Crowdy, S. H., Gardner, D., Grove, J. F. & Pramer, D. (1955). *J. exp. Bot.* **6**, 371.
 Crowdy, S. H., Green, A. P., Grove, J. F., McCloskey, P. & Morrison, A. (1959). *Biochem. J.* **72**, 230.
 Crowdy, S. H., Grove, J. F., Hemming, H. G. & Robinson, K. C. (1956). *J. exp. Bot.* **7**, 42.
 Davis, D. & Dimond, A. E. (1953). *Phytopathology*, **43**, 137.

Dimond, A. E. & Davis, D. (1953). *Phytopathology*, **43**, 43.
 Keyworth, W. G. & Dimond, A. E. (1952). *Phytopathology*, **42**, 311.
 Stokes, A. (1954). *Plant & Soil*, **5**, 132.
 Stubbs, J. (1952). *Ann. appl. Biol.* **39**, 439.
 van Raalte, M. H., Sijpesteijn, A. K., van der Kerk, G. J. M., Oort, A. J. P. & Pluygers, C. W. (1955). *Meded. LandbHoogesch. Gent*, **20**, 543.

The Metabolism of Glycerol in *Mycobacterium butyricum*

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Boissevain (1943) found a trace of pyruvic acid in Long's medium on which tubercle bacilli had been grown, and suggested that the acid came from glycerol. There is evidence that glycerol is phosphorylated by the mycobacteria and converted into pyruvate according to the Embden-Meyerhof scheme (Hunter, 1953*a*; Winder & Denneny, 1955). It is known also that the mycobacteria can oxidize pyruvate to acetate (Lindsay, O'Donnell & Edson, 1950; Goldman, 1958*a, b*) and that the reactions of the citric acid cycle can occur in cell-free extracts of these bacteria (Faine, Whiteside & Edson, 1951; Ochoa, Stern & Schneider, 1951; Blakley, 1952; Andrejew, 1954; Yamamura, Kusunose & Kusunose, 1954, 1955; Millman & Youmans, 1954, 1955; Millman & Darter, 1956; Goldman, 1956*a, b*, 1957; Morrison, 1956, 1957). These facts point to a terminal oxidation of glycerol in the citric acid cycle.

Mycobacterium butyricum grows well on a phosphate-buffered synthetic medium containing glycerol as the source of carbon and ammonium ion as the source of nitrogen, and excretes into the medium a considerable amount of 2-oxoglutaric acid together with small quantities of pyruvic and succinic acid (Hunter, 1953*b*). The origin of 2-oxoglutaric acid may be ascribed to the reactions shown in Fig. 1. Guided by this hypothesis we have used ^{14}C -tracer methods to study glycerol metabolism in cultures of *M. butyricum*.

MATERIALS AND METHODS

Organism. *Mycobacterium butyricum*, no. 337, was obtained from the National Collection of Type Cultures, Lister Institute, London. For many years the stock culture has been maintained on glycerinated Dorset's egg medium in this Laboratory. It was cultivated on the synthetic medium for several generations before use in

experiments. The inoculum (about 4 mg. wet wt.) was taken from a 4-day-old culture.

Medium. The synthetic medium contained 1.0 g. of $(\text{NH}_4)_2\text{SO}_4$, 0.2 g. of NaCl, 0.04 g. of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10.0 g. of KH_2PO_4 , 10.0 mg. of thiamine hydrochloride and approx. 30 g. of glycerol/l. of water. The pH was adjusted to 7.5 with carbonate-free 3*N*-NaOH solution. The required amount of MgSO_4 solution was added aseptically to the autoclaved medium. Glycerol and the inorganic salts were analytical reagents. Sterile solutions of radioactive substrates were added after the medium had been measured into the special culture flask used in the experiments. Solutions of radioactive glycerol and acetate were sterilized by steaming in sealed tubes for 0.5 hr. on 2 successive days; radioactive 0.5*M*- NaHCO_3 solution was sterilized by filtering it through a moist Seitz pad through which a solution of non-isotopic 0.5*M*- NaHCO_3 had been passed immediately beforehand.

Fig. 1

Description of the apparatus. The apparatus (Fig. 2) was a closed-circuit constant-pressure macrorespirometer similar to that described by Wood, Brewer, Mickelson & Werkman (1940). It was set up in a hot room at $38 \pm 1^\circ$.

The volume of the culture flask (*A*) was about 600 ml. and that of the medium 100 ml. The organisms grew as a surface pellicle. The ground-glass stopper of *A* carried a four-way connecting piece, one horizontal arm joining the culture flask to the gas burette (*B*) and manometer (*C*) and the parallel arm joining it to the humidifier (*D*). The short gas-inlet tube (*E*) was sealed at its lower end and provided with four holes situated about 0.5 in. from the sealed end. This arrangement prevented the down-draught from disturbing the bacterial pellicle. The upper vertical arm of the connecting piece, provided with a three-way tap and protected by a sterile cotton-wool filter (*F*), joined the closed system to a cylinder of O_2 . Before introduction of O_2 , the cylinder connexions and the cotton filter were flushed with the gas. The gas outlet emerged from *A* about 0.5 in. above the culture to connect with the absorbing train. The sterile zone of the flask and its immediate connexions were protected from contamination by sterile cotton-wool filters.

The 100 ml. burette (*B*), graduated to 0.2 ml., was provided with a levelling bulb (*G*), which contained a solution of NaCl saturated at 39° . The manometer was half-filled with Brodie's fluid. It was connected to a 1 l. compensating flask (*H*) to which 200 ml. of water was added to make the volume of air in *H* approximately equal to the volume of air in the closed system on the other side of the manometer.

The absorbing train consisted of (1) a glass-stoppered drying tube (*J*) containing dried $CaCl_2$ (analytical reagent) and anhydrous; (2) a CO_2 -absorbing bubbler (*K*) containing 10.0 ml. of carbonate-free 3*N*-NaOH solution and provided with a guard tube (*L*) filled with soda asbestos and anhydrous; and (3) a guard tube (*M*) also containing soda asbestos and anhydrous. The soda asbestos and the anhydrous, both 14–22 mesh, were microanalytical reagents. *J* and *K* + *L* could be replaced as required. There were no significant changes in the weight of *M* during any of the experiments.

