3. When a suspension of *Rsp. rubrum* was incubated in the light with an excess of propionate in the presence of $^{14}CO_2$ the residual propionate contained no significant radioactivity.

4. A similar experiment with succinate showed that the residual substrate contained radioactivity sufficient to account for only 2·8% of the total incorporation due to succinate.

5. The implication of these results is discussed.

I would like to thank Dr S. R. Elsden for his encouragement and interest in this work, Dr H. J. Saz for his advice on the use and assay of ^{14}C, and the Agricultural Research Council for the receipt of a Research Studentship. This work was supported in part by a grant from the Rockefeller Foundation.

REFERENCES

Barker, H. A. & Beck, J. V. (1942). *J. Bact.* **43**, 291.
Bassham, J. A., Benson, A. A., Kay, L. D., Harris, A. Z., Wilson, A. T. & Calvin, M. (1954). *J. Amer. chem. Soc.* **76**, 1760.
Bueding, E. & Yale, H. W. (1951). *J. biol. Chem.* **193**, 411.
Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M. & Yankwich, P. E. (1949). In *Isotopic Carbon*. New York: Wiley.
Cutinelli, C., Ehrensvärd, G., Reio, L., Saluste, E. & Stjernholm, R. (1951). *Ark. Kemi*, **3**, 315.
Delwiche, E. A. (1948). *J. Bact.* **56**, 811.
Eisenberg, M. A. (1953). *J. biol. Chem.* **203**, 815.
Elsden, S. R. & Gibson, Q. H. (1954). *Biochem. J.* **58**, 154.
Elsden, S. R. & Ormerod, J. G. (1956). *Biochem. J.* **63**, 691.
Gaffron, H. (1933). *Biochem. Z.* **260**, 1.
Gaffron, H. (1935). *Biochem. Z.* **275**, 301.
Glover, J. & Kamen, M. D. (1951). *Fed. Proc.* **10**, 190.
Henriques, F. C., Kistiakowsky, G. B., Margnetti, C. & Schneider, W. G. (1946). *Industr. Engng Chem.* (*Anal.*), **18**, 349.
Johns, A. T. (1951*a*). *J. gen. Microbiol.* **5**, 317.
Johns, A. T. (1951*b*). *J. gen. Microbiol.* **5**, 326.
Larsen, H. (1951). *J. biol. Chem.* **193**, 167.
Markham, R. (1942). *Biochem. J.* **36**, 790.
Ochoa, S., Mehler, A. & Kornberg, A. (1947). *J. biol. Chem.* **167**, 871.
Robertson, W. B. (1942). *Science*, **96**, 93.
Saz, H. J. & Krampitz, L. O. (1954). *J. Bact.* **67**, 409.
Swim, H. E. & Krampitz, L. O. (1954). *J. Bact.* **67**, 426.
van Niel, C. B. (1941). *Advanc. Enzymol.* **1**, 263.
van Niel, C. B. (1944). *Bact. Rev.* **8**, 1.
van Niel, C. B. (1949). In *Photosynthesis in Plants*, ed. by Franck, J. & Loomis, E.W. Ames: Iowa State College Press.
Van Slyke, D. D. & Folch, J. (1940). *J. biol. Chem.* **136**, 509.
Vernon, L. P. & Kamen, M. D. (1953). *Arch. Biochem. Biophys.* **44**, 298.
Westerkamp, H. (1933). *Biochem. Z.* **263**, 239.
Whiteley, H. R. (1953). *J. Amer. chem. Soc.* **75**, 1518.
Wood, H. G., Vennesland, B. & Evans, E. A. (1945). *J. biol. Chem.* **159**, 153.

The Biosynthesis of Penicillin

4. THE SYNTHESIS OF BENZYLPENICILLIN BY WASHED MYCELIUM OF *PENICILLIUM CHRYSOGENUM**

By W. J. HALLIDAY† and H. R. V. ARNSTEIN
National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 30 *April* 1956)

Although washed mycelial suspensions of *Penicillium chrysogenum* have been employed for the study of various metabolic properties of the mould, there have been few reports on the synthesis of penicillin by such preparations. Penicillin production by replacement cultures of *Penicillium notatum* was briefly reported by Abraham *et al.* (1941), who observed that maximum yields of penicillin were obtained more quickly when the original culture liquid was replaced by fresh nutrient medium. More recently Rolinson (1954) showed that *P. chrysogenum* could continue to produce penicillin at a maximum rate when the mycelium was washed and suspended in a fresh, nutrient medium. Rolinson's investigations indicated the possibility of performing short-term 'fermentations', which would yield penicillin in measurable quantities and be relatively free from the metabolic by-products of complete fermentations. We were therefore prompted to examine the effect of various nutrients and inhibitors on penicillin production by washed mycelium, with a twofold purpose: first, to see whether washed mycelium forms penicillin from the same precursors as does the mould under more usual conditions; secondly, to attempt the isolation and identification of the later intermediates in penicillin biosynthesis.

