for cocci later

Table 1. *Fractionation of cocci deficient and rich in phosphorus to show the distribution of ^{32}P under these conditions*

The relative amounts of phosphorus in the cocci were controlled by the phosphate content of the broth.

		Fractionation procedure					
		TCA 1 hr 4°	Ethanol, ethanol + ether (3 + 1)	TCA 5 min. 95°	Per- chloric acid 24 hr 4°	TCA 15 min. 95°	N- Sulphuric acid for 2 hr 100°
		Form of phosphorus in fraction					
		Poly- phosphate and inorganic phosphate	Lipids and poly- pep- tides	Teichoic acid, nucleic acid and polyphosphate	RNA	DNA	Insoluble
Phosphate content of broth ($\mu\text{g.}/\text{ml.}$)	Treatment of cocci after washing	Percentage of total coccal ^{32}P in each fraction. The values for percentage of polyphosphate in each fraction are in parentheses					
33	Albumin saline 1-5 hr	38 (2.3)	2.6	54	1.5	4.7	0.6
33	M/15 phos- phate buffer 1.5 hr	30 (26)	2.0	51 (92)	1.2	4.1	0.8
33		29 (4.0)	19	41 (50)	0.4	6.2	3.3
1000	None	79	0.7	14 (8.5)	1.4	1.4	0.1
2500	None	73 (4.5)	4.5	17 (4.8)	0.6	4.0	1.0

fractionated. Cocci grown in media with different PO_4 contents were analysed to discover the distribution of ^{32}P in the various cell compounds. Previous experiments showed that a coccus was saturated with phosphorus when it was grown in a medium containing more than $400\ \mu\text{g. PO}_4/\text{ml.}$ The proportion of ^{32}P in the various fractions was influenced by the available PO_4 (Table 1). When small amounts were available the hot TCA fraction contained the most; when $1000\ \mu\text{g. PO}_4$ or more were present in the medium the cold TCA fraction contained most ^{32}P . This would indicate that the hot TCA fraction was preferentially labelled and that when adequate phosphate was available then the cold TCA fraction was replenished with ^{32}P . This agrees with Albaum's *et al.* (1952) studies in plant seeds, when phosphorus was incorporated preferentially in certain compounds in states of phosphorus deficiency and appeared in greater amounts in other compounds in normal embryos. In phosphorus-rich cocci most of the ^{32}P was present in the inorganic fraction (cold TCA). Phosphorus-rich cocci contained proportionally less ^{32}P in the 'polyphosphate' fractions, especially in the acid-insoluble 'polyphosphate' fractions. In one experiment placing cocci in phosphate buffer caused an increase in ^{32}P in 'polyphosphate' but not in another (Table 1). Polyphosphate has been shown to increase and decrease according to the division cycle of the cocci (Sall *et al.* 1958) and may be influenced only at certain stages by immersion in phosphate buffer. Since the percentage of 'polyphosphate'- ^{32}P did not increase in phosphorus-rich cocci it does not appear to serve as a PO_4 reservoir.

*The uptake of $^{32}\text{PO}_4$ by non-dividing cocci and the distribution of ^{32}P
in coccal fractions under these conditions*

Mitchell & Moyle (1953) found that ^{32}P was taken up by non-dividing *Staphylococcus aureus* and that 91% of the ^{32}P appeared in the inorganic fraction. Since Mitchell & Moyle's growth medium was not treated to remove orthophosphate their cocci were probably not phosphorus deficient. Experiments were made to discover the effect of the concentration of coccal P on $^{32}\text{PO}_4$ uptake. Cocci after these experiments were washed with water at 4° since Hancock (1958) showed that washing with water did not cause a large release of intracellular compounds. The specific activity of a portion of the cocci was determined after washing with water.

Staphylococcus aureus when suspended in a phosphate solution containing carrier-free $^{32}\text{PO}_4$ incorporated ^{32}P into all coccal fractions. The rate at which $^{32}\text{PO}_4$ entered the cocci increased with decreasing cocci P (Table 2). When maximal $^{32}\text{PO}_4$ concentration was reached the ^{32}P in the cocci then decreased; how long absorption proceeded before release occurred was related to the concentration of $^{32}\text{PO}_4/\text{ml.}$ in the surrounding medium and the level of P in the cocci.

Cocci were fractionated after 1 hr in the suspending medium; excess medium was removed by washing twice with water. ^{32}P entered all the cell fractions, but most of it was present in the inorganic fraction soluble in cold TCA (Table 3). These observations are similar to those of Mitchell & Moyle (1953) but the rate of phosphate exchange depended on the concentration of cell P. The RNA phosphorus fraction was enriched more in the cocci which contained the smallest amount of P originally.