The air in the system was circulated at the rate of approx. 120 ml./min. by means of an all-metal bellows (*N*) and the bubbler valves (*P* and *Q*). The valves contained a saturated solution (39°) of NaCl. The bellows, which was made from four automobile petrol pumps, was driven at the

Fig. 2. Macrorespirometer used for the cultivation of *Mycobacterium butyricum* in media containing ^{14}C compounds. The lettering is the same as that in the descriptive text. 1, Glasswool packing; 2, sterile cotton wool; 3, $CaCl_2$ lumps; 4, soda asbestos; 5, anhydrous. The apparatus is not drawn to scale.

rate of 30 strokes/min. by an eccentric pulley and a geared motor.

The humidifier (*D*) contained 5 ml. of sterile water and the whole of this part of the apparatus was autoclaved before each assembly. Saturation of the air with water vapour was necessary to prevent concentration of the medium. There was no significant change in the volume of the medium in experiments lasting up to 11 days.

The ground-glass joints of the humidifier and the ground-glass surfaces within the sterile zone were lubricated with sterile rubber grease. All flexible connexions were made with thick rubber tubing.

Operation of the apparatus. The sterile medium (100 ml.) was measured into the culture flask and a solution (not more than 1 ml.) of the radioactive substrate added aseptically. After inoculation of the medium the apparatus was assembled and allowed to attain thermal equilibrium with all taps open to the air. The levelling fluid in the burette was adjusted approximately to the 100 ml. mark. After equilibration (1–2 hr.) all the taps, including those in the drying tube (*J*), were closed and the initial reading of the burette was taken with the fluid levels equalized on both sides of the manometer. All subsequent readings of the burette were taken in the same way. The barometric pressure was read at the end of the equilibration period.

The contraction of the volume (corrected to s.t.p.) of dry air in the system was the measurement of consumption of O_2 . During the first 72 hr. after inoculation the uptake of O_2 was very small and circulation of the air was unnecessary. Subsequently uptake of O_2 proceeded at a considerable rate and O_2 was restored to the system intermittently when the burette reading approached zero. At each addition of O_2 the burette reading was returned to about 100 ml.

Carbon dioxide formation was determined as the increase in weight of the bubbler (*K*) and its guard tube (*L*), which were weighed as a single unit. This unit was replaced as required within the limit of its absorbing capacity, the consumption of O_2 being used as a guide to the need for replacement. The drying-tube (*J*) was replaced before the anhydron showed any signs of moistness.

Preparation of the bacteria and the medium for analysis. At the end of an experiment the medium was decanted from the bacterial pellicle and centrifuged to remove a small quantity of bacteria. The pH was determined by a spot test with bromothymol blue. The bacterial pellicle was transferred to centrifuge tubes, broken up thoroughly and the organisms were washed three times with CO_2 -free water by centrifuging at 2000 *g*. The washings were added to the medium, the total volume was measured and a 5 ml. sample reserved for the estimation of glycerol, 2-oxoglutaric and pyruvic acid.

The combined medium and washings were placed in a three-necked flask provided with (1) a gas-inlet tube, (2) a dropping-funnel and (3) a connexion to a CO_2 -absorbing train similar to that described above (*J*, *K* and *L*), but of smaller capacity (3 ml. of liquid). Freshly prepared metaphosphoric acid solution (0.1 vol. of 10%, w/v, acid) was added and followed by 0.1–0.2 ml. of 10N- H_2SO_4 to bring the pH of the mixture to 3. Carbon dioxide-free air was bubbled through the flask and the bound CO_2 of the medium determined gravimetrically. The metaphosphoric acid was used to precipitate a small amount of material, designated 'protein', which the organisms release into the medium.

Its composition is uncertain but it gives protein colour tests (biuret, Sakaguchi and xanthoproteic reactions). The 'protein' was removed by centrifuging, washed twice with CO_2 -free water and the washings were added to the deproteinized medium.

The pH of the residual diluted medium was adjusted to 7.5 with carbonate-free 3N-NaOH solution, the volume measured and 3 ml. reserved for determination of total carbon and radioactivity. The medium was concentrated to 70–80 ml. in a rotary evaporator at 30°, acidified to pH 3 with 10N- H_2SO_4 and extracted continuously for 64 hr. with boiling peroxide-free diethyl ether. The ether was renewed at 16 and 40 hr. The ethereal extracts containing the organic acids of the medium were combined and neutralized (pH 7.5–8.0) with carbonate-free 0.5N-NaOH solution. The ether was removed *in vacuo* and the aqueous solution concentrated to 1 ml. in the rotary evaporator in preparation for chromatography.

The bacteria were dried to constant weight *in vacuo* at room temperature. A sample was reserved for combustion and measurement of radioactivity and the remainder defatted by boiling successively with 15 ml. of (a) ethanol-diethyl ether (1:1), (b) diethyl ether twice, (c) diethyl ether-chloroform (2:1, v/v), (d) ethanol-diethyl ether-conc. HCl (2:2:0.1, by vol.) and (e) diethyl ether. Each extraction lasted 1–2 hr. The combined extracts were shaken with water to remove HCl, dried over anhydrous Na_2SO_4 and evaporated to dryness *in vacuo*. The residual 'total lipids' were used for determination of carbon and radioactivity.

Materials. The inorganic chemicals, the acetic and succinic acid used as carriers and the benzene used as a solvent were analytical reagents. 2-Oxoglutaric acid (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) was recrystallized from ethyl acetate by addition of light petroleum. The purity of the product was at least 99% when it was assayed against the pure 2:4-dinitrophenylhydrazones of 2-oxoglutaric acid according to the method of Friedemann & Haugen (1943). [$1-^{14}C$]Acetate, [$2-^{14}C$]acetate, [$1-^{14}C$]succinic acid, sodium [^{14}C]bicarbonate and synthetic [$1-^{14}C$]glycerol were obtained from The Radiochemical Centre, Amersham, Bucks, England.

Analytical methods

Glycerol was estimated by the method of Lambert & Neish (1950), and 2-oxoglutaric and pyruvic acid by the method of El Hawary & Thompson (1953). Carbon was determined by drying samples of organic material at 105° and performing wet combustion with the reagent of Van Slyke & Folch (1940) in the apparatus described by Sakami (1955). The CO_2 was trapped in carbonate-free 3N-NaOH solution.