This paper describes the preparation and nutrient requirements of the washed mycelium of *P. chrysogenum*, and shows that the precursors of penicillin in complete fermentations are used in a similar way by washed mycelium.

* Part 3: Arnstein, Clubb & Grant (1954).

† Present address: Bacteriology Department, The University of Queensland, Brisbane, Australia.

EXPERIMENTAL

Many of the experimental methods used have been described in previous communications from this Laboratory (Arnstein & Grant, 1954*a*, *b*).

Preparation of washed mycelium

The growth medium was essentially that of Jarvis & Johnson (1950), supplemented by yeast extract, but the metal sulphates were replaced by the chlorides or acetates, with the exception of sodium sulphate, which supplied all the inorganic S of the medium. This modification was designed to allow the S content of the medium to be easily varied. The ingredients of the growth medium for a 1 l. fermentation were as follows: ammonium lactate, 6·0 g.; ammonium acetate, 3·5 g.; KH_2PO_4, 3·0 g.; Na_2SO_4, 0·5 g.; $Mg(O_2C.CH_3)_2,4H_2O$, 0·2 g.; $CaCl_2$, 0·04 g.; $FeCl_2$, 0·03 g.; $ZnCl_2$, 0·01 g.; $MnCl_2,4H_2O$, 0·01 g.; $Cu(O_2C.CH_3)_2,H_2O$, 0·004 g.; Difco yeast extract, 0·1 g.; lactose, 30 g.; glucose, 10 g.; silicone antifoam emulsion, about 0·5 g. These ingredients, with the exception of the sugars, were dissolved in 900 ml. of water in a small stirred aerated jar fermenter (Arnstein & Grant, 1954*b*), adjusted to pH 5·8 with H_3PO_4, and autoclaved to sterilize. The sugars were dissolved in a further 100 ml. of water, autoclaved separately, and added to the sterile, cooled fermenter. The spore inoculum has been described by Arnstein & Grant (1954*b*). Sterile potassium phenylacetate (20%, w/v) was added to a final concentration of 0·1% (w/v), as a single addition about 40 hr. after the fermenter had been inoculated.

Penicillium chrysogenum WIS 51–20 was used in most of the work, and some of the results were confirmed with strain WIS 48–701. Both strains were obtained through the courtesy of Dr M. Lumb of Boots Pure Drug Co. Ltd., Nottingham.

The mycelium was harvested 68–70 hr. after inoculation, at which time the penicillin concentration in the broth was usually between 150 and 300 units/ml., and was increasing at the maximum rate of about 10 units/ml./hr. The pH had then risen to about 7·5, and required no adjustment during the fermentation. Washing of the mycelium was accomplished by collecting it on a large sintered-glass funnel with moderate suction, resuspending it in the original volume of 0·01 M potassium phosphate buffer at pH 7, and repeating this procedure until the mycelium had been washed four times. The 0·01 M buffer could be replaced by water, with identical results. The final pad of mycelium, containing about 88% of water, was used for the production of penicillin in short-term experiments in shake flasks. Portions of the mycelial pad were weighed into the shake flasks, and thus errors arising from the pipetting of viscous suspensions were avoided. From the time of harvesting the mycelium, no aseptic precautions were taken.

Use of washed mycelium

Conical flasks of 100 ml. capacity were generally used, but the size of the vessels and the volume of their contents were occasionally increased for preparative work. Into each 100 ml. flask were put 1·0 g. of the washed mycelial pad, and liquid to a total volume of 10 ml. The mycelial concentration (dry basis) was then about 12 mg./ml., as in the fermenter at the time of harvesting. Fresh growth medium was used initially, with good yields of penicillin, but it was not satisfactory for the work on cyanide inhibition (probably as a result of the content of Cu). Therefore, a nutrient medium similar to that of Rolinson (1954) was used to suspend the mycelium. This medium contained, per l.: lactose, 30 g.; $NaNO_3$, 3·0 g.; Na_2SO_4, 1·0 g.; KH_2PO_4, 0·5 g.; phenylacetic acid, 0·1 g. (as K salt); corn-steep liquor, 50 ml.; the pH was adjusted to 7·0. The flasks were incubated for periods of 1–5 hr. on a rotary shaker (250 rev./min.) at 24°. At the end of this period, the flask contents were filtered through paper, and the filtrates kept for examination.