Table 2. *The relationship between the coccal phosphorus content and the uptake of $^{32}\text{PO}_4$ from non-nutrient solutions for phosphorus-deficient cocci*

The concentration of coccal phosphorus was related to the phosphate content of the culture fluid. See Fig. 1.

Phosphate concentration of broth ($\mu\text{g./ml.}$)	Uptake of ^{32}P by cocci from a non-nutrient solution of $^{32}\text{PO}_4$		
	Concentration of ^{32}P ($\mu\text{c./ml.}$)	Period of suspension in ^{32}P solution (min.)	^{32}P uptake by cocci (counts/min./mg. dry wt.)
< 1.0	0.01	15	9.65×10^2
< 1.0	0.01	60	1.73×10^3
< 1.0	5.5	15	8.30×10^5
< 1.0	5.5	60	3.83×10^5
16.5	0.01	15	1.85×10^3
16.5	0.01	60	1.83×10^3
16.5	5.5	15	8.66×10^4
16.5	5.5	60	4.54×10^4
33	0.01	15	58
33	0.01	60	1.27×10^3
33	5.5	15	3.07×10^4
33	5.5	60	1.26×10^4

The addition of 10 $\mu\text{g./ml.}$ of phosphate to the 0.01 $\mu\text{c./ml.}$ $^{32}\text{PO}_4$ solution resulted in the coccal ^{32}P content reaching a maximum in 15 min.

Table 3. *The fractionation of cocci to obtain the distribution of ^{32}P taken up by non-dividing cocci containing different amounts of phosphorus*

The ^{32}P incorporated by the cocci was proportional to the coccal phosphorus content. The fractionation procedure is as given in Table 1.

Phosphate content of broth ($\mu\text{g./ml.}$)	Concentration of ^{32}P in cocci (counts/min./mg. dry wt.)	Fraction					
		Poly-phosphate and inorganic phosphate	Lipids and poly-peptides	Teichoic acid, nucleic acid and poly-phosphate	RNA	DNA	Insoluble
		Percentage of total coccal ^{32}P in each fraction. The values in parentheses are the percentages of ^{32}P in the fraction as polyphosphate					
1	2.3×10^5	84	1.5	11.4 (16)	0.41	1.7	0.93
16.5	1.5×10^5	85	1.2	10.2 (2.5)	0.22	0.75	0.85
33	1.14×10^5	86	1.5	8.2 (6.0)	0.18	0.92	0.86
400	4.0×10^4	92 (2.0)	2.5	2.5 (5.1)	0.09	1.1	0.90
400	4.3×10^4	83 (2.4)	2.3	8.8 (9.3)	0.12	3.9	0.80

*Release of ^{32}P from the cocci**(a) Release in solutions not supporting growth*

Cocci placed in M/15 phosphate buffer for $1\frac{1}{2}$ hr at 37° released between 13 and 30 % of the ^{32}P they contained. When the cocci were washed and resuspended in M/15 phosphate buffer they lost about 5–20 % more of the ^{32}P they contained. This subsequent release of ^{32}P after $1\frac{1}{2}$ hr in M/15 phosphate buffer was not prevented by suspending in 2 % glucose solution, borate buffer or 0.85 % NaCl solution containing 0.025 % serum albumin. Phosphate buffer (M/15) caused more ^{32}P to be released from the cocci in the first 2 hr than albumin saline (13 % compared with 1 %). Only 1.6–1.8 % of the ^{32}P in cocci was released into human serum during this period. Phosphate exchange with the external medium accounted for some of the release of ^{32}P as indicated by increased ^{32}P in the presence of PO_4 .

The phosphate buffer in which cocci had been suspended was dialysed and treated with barium acetate solution at pH 7.3 to discover how much ^{32}P was present in the form of orthophosphate. Dialysis showed that only a proportion of the ^{32}P was diffusible; the amount diffusible varied with the experiment from about 90 to 50 %. Precipitation of the phosphate buffer with barium acetate showed that the $^{32}\text{PO}_4$ released also varied in a similar way. Precipitation figures for $^{32}\text{PO}_4$ were usually 5–10 % higher than the dialysis figure, which would indicate the presence of other

Table 4. *The effect of the specific activity of growth medium on the release of ^{32}P from labelled cocci into M/15 PO_4 buffer, pH 7.2*

Cocci were grown in broth containing 33 μg . PO_4/ml . with $^{32}\text{PO}_4$, washed three times and resuspended in non-radioactive buffer.