Chromatographic isolation of organic acids. The stationary phase was prepared by mixing 20 g. of acid- and ether-washed Celite (no. 545, Johns Manville Corp., U.S.A.) with 20 g. of silicic acid powder (Mallinckrodt Chemical Works, New York, U.S.A.; reagent for chromatography, 100 mesh) and adding 27 ml. of 0.05N- H_2SO_4 . The column was packed into a tube (2 cm. $\times$ 50 cm.) half-filled with benzene which had been equilibrated with 0.05N- H_2SO_4 and was washed with 100 ml. of benzene. The aqueous solution (1 ml.) of the salts of the organic acids extracted from the medium (see above) was acidified to pH 2–3 with 10N- H_2SO_4 ,

absorbed on 3 g. of the Celite-silicic acid mixture and packed on to the top of the column, which was developed with benzene and mixtures of benzene and diethyl ether containing successively greater proportions of ether. All the solvents were equilibrated with 0.05N-H₂SO₄ and passed through filter paper before use. The fractions (10 ml.) were titrated with carbonate-free 35 mM-NaOH solution with phenol red as indicator.

This procedure, which is similar to that of Kinnory, Takeda & Greenberg (1955), was developed for the purpose of obtaining a satisfactory separation of succinic and 2-oxoglutaric acid, which did not separate satisfactorily on the Celite column of Swim & Krampitz (1954) when they were present in the proportions encountered in this work. Fig. 3 shows the degree of separation achieved from a mixture of acids (Table 1) made up in proportions which were likely to be encountered in the culture medium. In fact the cultures never contained detectable amounts of

Fig. 3. Chromatographic separation of organic acids on a Celite-silicic acid column. The composition of the acid mixture is given in Table 1. Solvent: benzene, 0–130 ml.; benzene-diethyl ether (80:20, v/v) 130–190 ml., (60:40, v/v) 190–250 ml., (40:60, v/v) 250–310 ml., (30:70, v/v) 310–370 ml., (20:80, v/v) 370–430 ml., (15:85, v/v) 430–490 ml., (10:90, v/v) 490–590 ml.; diethyl ether 590–810 ml.

Table 1. *Chromatographic separation of organic acids*

Celite-silicic acid column; solvent, benzene-diethyl ether mixtures.		
Acid mixture	Amount present (μmoles)	Recovery (%)
Butyric acid	93	87
Propionic acid	50	90
Acetic acid	41	107
Pyruvic acid	35	98
Succinic acid	79	
2-Oxoglutaric acid	590	91

butyric and propionic acid. The recoveries are given in Table 1. There is a small overlap between pyruvic and succinic acid but 2-oxoglutaric acid is separated satisfactorily. The separation of 2-oxoglutaric and succinic acid was tested by applying a mixture of [1,4-¹⁴C]₂succinic acid (0.1 m-mole, specific activity 14 300 counts/min./m-mole of C) and 2-oxoglutaric acid (0.83 m-mole) to the column. The succinic acid fraction contained no 2-oxoglutaric acid detectable by the method of Friedemann & Haugen (1943) and the specific activity was 14 600 counts/min./m-mole of C. Three fractions of 2-oxoglutaric acid (early, peak and late fractions) had specific activities of 23, 24 and 25 counts/min./m-mole of C. These data show that the 2-oxoglutaric acid was not significantly contaminated with succinic acid.

Degradation of the organic acids separated chromatographically. The acetate fraction was acidified, distilled in steam and titrated. Carrier sodium acetate was added if necessary. Portions of the solution were evaporated to dryness and used for total combustion and for the Schmidt degradation according to Martin & Batt's (1957) modification of the method of Phares (1951).

Since pyruvic acid and succinic acid are not completely separated by the chromatographic column, the pyruvate and succinate fractions were pooled and the pyruvate decarboxylated with ceric sulphate (Krebs & Johnson, 1937). The CO₂ derived from the carboxyl group of pyruvic acid was collected in a bubbler of NaOH soln. to give a measurement of the amount of pyruvic acid present, and this value was checked by a colorimetric determination on the original pooled fractions. Ferrous sulphate was added to remove any excess of ceric sulphate and the solution extracted with diethyl ether for 24 hr. The ethereal extract was titrated, the sodium salts were taken to dryness and chromatographed on a 10 g. column of the Celite-silicic acid mixture. The acetate fraction was degraded as above to give C-2 and C-3 of the original pyruvic acid. The succinate fraction was diluted with carrier, acidified to pH 2.3, extracted with diethyl ether for 24 hr. and the ether removed. The succinic acid was purified by sublimation *in vacuo* at 115–120°. A sample was subjected to total combustion and the rest degraded according to Phares & Long (1955) except that the ethylenediamine was trapped in 0.5N-H₂SO₄. The recovery of the methylene carbon was only 5–10%.

The 2-oxoglutarate fraction was diluted with carrier to give about 4 m-moles of the acid. A portion was degraded with ceric sulphate to give the α-carboxyl group as CO₂. The rest was totally degraded by the method of Mosbach, Phares & Carson (1951) as modified by Koeppel & Hill (1955). The specific activities of the α-carboxyl group as determined by the ceric sulphate method and by the ninhydrin reaction used in the procedure of Mosbach *et al.* (1951) agreed within 3–8%.

In general the procedures and the apparatus used were those described by Sakami (1955). The accuracy of each step was checked by calculating the specific activity of the original compound from the sum of the specific activities of its fractions and comparing this value with the specific activity found by combustion of the original compound.

Measurement of radioactivity. All samples were plated in triplicate on filter paper as Ba¹⁴CO₃ and counted with an end-window Geiger-Müller tube. The counts were corrected for background and for self-absorption (to infinite thinness).

At least 5000 counts (standard error of less than $\pm 1\%$) were recorded for each plate. The corrected counts of triplicates differed by less than 10%. The value given for each sample is the mean of the corrected counts for the three plates.

RESULTS

Utilization of glycerol and acetate

Growth of the bacteria. *M. butyricum* was grown on a synthetic medium (see Methods), a thin pellicle covering the surface by the end of the third day; subsequently the pellicle thickened and became deeply pigmented. In cultures 1, 2 and 3 non-isotopic glycerol was the main carbon source and smaller amounts of ^{14}C compounds were added. Culture 1 was grown in the presence of $[^{14}\text{C}]$ -bicarbonate, culture 2 in the presence of $[1\text{-}^{14}\text{C}]$ -acetate and culture 3 in the presence of $[2\text{-}^{14}\text{C}]$ -acetate. In culture 4 $[1\text{:}3\text{-}^{14}\text{C}_2]$ glycerol was used without the addition of another carbon source (Table 2).