Respiration of mycelial suspensions was measured in the conventional Warburg apparatus, and the penicillin produced at the same time was assayed biologically. Inhibition of penicillin formation and respiration by cyanide was studied by adding KCN to the KOH normally present in the centre wells of the Warburg flasks (Rolinson, 1954; Robbie, 1946).

Assay and isolation of penicillin

Penicillin was assayed by the cup-plate method of Humphrey & Lightbown (1952), with *Bacillus subtilis* (NCTC 8241) as test organism and benzylpenicillin (sodium salt; 4, 8 and 16 units/ml.) as standard. When benzylpenicillin *N*-ethylpiperidine salt was assayed, a pure sample of this substance was used as standard.

Radioactive penicillin was isolated from filtrates after the addition of non-radioactive sodium benzylpenicillin as carrier. The filtrate was cooled in ice and the pH was adjusted to 2–2·5 with 25% (v/v) H_3PO_4. The penicillin was then extracted into ether, back into 0·1 M phosphate buffer at pH 7, and finally into ether after reacidifying. All these operations were carried out at 2°. The final ethereal solution (about 20 ml.) was evaporated to about 5 ml. in a centrifuge tube, and cooled in an ethanol–solid CO_2 bath to freeze out the water as ice, which was removed by centrifuging. *N*-Ethylpiperidine (10%, v/v, in dry ether) was added in slight excess, and the precipitated penicillin salt of this base was then recrystallized by dissolving in a minimum of warm, dry $CHCl_3$ and adding several volumes of dry acetone. The purity of the preparations was confirmed by recrystallization to constant radioactivity, and by comparing the antibiotic potency with genuine *N*-ethylpiperidine salt of benzylpenicillin.

Estimation of ^{14}C *and* ^{35}S

Radioactivity determinations were carried out with a thin end-window Geiger–Müller counter by using infinitely thick samples as described by Arnstein & Grant (1954*b*). The standard error was less than 5% in all determinations.

RESULTS

Effect of conditions on the yield of penicillin. When washed mycelium was resuspended in the lactose corn-steep medium as described above, penicillin was produced at a uniform rate for at least 5 hr. This has been reported by Rolinson (1954). The rate of production was the same as in the fermenter at the time of harvesting, and was usually between 8 and 12 units/ml./hr. The yield from duplicate flasks was remarkably constant (see Table 1).

Oxygen was required for penicillin synthesis, since mycelium under an atmosphere of nitrogen produced negligible amounts of the antibiotic ($< 0·2$ unit/ml./hr.). Half the concentration of washed mycelium produced half the amount of penicillin, when shaken in air; hence the supply of oxygen was not limiting under the conditions described. In the same nutrient medium, and also

in the simpler media described below, adjustment of the pH between the limits 5·8 and 7·8 had no appreciable effect on the yield of penicillin. A pH value of 7·0 was chosen, since penicillin is most stable in the neutral region.

The requirement for nutrients was examined by omitting single ingredients from the nutrient medium, and assaying for penicillin after a standard incubation time of 3 hr. The results are summarized in Table 1. It appears that corn-steep liquor was dispensable, except in the absence of phenylacetate; presumably the corn-steep liquor provided precursors for the side chain of the penicillin molecule, and its nutritive role in the washed mycelium could be filled by phenylacetate. In the presence of phenylacetate (with or without corn-steep liquor), nitrate, sulphate or phosphate could be omitted without decreasing the yield of penicillin. The omission of lactose and corn-steep liquor resulted in a 20 % decrease in yield, which could be restored by adding either of these constituents. Thus the only indispensable nutrient was the side-chain precursor, and it was found subsequently that washed mycelium suspended in aqueous potassium phenylacetate (0·01 %, pH 7) produced penicillin at a rate of 6–10 units/ml./hr. This yield could be increased to the maximum of 8–12 units/ml./hr. by the addition of lactose or corn-steep liquor. Most of the experiments reported below were conducted with aqueous potassium phenylacetate as the supporting medium for the washed mycelium.