Specific activity of the culture medium ($\mu\text{c}/\text{mg}$.)	^{32}P released (% total) after	
	1 hr	24 hr
5.8	16.0	—
0.58	24.0	57.0
0.13	23.0	49.0
0.0003	21.0	57.0

materials in the PO_4 buffer which reacted similarly to PO_4 with barium acetate but had a high molecular weight. These results indicated that orthophosphate and other P-containing compounds were released from cocci into phosphate buffer, but that the amount of non-orthophosphate-P was variable. When cocci were suspended in an albumin-saline solution the proportion of dialysable ^{32}P released was lower than in phosphate buffer, 10–11 % compared with 50–90 %. Solutions which contained glucose and released ^{32}P were Nesslerized after digestion with sulphuric acid and potassium persulphate to test for the release of intracellular N as well as ^{32}P . All solutions contained about 30 μg . N/ml., whether glucose was present with phosphate buffer or not.

Mitchell & Moyle (1953) found that the movement of $^{32}\text{PO}_4$ into the medium ceased when the cocci were respiring on glucose added to a buffer solution. In these

experiments the addition of glucose to the phosphate buffer solution did not prevent the outward movement of PO_4 .

The simultaneous release of $^{32}\text{PO}_4$ and substances absorbing light at $260\text{ m}\mu$ from *Staphylococcus aureus* treated with CTAB has been described by Salton (1951). Hancock (1958) showed that these substances were purines and pyrimidines and their corresponding nucleosides and nucleotides. Amino acids and phosphorus were also released from cells. A similar release of nitrogenous material with $^{32}\text{PO}_4$ occurred when cocci were suspended in phosphate buffer.

The effect of the specific activity of cocci on the release of ^{32}P compounds into PO_4 buffer $\text{M}/15$ was investigated. The amount of radioactivity in the coccus did not appear to influence the amount of ^{32}P released (Table 4).

The effect of total amount of P a coccus contained on the release of phosphorus was determined (Table 5). Analysis of the variance shows that the P content of the coccus had no significant effect on the amounts of ^{32}P released.

Table 5. *The effect of coccal phosphorus content on the release of ^{32}P into phosphate buffer*

Cocci were grown in broth with $1\text{--}10\text{ }\mu\text{c. }^{32}\text{PO}_4/\text{ml.}$, washed and resuspended in $\text{M}/15\text{ PO}_4$ buffer $\text{pH } 7.2$. The ^{32}P level in the cocci increased with increasing $^{32}\text{PO}_4/\text{ml.}$ in the broth (Fig. 1). Each result was calculated from five experiments.

$^{32}\text{PO}_4$ in broth ($\mu\text{g.}/\text{ml.}$)	Total ^{32}P released in 1 hr (%: mean and S.E.M.)
33	26.5 ± 12.8
100	24.5 ± 10.9
400	35 ± 15.7

Variance estimate, $F = 0.631$

(b) *Release of ^{32}P from dividing cocci*

Cocci labelled with ^{32}P by growing them in broth containing $150\text{--}200\text{ }\mu\text{c. }^{32}\text{P}/\text{ml.}$ and $33\text{ }\mu\text{g. PO}_4/\text{ml.}$ were inoculated into nutrient broth which had not been dephosphated. After washing with albumin-saline solution the distribution of ^{32}P between the cocci and the medium was measured. $80\text{--}81\%$ of the ^{32}P originally present in the cells appeared in the medium after overnight growth at 37° . Only $20\text{--}21\%$ of the ^{32}P released was diffusible on dialysis. Analysis of the ^{32}P distribution after growth of the labelled inoculum in the unlabelled medium showed small changes had occurred in the relative amounts of ^{32}P in each fraction. The hot TCA fraction contained less ^{32}P , 41% compared with 54% in the original inoculum; the cold TCA fraction contained 35% after growth; the original inoculum contained 38% . The content of the DNA fraction increased after growth from 4.7 to 9% ; the phospho-protein and lipid fraction was 9.0% after growth and 2.6% in the original inoculum. The organic fraction was most severely depleted.

Autoradiography of labelled cocci. Good autoradiographs of *Staphylococcus aureus* labelled with ^{32}P in the skin of the mouse were obtained when AR 10 (Kodak) stripping film was exposed to cocci containing $3.3 \times 10^{-8}\text{ }\mu\text{c.}/\text{coccus}$ for 3 days.