Although acetate is oxidized rapidly by washed suspensions of *M. butyricum* (Hunter, 1953b) the

organism grows very poorly when the glycerol of the medium is replaced by acetate, but it grows in the normal way when both acetate and glycerol are present. Acetate is utilized under these conditions (Tables 2–4). The experiments were designed to give a sufficient yield of 2-oxoglutaric acid with a specific activity high enough to permit the operations involved in total degradation. Table 2 shows the duration of the experiments, the yields of bacteria, the changes in the pH of the media, oxygen consumption, carbon dioxide production and the quantities of organic acids recovered from the media. The quantities of glycerol present initially and the amounts used are also given. Preliminary experiments showed that the bacteria attained their maximum growth in 7–8 days, after which slight autolysis occurred. Accumulation of 2-oxoglutaric acid, which was not significant until the fifth day after inoculation, continued for 5 days after growth had ceased. When $[^{14}\text{C}]$ acetate was added to the medium the duration of the experiments was curtailed. It was desirable that approximately 1 m-mole of acetate should remain in the

Table 2. *Growth of Mycobacterium butyricum on a synthetic medium containing ^{14}C -labelled compounds*

The medium (100 ml.) contained $(\text{NH}_4)_2\text{SO}_4$, a salt mixture and thiamine (see Methods). In cultures 2 and 3 the carbon sources were non-isotopic glycerol and sodium $[^{14}\text{C}]$ acetate; in culture 1 non-isotopic glycerol and $[^{14}\text{C}]$ bicarbonate; and in culture 4 the carbon source was $[^{14}\text{C}]$ glycerol. Initial pH of the medium, 7.5. Temp., $38 \pm 1^\circ$. Inoculum, approx. 4 mg. wet wt. of bacteria.

Culture no.	1	2	3	4
Labelled compound	$[^{14}\text{C}]\text{NaHCO}_3$	$[1\text{-}^{14}\text{C}]\text{Acetate}$	$[2\text{-}^{14}\text{C}]\text{Acetate}$	$[1\text{:}3\text{-}^{14}\text{C}_2]\text{Glycerol}$
Time (hr.)	262	168	167	283
Yield of bacteria (mg. dry wt.)	212	215	240	207
Final pH of medium	6.0	7.3	7.4	6.5
Radioactivity added (counts/min.)	4 610 000	1 770 000	1 520 000	5 550 000
Radioactivity used* (counts/min.)	504 000	1 540 000	1 490 000	1 880 000
	m-moles			
Oxygen uptake	24.1	16.5	19.4	19.9
Carbon dioxide production				
Total	17.6	13.2	15.6	15.8
Bound	0.04	1.56	2.0	0.05
Bacterial carbon	8.7	8.7	9.9	8.2
Bacterial lipid carbon	1.9	3.2	2.5	2.9
Protein carbon in the medium	0.17	0.15	0.16	0.38
Initial non-isotopic glycerol	28.6	31.7	32.0	0
Labelled compound added	0.5	7.15	6.6	32.9
Glycerol used	12.0	6.0	7.3	11.1
Acids in the medium				
2-Oxoglutaric acid	0.44	0.73	0.68	0.63
Succinic acid	0.08	0.12	0.13	0.11
Pyruvic acid	Trace	0.32	Trace	0.02
Acetic acid	0.02	1.23	0.14	0.05
	%			
Recovery of carbon†	90	97	94	90
Recovery of radioactivity‡	77	86	93	90

* In cultures 2, 3 and 4 the radioactivity used equals the sum of the counts/min. found in the CO_2 , the bacteria and the medium minus the counts/min. in the residue of the added ^{14}C compound. In culture 1 the counts/min. in the CO_2 (86% of the counts/min. added) have been omitted from the sum because much of this radioactivity was expelled from $[^{14}\text{C}]$ -bicarbonate by non-metabolic processes (see text).

† Sum of the carbon in the total CO_2 , bacteria, protein of the medium and the residual medium.

‡ Sum of the counts/min. found in the same fractions of the cultures.

* These figures are expressed as percentages of the radioactivity fixed in organic compounds only, because the radioactivity in the carbon dioxide does not have the same metabolic significance as in the other cultures.

were shorter than those with glycerol alone (cultures 1 and 4). The data suggest that acetate may be oxidized faster than glycerol.

In general the distribution of radioactivity is in parallel with that of carbon (Tables 3 and 4). One difference is conspicuous: the radioactivity derived from the methyl group of acetate is fairly evenly distributed between the bacterial carbon and the carbon dioxide (32.5 and 27.5 % respectively), whereas that derived from the carboxyl group is unequally distributed between these two fractions, 51.6 % appearing in carbon dioxide and 17.8 % entering the bacteria. There are also differences in the specific activities of the non-lipid bacterial carbon formed from the two kinds of acetate. These distributions are consistent with the operation of a citric acid cycle that is performing synthetic functions (Weinman, Strisower & Chaikoff, 1957).

*Fixation of carbon dioxide by
Mycobacterium butyricum*

Since the incorporation of carbon dioxide into oxaloacetate is a crucial feature of the scheme for glycerol oxidation (Fig. 1), this point was tested by adding a single dose of 0.5 m-mole of sodium [^{14}C]bicarbonate to the glycerol-containing medium before inoculation. It was expected that a substantial part of the radioactive bicarbonate would be decomposed and removed from the medium through operation of the low partial pressure of carbon dioxide in the circulating air and the development of acidity in the medium. These effects were discernible in the carbon dioxide collected from culture 1: the first sample (14.3 m-moles, 0–194 hr.) had a specific activity of 213 000 counts/min./m-mole, and the second (3.2 m-moles, 194–262 hr.) only 3150 counts/min./m-mole. In order to compensate for the anticipated removal of

radioactivity by non-metabolic factors and for dilution by respiratory carbon dioxide the initial specific activity of the bicarbonate was made very high (9.23×10^6 counts/min./m-mole). The need for this precaution when a single dose of bicarbonate is employed is apparent in the relatively low specific activities of the acids which accumulate in the medium after 120 hr. (Table 4).

At the end of the experiment there was practically no bicarbonate left in the medium. About 11 % of the radioactivity was recovered in the bacteria and the organic residues of the medium and about 66 % in the respiratory carbon dioxide. Analytical errors alone will not account for the deficit. Of 0.5 m-mole of $^{14}\text{CO}_2$ added about 0.05 m-mole was fixed in organic compounds. The total yield of carbon dioxide was 17.6 m-moles, which would represent a 39-fold dilution of the remaining 0.45 m-mole of $^{14}\text{CO}_2$. The 53-fold dilution which was observed (Tables 2 and 4) could be accounted for by assuming that approximately 0.1 m-mole of $^{14}\text{CO}_2$ was lost during equilibration of the apparatus.