Table 1. *Effect of nutrient medium on yield of penicillin from washed mycelium*

Each flask contained 1·0 g. of washed mycelium of *P. chrysogenum* WIS 51–20, in a total volume of 10 ml. Final concentration of mycelium (dry basis), 12 mg./ml. For ingredients of complete nutrient medium, see Experimental section. Results refer to duplicate flasks.

Medium	Yield of penicillin (units/ml./3 hr.)
Complete nutrient medium	25, 25
Corn-steep liquor omitted	26, 25
Lactose omitted	24, 24
Nitrate omitted	25, 24
Sulphate omitted	27, 24
Phosphate omitted	26, 25
Phenylacetate omitted	16, 13
Corn-steep liquor and lactose omitted	21, 20
Corn-steep liquor and nitrate omitted	26, 26
Corn-steep liquor and sulphate omitted	27, 25
Corn-steep liquor and phosphate omitted	24, 26
Corn-steep liquor and phenylacetate omitted	<1
All nutrients omitted (mycelium plus water)	<1

Table 2. *Yield of penicillin from various concentrations of phenylacetate*

Conditions as for Table 1, except that corn-steep liquor was omitted entirely, and the amount of potassium phenylacetate was varied. Concentrations of phenylacetate are expressed as g. of phenylacetic acid/l.

Concn. of phenylacetate (g./l.)	Yield of penicillin (units/ml./1·5 hr.)	Molar conversion of phenylacetate into penicillin (%)
0	<0·5	—
0·0025	3·5, 3·6	30
0·005	6·2, 5·5	23
0·01	8·5, 8·0	17
0·025	12·1, 11·3	9·5
0·05	13·5, 11·5	5·1
0·1	11·2, 10·5	2·2

The effect of varying the concentration of phenylacetate was determined by incubating washed mycelium with the complete nutrient medium (minus corn-steep liquor and phenylacetate) to which various amounts of phenylacetate were added. Table 2 shows the results obtained.

Several attempts were made to find conditions under which washed mycelium could be stored, and yet retain its activity. Storage at 2° in a mass or under nitrogen brought about a rapid degeneration, only 20 % of the activity remaining after 2 days. When the mycelial pad was finely divided and kept in air or oxygen at 2°, it remained fully active for 1–2 days, but only 30 % of the activity remained after 6 days. A final washing of the mycelium with 0·1 M phosphate buffer at pH 7 improved the keeping qualities slightly, probably by controlling the rise in pH which otherwise occurred. Freeze-drying or freezing and thawing, even in the presence of glycerol (20–50 %, v/v), completely destroyed the ability to synthesize penicillin, as also did grinding with sand or drying with acetone. Freshly harvested mycelium was used in all the experiments reported here.

Utilization of labelled precursors. Some substances known to be effective precursors of penicillin in complete fermentations were tested for their ability to serve as precursors with washed mycelium. The radioactive substances were added to the mycelium, which was suspended in water containing phenylacetate, and the penicillin formed was isolated as the *N*-ethylpiperidine salt after the addition of carrier penicillin. The experimental details and results are shown in Table 3. These results confirm that the washed mycelium of *P. chrysogenum* can use several exogenous precursors to form penicillin. The molar radioactivity of phenylacetate was diluted to only a small degree, which is in accord with the obligatory requirement of this precursor. L-Cystine was also used efficiently, but we were not able to demonstrate convincingly that the supply of cystine ever became a limiting factor in penicillin biosynthesis. The added L-valine was incorporated into penicillin with moderate efficiency. Sulphate was diluted by the endogenous sulphur of the mycelium to a much greater extent than was

cystine, probably mainly because the amount of labelled sulphate added was much smaller than that of cystine. A comparison of the amount of radioactivity incorporated into penicillin suggests that sulphate is utilized less efficiently than cystine for penicillin biosynthesis.

Effect of cyanide on penicillin yields and on respiration. Rolinson (1954) reported that penicillin synthesis by washed mycelium was inhibited by low concentrations of cyanide which did not affect respiration. We have attempted to repeat this work under our experimental conditions, since knowledge of specific inhibitors of penicillin synthesis may give insight into the mechanism of the normal process.

Mycelium prepared in the usual way was suspended in both the complete nutrient medium (minus corn-steep liquor) and in aqueous phenylacetate in Warburg flasks. In the centre wells of the flasks was placed potassium hydroxide with potassium cyanide to give the required level of cyanide in the surrounding vessel (Robbie, 1946). Uptake of oxygen and production of penicillin were determined after 1 hr., and the results obtained with duplicate flasks are shown in Table 4.