DISCUSSION

Staphylococcus aureus may be satisfactorily labelled with ^{32}P for autoradiographic purposes and tracer experiments in animals by growing it in broth containing 150–200 $\mu\text{c. } ^{32}\text{P}/\text{ml.}$ and 33 $\mu\text{g. orthophosphate}/\text{ml.}$ The specific activity of the bacteria was about $2.0\text{--}8.5 \times 10^{-8} \mu\text{c.}/\text{coccus.}$ It is not possible to label the cocci more heavily without severely decreasing their growth and viability. Cocci labelled in this way are as virulent in mice as non-labelled ones. These findings are in agreement with Stonier's (1956) *Agrobacterium tumefaciens* labelled with ^{32}P . He found a similar specific activity compatible with normal viability in bacteria grown on a defined medium. It would appear that in both *S. aureus* and *A. tumefaciens* the specific activity of the organism decides whether they will survive. The level of radioactivity per ml. (150–200 $\mu\text{c.}$) did not affect growth when sufficient non-radioactive phosphate was present (about 67 $\mu\text{g.}/\text{ml.}$); metabolic disturbance was probably caused by the decay of ^{32}P to ^{32}S .

The interrelation of phosphate in the growth medium and phosphorus in fungi was previously recorded by Rennerfelt (1934), who worked with *Aspergillus niger*. As the phosphate concentration of the medium was increased from 25 $\mu\text{g.}/\text{ml.}$ to 750 $\mu\text{g.}/\text{ml.}$ the phosphorus in the mycelium increased almost linearly. Maximum uptake of phosphate from the medium was 20 %, decreasing to 17 % when maximal amounts of phosphate were present. The relationship between phosphorus in the cells and phosphate in the medium is very similar for *Staphylococcus aureus*. The maximal amount of phosphate in the medium is about half the maximal amount for *A. niger* and the percentage uptake is also less.

Park & Johnson (1949) analysed *Staphylococcus aureus* and found that 60 % of the P was present as inorganic phosphate, 5 % as labile phosphate and 30–35 % as stable phosphate. This analysis is similar to the one for P-rich cocci in these experiments. The cold TCA fraction contained 73–78 % of coccal ^{32}P , 80 % of which gave an orthophosphate reaction by forming an insoluble Ba salt at pH 7.4. Polyphosphate has not been described in *S. aureus* but has been found in other organisms, in *Corynebacterium diphtheriae* by Sall *et al.* (1958), in Mycobacteria by Winder & Denny (1957) and in *Azotobacter vinelandii* by Zaitseva, Belozerskii & Novozhilova (1960). In these organisms it serves as a reservoir of P for RNA synthesis. In *S. aureus* part of the cold TCA and hot TCA fractions formed an insoluble Ba salt at pH 4, a reaction which is typical of metaphosphate. However, *S. aureus* contains large amounts of teichoic acid (Archibald, Armstrong & Baddiley, 1961), and if this compound forms an acid-insoluble Ba salt it might have been confused with metaphosphate.

The release of non-diffusible ^{32}P compounds on dialysis into phosphate buffer from *Staphylococcus aureus* may have been the result of osmotically fragile cells autolysing (Mitchell & Moyle, 1957). This release of large molecular weight materials may also be the result of degradation of RNA and other phosphorus-containing compounds present in non-viable cocci. The release from dividing cocci might be the result of an exchange reaction, autolysis and the degradation of P-containing compounds. The relatively low amounts of $^{32}\text{PO}_4$ in the broth, about 20 % compared with the original content of the cocci, about 35 %, suggests that some of the released material might have been re-utilized by the growing population. If this has occurred the cocci

utilizing the ^{32}P would have a higher proportion of ^{32}P in the cold TCA fraction and less in the hot TCA fraction, since the broth contained about 700 μg . PO_4/ml . The population would then contain less ^{32}P in the hot TCA fraction than the original inoculum. This change did take place but may also be explained on the basis of greater dilution of the hot TCA fraction with ^{32}P taken from the medium and the movement of ^{32}P from the organic fraction to compounds with a low P turnover. Possibly several factors caused a depletion of ^{32}P in the hot TCA fraction after growth in an unlabelled medium. Stonier (1960) found that the release of ^{32}P from *Agrobacterium tumefaciens* was caused by a lytic phage. No such phage was demonstrated in this strain of *S. aureus*.

Elek (1959) described the formation of extracellular compounds by *Staphylococcus aureus*: some of the ^{32}P appearing in the growth medium could be present in the extracellular enzymes and proteins excreted as the result of non-autolytic processes.

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