Tables 3 and 4 show that $^{14}\text{CO}_2$ was incorporated into the organic constituents of *M. butyricum* and into the protein and other extracellular products found in the medium. The bacterial lipid C has a much lower specific activity than the non-lipid C, whereas the protein C of the medium has a specific activity of the same order as that of the bacterial non-lipid C. Similar demonstrations of the incorporation of carbon dioxide into the substance and extracellular products of tubercle bacilli have been reported (Long, Anderson, Rittenberg, Karnovsky & Henderson, 1955; Karnovsky, Stählen, Suter, Long & Henderson, 1956); and Wright (1957), using the somatic proteins, has identified the amino acids which were labelled

Table 4. *Specific activities of the bacteria (Mycobacterium butyricum) and other products*

These results refer to the same cultures as in Table 2.

Radioactive substrate added	[^{14}C]Bicarbonate	[1- ^{14}C]Acetate (counts/min./m-mole of C)	[2- ^{14}C]Acetate	[1:3- ^{14}C]Glycerol*
Labelled atom(s) of the substrate	9.23×10^6	248 000	230 000	84 500
Bacterial carbon	36 400	31 500	48 000	48 500
Bacterial lipid carbon	3 800	46 300	53 500	37 700
Bacterial non-lipid carbon (calc.)	52 700	22 900	46 300	54 200
Total carbon dioxide (weighted average of successive samples)	175 000	60 300	32 600	62 600
Bound carbon dioxide	—	54 400	21 300	—
Protein of the medium	64 000	20 900	60 400	46 900
Residual acids in the medium:				
2-Oxoglutaric acid	7 600	28 200	42 800	47 000
Succinic acid	2 270	31 000	61 000	53 400
Pyruvic acid	—	7 130	—	—
Acetic acid	—	94 000	111 000	24 000

* The glycerol is assumed to be equally labelled in C-1 and C-3.

Table 5. *Isotope distribution in organic acids formed by Mycobacterium butyricum in the presence of [¹⁴C]bicarbonate*

The medium contained non-isotopic glycerol and sodium [¹⁴C]bicarbonate (initial specific activity, 9.23×10^6 counts/min./m-mole). See culture 1, Table 2.

Metabolite		Carbon atom(s)	Specific activity (counts/min./m-mole of C)
2-Oxoglutaric acid	1	CO ₂ H	33 900
	2	C=O	
	3	CH ₂	1 040
	4	CH ₂	
	5	CO ₂ H	
Succinic acid	1, 4	CO ₂ H	4 030
	2, 3	CH ₂	510

during the growth of *M. butyricum* on a synthetic medium containing glycerol and [¹⁴C]bicarbonate. Aspartic and glutamic acids were heavily labelled.

According to Fig. 1 carbon dioxide is fixed in the β -carboxyl group of oxaloacetate, which subsequently enters the citric acid cycle. If citrate is metabolized asymmetrically in the manner characteristic of animal tissues (Wood, Werkman, Hemingway & Nier, 1941; Evans & Slotin, 1941; Ogston, 1948; Potter & Heidelberger, 1949), the isotope will appear predominantly in the α -carboxyl group of 2-oxoglutaric acid. Table 5 shows that the addition of [¹⁴C]bicarbonate to the glycerol-containing medium leads to the formation of 2-oxoglutaric acid, in which the carbon atom of the α -carboxyl group has a specific activity about 30 times as great as the average of that found in the other carbon atoms. In this respect the result is consistent with the hypothesis. It is possible that the low concentrations of the isotope found in succinic acid and in C-2 to C-5 of oxoglutaric acid were introduced by means of pyruvate, which had been labelled in all three carbon atoms by the following process: ¹⁴CO₂ can be introduced into the carboxyl group of pyruvate by way of a symmetrical dicarboxylic acid, e.g. fumaric or succinic. Incorporation of carboxyl-labelled pyruvate into hexose, followed by metabolism of the hexose in the pentose phosphate cycle, which occurs in the mycobacteria (Crowley, 1955), would yield pyruvate labelled in all its carbon atoms.

Succinic and 2-oxoglutaric acid are not in isotopic equilibrium with each other.

Metabolism of acetate

Another essential part of the hypothesis (Fig. 1) is the postulate that acetate derived from glycerol is oxidized in the citric acid cycle. The oxidation of acetate was studied by adding carboxyl- or methyl-labelled acetate to the glycerol-containing medium and determining the isotope distribution in 2-oxoglutaric acid and other acids recovered from the medium (Table 6). It will be seen that the residual

extracellular acetate has acquired a low concentration of ¹⁴C in the carbon atom which was originally unlabelled and that the specific activity of the carbon atom bearing the original label has diminished. This effect shows that the external acetate has exchanged with intracellular acetate, but the specific activities indicate that the external acetate has not attained isotopic equilibrium with the internal acetate pool.

The isotope distributions in the 2-oxoglutaric and succinic acid formed in the presence of [1-¹⁴C]- and [2-¹⁴C]-acetate conform to the well-known patterns of labelling which can be deduced from the theory of the citric acid cycle by application of principles enunciated by Strisower, Kohler & Chaikoff (1952) and Weinman *et al.* (1957). These principles assume that acetate of constant specific activity enters the cycle at a constant rate. Such conditions do not hold for cultures of *M. butyricum*. The mass of bacteria increases to reach a maximum about the eighth day and the concentration of external acetate diminishes progressively, most of this acetate vanishing by the end of the experiment. The excess of glycerol, however, is so great that the final concentration in the medium is about four-fifths of the initial concentration (Table 2). In these circumstances there will be a progressive decline in the specific activity of the internal acetate pool owing to dilution with non-isotopic acetate formed from glycerol. An indication of the correctness of this supposition is to be found in the specific activity of the carbon dioxide, the later samples having a significantly lower isotope concentration (Table 6).

If acetate labelled in one carbon atom only enters the citric acid cycle, the distribution of the isotope in the intermediates of the cycle and in carbon dioxide can be calculated from the equations of Strisower *et al.* (1952). After an infinite number of cycles the distribution would approach the limits given in Table 7 if there were no dilution by unlabelled oxaloacetate. Although dilution does occur, Table 7 is a guide to the expected ¹⁴C ratios between the carbon atoms.

Table 6. *Isotope distribution in the carbon dioxide and organic acids formed by Mycobacterium butyricum in the presence of [¹⁴C]acetate*

The medium contained non-isotopic glycerol and either [^{1-¹⁴C}]acetate (248 000 counts/min./m-mole of C-1) or [^{2-¹⁴C}]acetate (230 000 counts/min./m-mole of C-2). See cultures 2 and 3, Table 2.