Both strains of *P. chrysogenum* (WIS 51–20 and WIS 48–701) responded in the same way to cyanide, although the actual yields of penicillin were greater with WIS 51–20. Penicillin biosynthesis was more sensitive to poisoning by cyanide than was respiration, irrespective of the medium used to suspend the mycelium. The results appear to differ quantitatively from those of Rolinson, and this difference is discussed further in the next section.

DISCUSSION

The use of washed mycelium offers several advantages in the study of penicillin biosynthesis. The effect of many different conditions may be determined in experiments lasting only a few hours, and use is made of mycelium which is quite uniform since it comes from one original fermenter. Experiments may be of such short duration as to make separable the two phenomena of mycelial growth and penicillin production. As the mycelium is able

Table 3. *Incorporation of radioactive precursors into penicillin synthesized by washed mycelium*

Except in the experiment with $Na_2{}^{35}SO_4$, washed mycelium of *P. chrysogenum* WIS 51–20 (5·0 g.) was added to water (50 ml.) containing potassium phenylacetate (0·05 g./l.) and the radioactive substance under investigation. The mixtures were shaken for 3 hr. in 500 ml. flasks and filtered, and the filtrates assayed for penicillin. In the [^{35}S]sulphate experiment, only 1·0 g. of washed mycelium was used in a 100 ml. conical flask, and the final volume was therefore reduced to 10 ml.

Sodium benzylpenicillin (50 mg.) was added to each flask; *N*-ethylpiperidine salt of benzylpenicillin was isolated and its radioactivity determined as described in the text.

Radioactive substance	Amount added (μmoles)	Amount added (μc)	Specific radioactivity (μc/m-mole) *A*	Calc. radioactivity of penicillin before dilution with carrier (μc/m-mole) *B*	Dilution of molar radioactivity *A/B*	Radioactivity incorporated into penicillin (%)
Potassium [1-^{14}C]phenylacetate	17·6	21·5	1220	1140	1·07	3·7
L-[^{14}C]Valine (uniformly labelled)	4·27	14·2	3320	169	19·6	0·27
L-[^{35}S]Cystine (calc. as cysteine)	83·3	0·026	0·31	0·17	1·82	2·5
$Na_2{}^{35}SO_4$ ('carrier-free')	0·01 (approx.)	10	10^6	430	2300	0·83

Table 4. *Effect of cyanide on penicillin synthesis and on respiration*

Each Warburg flask contained 3·5 ml. of mycelial suspension (0·15 g. of mycelial pad; final dry wt. of mycelium, 6 mg./ml.) and 0·2 ml. of 0·5 M-KOH. The KOH solution also contained KCN, in the amounts derived by Robbie (1946). Flasks were shaken for 1 hr. at 25°. The percentage inhibition is calculated from the mean of the duplicate flasks.

Strain	Suspending fluid	Concn. of cyanide (μM)	Total oxygen uptake (μl.)	Total oxygen uptake % inhibition	Penicillin synthesis (units/ml.)	Penicillin synthesis % inhibition
WIS 48–701	Complete nutrient medium without corn-steep liquor	0	548, 524	0	7·3, 6·5	0
		2	536, 444	9	5·2, 4·6	29
		10	387, 363	30	2·7, 4·0	52
		50	329, 204	50	2·9, 1·5	68
		200	133, 125	76	1·0, 0·7	87
WIS 51–20	Aqueous potassium phenylacetate (0·05 g./l.)	0	190, 198	0	11·5, 11·5	0
		2	202, 208	0	10·0, 11·0	9
		10	211, 211	0	9·8, 8·9	19
		50	169, 144	20	4·8, 5·3	57
		200	75, 97	56	3·4, 3·6	70

to synthesize penicillin in a very simple medium (aqueous potassium phenylacetate), the antibiotic may be extracted with a minimum of interference from nutrients and end products of the fermentation.

In the absence of an exogenous side-chain precursor such as phenylacetate, little or no penicillin is produced, and washed mycelium may therefore be used as a convenient system for testing the utilization of potential precursors for the biosynthesis of penicillins with different side chains.

We may deduce, from the fact that washed mycelium continues to produce penicillin if only phenylacetate and oxygen are supplied, that the synthesis does not depend on diffusible intermediates formed early in the fermentation. Intracellular substances which withstand washing seem to be readily available for penicillin synthesis; the latter occurs at a rate which is increased only by the addition of lactose, which may function as a source of energy.