Labelled substrate added	...	...	...	...	Specific activity (counts/min./m-mole of C)	
					[1- ¹⁴ C]Acetate	[2- ¹⁴ C]Acetate
Metabolite	Carbon atom(s)					
2-Oxoglutaric acid	1		CO ₂ H		57 500	21 000
	2		C=O		530	50 800
	3		CH ₃		980	49 200
	4		CH ₂		370	62 000
	5		CO ₂ H		84 400	12 900
Succinic acid	1, 4		CO ₂ H		58 800	19 600
	2, 3		CH ₂		880	46 400
Pyruvic acid	1		CO ₂ H		20 100	—
	2		C=O		1 060	—
	3		CH ₃		235	—
Acetic acid (residual)	1		CO ₂ H		184 000	8 960
	2		CH ₃		1 730	205 000
CO ₂	—		—		65 600	46 800
					(0-129 hr.)	(0-126 hr.)
					56 100	21 300
				(129-168 hr.)	(126-167 hr.)	
Bound CO ₂	—		—		54 400	21 300

Table 7. *Theoretical distribution of acetate carbon entering the citric acid cycle or the glyoxylate cycle*

The specific activity of the labelled carbon atom of acetate is taken to be 1.0. It is assumed that there is no dilution either by non-isotopic acetate or by non-isotopic oxaloacetate and that isotope equilibrium has been reached.

Cycle	...	...	Limit of specific activity			
			[1- ¹⁴ C]Acetate		[2- ¹⁴ C]Acetate	
			Citric acid	Glyoxylate	Citric acid	Glyoxylate
Oxaloacetate						
		CO ₂ H	0.5	1.0	0.5	0
		C=O	0	0	1.0	1.0
		CH ₃	0	0	1.0	1.0
		CO ₂ H	0.5	1.0	0.5	0
2-Oxoglutarate*						
		CO ₂ H	0.5	1.0	0.5	0
		C=O	0	0	1.0	1.0
		CH ₃	0	0	1.0	1.0
		CH ₂	0	0	1.0	1.0
		CO ₂ H	1.0	1.0	0	0
Succinate						
		CO ₂ H	0.5	1.0	0.5	0
		CH ₃	0	0	1.0	1.0
		CH ₂	0	0	1.0	1.0
		CO ₂ H	0.5	1.0	0.5	0
		CO ₂ from cycle	0.5	—	0.5	—
Pyruvate						
		CO ₂ H	0.5	1.0	0.5	0
		C=O	0	0	1.0	1.0
		CH ₃	0	0	1.0	1.0

* 2-Oxoglutaric acid is not a catalytic component of the glyoxylate cycle but is taken to be a product of it.

The specific activities of C-4 and C-5 of 2-oxoglutaric acid (Table 6) may be regarded as the mean specific activities of the methyl and carboxyl groups of the intracellular acetate during the period over which oxoglutaric acid was excreted into the medium, i.e. from about 120 hr. until the end of the experiments. This assumption about the status of the intracellular acetate pool is consistent with the observed distribution of ^{14}C in both samples of 2-oxoglutaric acid. The virtual exhaustion of the added $[2\text{-}^{14}\text{C}]\text{acetate}$ in culture 3 (Table 2) will account for the fact that the ratio of the specific activities of C-4 and C-5 in 2-oxoglutaric acid is only 5:1; whereas in culture 2, in which the residual $[1\text{-}^{14}\text{C}]\text{acetate}$ was 1.23 m-moles, the ratio of the specific activities of C-5 and C-4 is 240:1.

It will be noticed that the α -carboxyl group of 2-oxoglutaric acid is in isotopic equilibrium with the later sample of carbon dioxide (Table 6). This is to be expected because most of the oxoglutaric acid is formed after 120 hr. The succinic acid is in isotopic equilibrium with the moiety of oxoglutaric acid (C-1, C-2 and C-3) which is derived from oxaloacetate. Culture 2 alone gave a sufficient yield of pyruvic acid to permit degradation. All the carbon atoms were labelled but the pyruvic acid is not in isotopic equilibrium with the succinic acid.

Neither sample of 2-oxoglutaric acid has reached a theoretical equilibrium like that discussed by Strisower *et al.* (1952) or shown in Table 7. The specific activity of the α -carboxyl group in the sample derived from $[1\text{-}^{14}\text{C}]\text{acetate}$ is significantly greater than the predicted value (about 42 000 counts/min./m-mole of C); and in the sample derived from $[2\text{-}^{14}\text{C}]\text{acetate}$ it is significantly lower

than the predicted 37 000 counts/min./m-mole of C. Moreover, the equal specific activities of C-2 and C-3 in the sample derived from $[2\text{-}^{14}\text{C}]\text{acetate}$ are significantly lower than the specific activity of C-4. These facts may reflect the complex kinetics of the cultures in which the rates of growth, substrate disappearance and acid liberation are important variables. They may also imply the operation of a metabolic process additional to the classical steps of the citric acid cycle.

Metabolism of glycerol

Tables 4 and 8 give the specific activities of the products formed by *M. butyricum* in a medium containing $[1\text{-}^{14}\text{C}_2]\text{glycerol}$. Since the substrate is deemed to be equally labelled in C-1 and C-3, the subsequent distribution of the isotope will not be influenced by asymmetric metabolism of glycerol (Schambye, Wood & Popják, 1954; Swick & Nakao, 1954) if this process occurs in *M. butyricum*. According to hypothesis (Figs. 1, 4), the acetate formed from glycerol should be labelled more heavily in the methyl than the carboxyl group; carbon dioxide released in the oxidative decarboxylation of pyruvate should be labelled; and carbon dioxide fixation should give rise to oxaloacetate labelled in both carboxyl groups and more heavily in the methylene than in the carbonyl group. Since there was a considerable excess of labelled glycerol in the medium at the termination of the experiment, one of the complications of previous experiments was avoided, the only ^{12}C available to dilute the isotope being C-2 of glycerol.

It follows that the isotope distribution in 2-oxoglutaric acid should resemble the pattern obtained when $[2\text{-}^{14}\text{C}]\text{acetate}$ is metabolized but

Table 8. *Isotope distribution in the carbon dioxide and organic acids formed by Mycobacterium butyricum in the presence of $[^{14}\text{C}]\text{glycerol}$*

The medium contained $[1\text{-}^{14}\text{C}_2]\text{glycerol}$ (specific activity, 56 300 counts/min./m-mole of C or 84 500 counts/min./m-mole of C in each labelled atom). See culture 4, Table 2.

Metabolite	Carbon atom(s)	Specific activity (counts/min./ m-mole of C)
2-Oxoglutaric acid	1 CO_2H	39 600
	2 $\text{C}=\text{O}$	66 500
	3 CH_2	47 000
	4 CH_2	73 400
	5 CO_2H	9 380
Succinic acid	1, 4 CO_2H	40 600
	2, 3 CH_2	60 200
Acetic acid	1 CO_2H	8 100
	2 CH_3	48 600
CO_2	—	73 700
	—	(0–126 hr.)
	—	59 300
	—	(126–190 hr.)
	—	53 600
	—	(190–283 hr.)

The earliest sample of carbon dioxide collected during the phase of rapid growth has a high specific activity which can be explained by assuming that a significant fraction of this carbon dioxide has arisen from the oxidative decarboxylation of pyruvate, whilst an equivalent amount of the resulting acetate has been used for synthetic purposes. The later samples of carbon dioxide have specific activities consistent with complete combustion of glycerol. Neither the carboxyl group of succinic acid nor the α -carboxyl group of 2-oxoglutaric acid is in isotopic equilibrium with the carbon dioxide; yet both these carboxyl groups possess isotope concentrations which would be predicted from the observed specific activities in C-4 and C-5 of 2-oxoglutaric acid (see Fig. 4).

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DISCUSSION

It has been established that $^{14}\text{CO}_2$ is fixed into 2-oxoglutaric acid in the position to be expected if a citric acid cycle operates in *M. butyricum* (Table 5). Further, the metabolism of $[1\text{-}^{14}\text{C}]\text{acetate}$ and $[2\text{-}^{14}\text{C}]\text{acetate}$ has led to the production of 2-oxoglutaric and succinic acid in which the isotope distribution (Table 6) is consistent with the view that these substances are formed by way of the citric acid cycle.

The results obtained with $[1\text{-}3\text{-}^{14}\text{C}_2]\text{glycerol}$ permit amplification of the original hypothesis outlined in Fig. 1. The model shown in Fig. 4 depicts a steady-state mechanism by means of which 2-oxoglutarate and succinate with an isotope distribution similar to that observed could be formed from $[1\text{-}3\text{-}^{14}\text{C}_2]\text{glycerol}$. Whole numbers adjacent to the carbon atoms represent the approximate specific activities ($\times 10^{-3}$). The specific activity of each labelled carbon atom in $[1\text{-}3\text{-}^{14}\text{C}_2]\text{glycerol}$ is taken to be 80 in order that the resultant specific activities may be comparable with those observed (Table 8). The numbers beside the arrows denote molar quantities of the molecular species mixed in the metabolic pools. These values have been chosen arbitrarily to fit the observed isotope distribution. The outflow of carbon from the citric acid cycle for the synthesis of carbohydrates, amino acids and lipids has been neglected and only the production of 2-oxoglutaric acid has been considered.

Essentially the scheme shows the distribution of ^{14}C from a species of acetate which is labelled predominantly in the methyl group. The acetate is in isotopic equilibrium with the pyruvate pool, one-quarter of which has arisen from oxaloacetate and three-quarters directly from glycerol. Since the mechanism of carbon dioxide fixation is not known, it has been assumed that fixation occurs by way of malate, which mixes with cycle malate in the ratio of 12:28, and that oxaloacetate arises solely from the malate pool. The possibility of randomization in a malate-fumarate equilibrium has been omitted. The resulting isotope distribution in 2-oxoglutaric acid is very similar to that found by experiment, the distinctive feature being the unequal distribution of ^{14}C between C-2 and C-3 (cf. Tables 6 and 8).

In Table 6 the specific activity of C-1 of the 2-oxoglutaric formed in the presence of $[1\text{-}^{14}\text{C}]\text{acetate}$ is higher than would be predicted from the citric acid cycle equilibrium (Table 7); and in the 2-oxoglutaric acid formed in the presence of $[2\text{-}^{14}\text{C}]\text{acetate}$ the corresponding specific activity is lower than would be predicted. Such effects might be expected if the recently described glyoxylate cycle (Kornberg & Madsen, 1957; Kornberg & Krebs,

1957; Kornberg & Quayle, 1958) were operating in *M. butyricum* (see also Table 7). Although isocitratase has been found in this organism (Morrison & Wright, 1958), the occurrence of a glyoxylate cycle is uncertain because the presence of malate synthetase has not been demonstrated.

The scheme of Fig. 4 illustrates an attempt to find one way in which the observed distribution could have arisen, but the complicated dynamics of growing cultures make it difficult to interpret metabolic events from the observation of final isotope distributions. Despite this obstacle, a key to interpretation has been found in the isotopic composition of an unusually large excretion of 2-oxoglutaric acid, the distinctive labelling pattern of which strongly supports the view that the citric acid cycle plays a major part in the intermediary metabolism of glycerol.

SUMMARY

1. The metabolism of glycerol has been studied quantitatively in cultures of *Mycobacterium butyricum* grown on a synthetic medium containing $[^{14}\text{C}]\text{glycerol}$ or non-isotopic glycerol accompanied by $[^{14}\text{C}]\text{bicarbonate}$ or $[^{14}\text{C}]\text{acetate}$. The organism excreted a considerable amount of 2-oxoglutaric acid into the medium.

2. The incorporation of ^{14}C into the bacteria and some of their extracellular products has been determined.

3. Total degradation of the 2-oxoglutaric acid formed in the presence of sodium $[^{14}\text{C}]\text{bicarbonate}$ gave a labelling pattern consistent with the fixation of carbon dioxide in the β -carboxyl group of precursor oxaloacetate; and the patterns found in the presence of $[1\text{-}^{14}\text{C}]\text{-}$ or $[2\text{-}^{14}\text{C}]\text{-acetate}$ are consistent with oxidation of acetate in the citric acid cycle.

4. The distribution of ^{14}C in the 2-oxoglutaric acid formed in the presence of $[1\text{-}3\text{-}^{14}\text{C}_2]\text{glycerol}$ is consistent with the scheme: 2 glycerol $\rightarrow$ 2 pyruvate $\rightarrow$ oxaloacetate + acetate $\rightarrow$ 2-oxoglutarate + carbon dioxide. The scheme is discussed in detail.