Radioactive sulphate, cystine and valine were incorporated into penicillin by washed mycelium, although the supply of these substances did not appear to be rate-limiting as with phenylacetate. Added sulphate was less efficiently utilized than cystine, indicating that synthesis of penicillin was proceeding largely from more complex forms of sulphur, such as cystine, derived from the mycelial protein. Other investigators have obtained similar results for radioactive substances added to complete fermentations. Labelled phenylacetate (Sebek, 1953), sulphate (Smith & Hockenhull, 1952), cystine (Arnstein & Grant, 1954*b*) and valine (Arnstein & Grant, 1954*a*) have been shown to give rise to labelled penicillin, with dilutions of radioactivity differing slightly from those shown in Table 3. The differences probably result from the fact that in the complete fermentations the precursors were added at different levels and were available to the mould over a period of several days.

The practical advantages of the use of washed mycelium would be greatly increased if the material could be preserved in an active state. At present, the results of any set of experiments are not known until the following day, when further experiments with the original mycelium may not be strictly comparable. Further work on the preservation of the mycelium is in progress. The possibility of obtaining a cell-free system capable of synthesizing penicillin appears to be remote, since any manipulation which damaged the cell wall (freezing, drying, grinding) destroyed the synthetic ability of the cells.

The difference between the cyanide sensitivities of penicillin synthesis and of respiration, although significant, is not as pronounced as that reported by Rolinson (1954). He found, for instance, that 2×10^{-6} M cyanide depressed the rate of penicillin production to 25% of normal, while causing no significant inhibition of respiration. In calculating the equilibrium concentration of cyanide in contact with the mycelium, Rolinson used the information given by Umbreit, Burris & Stauffer (1945), which has been shown by Robbie (1946) to be in error. Thus what was reported as 2×10^{-6} M cyanide was probably ten times this concentration. A recalculation of Rolinson's data by the use of Robbie's figures reduces the discrepancy mentioned above. The difference then remaining between our results and those of Rolinson could well be due to the different growth media used and to possible differences in the strains of *P. chrysogenum*.

SUMMARY

1. Washed mycelium of *Penicillium chrysogenum* (strains WIS 48–701 and WIS 51–20), resuspended in water, required only oxygen and phenylacetate for the continued synthesis of penicillin. The pH of the medium had no significant effect between the limits 5·8 and 7·8. The synthetic ability of the mycelium degenerated after 1–2 days at 2°.

2. Substances known to give rise to penicillin in complete fermentations were also utilized for the synthesis of penicillin by washed mycelium. The incorporation of potassium [1-^{14}C]phenylacetate, [^{35}S]sulphate, L-[^{35}S]cystine and L-[^{14}C]valine into penicillin by incubation with washed mycelium for 3 hr. was 3·7, 0·8, 2·5 and 0·27% respectively.

3. Penicillin synthesis was more sensitive than respiration to poisoning by cyanide. The results obtained differ quantitatively from those of Rolinson (1954), and the reasons for this are discussed.

4. The use of washed mycelium provides a convenient method for the investigation of penicillin biosynthesis, since many variables may be tested in a short time in a medium free from interfering substances.

REFERENCES

Abraham, E. P., Chain, E., Fletcher, C. M., Florey, H. W., Gardner, A. D., Heatley, N. G. & Jennings, M. A. (1941). *Lancet*, **2**, 177.

Arnstein, H. R. V., Clubb, M. & Grant, P. T. (1954). *Proc. 2nd Radioisotope Conf., Oxford*, p. 306. London: Butterworth's Scientific Publications.

Arnstein, H. R. V. & Grant, P. T. (1954*a*). *Biochem. J.* **57**, 353.

Arnstein, H. R. V. & Grant, P. T. (1954*b*). *Biochem. J.* **57**, 360.

Humphrey, J. H. & Lightbown, J. W. (1952). *J. gen. Microbiol.* **7**, 129.

Jarvis, F. G. & Johnson, M. J. (1950). *J. Bact.* **59**, 51.

Robbie, W. A. (1946). *J. cell. comp. Physiol.* **27**, 181.

Rolinson, G. N. (1954). *J. gen. Microbiol.* **11**, 412.

Sebek, O. K. (1953). *Proc. Soc. exp. Biol., N.Y.*, **84**, 170.

Smith, E. L. & Hockenhull, D. J. D. (1952). *J. appl. Chem.* **2**, 287.

Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1945). *Manometric Techniques and Related Methods for the Study of Tissue Metabolism.* Minneapolis: Burgess Publ. Co.