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REFERENCES

- Andrejew, A. (1954). D.Sc. Thesis: Faculty of Sciences, University of Paris.
Blakley, R. L. (1952). *J. Bact.* **64**, 609.
Boissevain, C. H. (1943). *Proc. Soc. exp. Biol., N.Y.*, **54**, 342.
Crowley, G. M. (1955). *Proc. Univ. Otago med. Sch.* **33**, 22.
El Hawary, M. F. S. & Thompson, R. H. S. (1953). *Biochem. J.* **53**, 340.

- Evans, E. A. & Slotin, L. (1941). *J. biol. Chem.* **141**, 439.
- Faine, S., Whiteside, M. & Edson, N. L. (1951). *Proc. Univ. Otago med. Sch.* **29**, 18.
- Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
- Goldman, D. S. (1956a). *J. Bact.* **71**, 733.
- Goldman, D. S. (1956b). *J. Bact.* **72**, 401.
- Goldman, D. S. (1957). *J. Bact.* **73**, 602.
- Goldman, D. S. (1958a). *Biochim. biophys. Acta*, **27**, 506.
- Goldman, D. S. (1958b). *Biochim. biophys. Acta*, **27**, 513.
- Hunter, G. J. E. (1953a). *Biochem. J.* **55**, 320.
- Hunter, G. J. E. (1953b). *J. Bact.* **66**, 676.
- Karnovsky, M. L., Stählen, H., Suter, E., Long, E. R. & Henderson, H. J. (1956). *Proceedings of the International Conference on the Peaceful Uses of Atomic Energy*, vol. 10, p. 509. New York: United Nations.
- Kinnory, D. S., Takeda, Y. & Greenberg, D. M. (1955). *J. biol. Chem.* **212**, 379.
- Koepe, R. E. & Hill, R. J. (1955). *J. biol. Chem.* **216**, 813.
- Kornberg, H. L. & Krebs, H. A. (1957). *Nature, Lond.*, **179**, 988.
- Kornberg, H. L. & Madsen, N. B. (1957). *Biochim. biophys. Acta*, **24**, 651.
- Kornberg, H. L. & Quayle, J. R. (1958). *Biochem. J.* **68**, 542.
- Krebs, H. A. & Johnson, W. A. (1937). *Enzymologia*, **4**, 148.
- Lambert, M. & Neish, A. C. (1950). *Canad. J. Res. B*, **28**, 83.
- Lindsay, M., O'Donnell, T. V. & Edson, N. L. (1950). *Biochem. J.* **46**, 248.
- Long, E. R., Anderson, R. J., Rittenberg, D., Karnovsky, M. L. & Henderson, H. J. (1955). *Amer. Rev. Tuberc.* **71**, 609.
- Martin, J. K. & Batt, R. D. (1957). *J. Bact.* **74**, 359.
- Millman, I. & Darter, R. W. (1956). *Proc. Soc. exp. Biol. N.Y.*, **91**, 271.
- Millman, I. & Youmans, G. P. (1954). *J. Bact.* **68**, 411.
- Millman, I. & Youmans, G. P. (1955). *J. Bact.* **69**, 320.
- Morrison, N. E. (1956). *Proc. Univ. Otago med. Sch.* **34**, 23.
- Morrison, N. E. (1957). *Proc. Univ. Otago med. Sch.* **35**, 35.
- Morrison, N. E. & Wright, D. E. (1958). *Proc. Univ. Otago med. Sch.* **36**, 15.
- Mosbach, E. H., Phares, E. F. & Carson, S. F. (1951). *Arch. Biochem. Biophys.* **33**, 179.
- Ochoa, S., Stern, J. R. & Schneider, M. C. (1951). *J. biol. Chem.* **193**, 691.
- Ogston, A. G. (1948). *Nature, Lond.*, **162**, 963.
- Phares, E. F. (1951). *Arch. Biochem. Biophys.* **33**, 173.
- Phares, E. F. & Long, M. V. (1955). *J. Amer. chem. Soc.* **77**, 2556.
- Potter, V. R. & Heidelberger, C. (1949). *Nature, Lond.*, **164**, 180.
- Sakami, W. (1955). *Handbook of Isotope Tracer Methods*. Ed. by Sakami, W., Dept. Biochem., Western Reserve University School of Medicine, Cleveland, Ohio, U.S.A.
- Schambye, P., Wood, H. G. & Popják, G. (1954). *J. biol. Chem.* **206**, 875.
- Strisower, E. H., Kohler, G. D. & Chaikoff, I. L. (1952). *J. biol. Chem.* **198**, 115.
- Swick, R. W. & Nakao, A. (1954). *J. biol. Chem.* **206**, 883.
- Swim, H. E. & Krampitz, L. O. (1954). *J. Bact.* **67**, 419.
- Van Slyke, D. D. & Folch, J. (1940). *J. biol. Chem.* **136**, 509.
- Weinman, E. O., Strisower, E. H. & Chaikoff, I. L. (1957). *Physiol. Rev.* **37**, 252.
- Winder, F. G. & Denny, J. M. (1955). *Nature, Lond.*, **175**, 636.
- Wood, H. G., Brewer, C. R., Mickelson, M. N. & Werkman, C. H. (1940). *Enzymologia*, **8**, 314.
- Wood, H. G., Werkman, C. H., Hemingway, A. & Nier, A. O. (1941). *J. biol. Chem.* **139**, 483.
- Wright, D. E. (1957). *Proc. Univ. Otago med. Sch.* **35**, 37.
- Yamamura, Y., Kusunose, M. & Kusunose, E. (1954). *J. Biochem., Tokyo*, **39**, 513.
- Yamamura, Y., Kusunose, M. & Kusunose, E. (1955). *J. Biochem., Tokyo*, **42**, 785.

Enzymic Studies of X-Irradiated Cornea and Lens with Special Reference to Glutathione Reductase

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Nordmann & Mandel (1955) and Mandel & Schmitt (1956) have reported a decrease in lens hexokinase activity to be an early change after X-irradiation of the eye. van Heyningen & Pirie (1957) have not been able to confirm these results. The question of whether hexokinase activity is affected in the first few days after exposure to irradiation is important because hexokinase has been shown by Green, Bocher & Leopold (1955) to be a limiting system in the glycolytic pathway of lens tissue. The relatively

low levels found in cornea suggest that it may be limiting there also (Kuhlman & Resnik, 1958; Kuhlman, 1959). A decrease in the activity of this or any other system related directly to metabolism might be expected to produce a morphological change in the tissue. Attempts to correlate lens damage induced by radiation with early enzymic changes have not been successful (Pirie, van Heyningen & Boag, 1953; van Heyningen, Pirie & Boag, 1954). With the exception of an early fall of