ENDOGENOUS METABOLISM OF FILAMENTOUS FUNGI*

Harold J. Blumenthal

Department of Bacteriology, The University of Michigan
Ann Arbor, Mich.

Among the filamentous fungi the rate of the endogenous respiration, which often is a useful measure of endogenous metabolism, usually is high as compared to the total respiration in the presence of exogenous substrates. This feature is not limited to the fungi alone and may be found under certain conditions in bacteria (Dawes and Holms, 1958) and in animal tissues as well (Allen *et al.*, 1955).

Two techniques have been used to determine the status of the endogenous respiration during concurrent utilization of externally furnished substrates: Barker's indirect manometric method (1936) and the more direct isotopic methods involving either the use of uniformly-C^{14}-labeled cells and an unlabeled substrate (Burris, 1949) or unlabeled cells and an uninformly-C^{14}-labeled substrate (Blumenthal, Koffler, and Heath, 1957).

In the indirect manometric method, which is based on the premise that over a wide range of substrate/cell ratios a relatively constant proportion of the substrate is oxidized and assimilated, data corrected for the endogenous respiration are compared with uncorrected data. The practice which gives more constant values is considered to be correct even though the basic assumption has not been adequately tested (Blumenthal *et al.*, 1957). A number of modifications of the indirect manometric method have appeared differing in the manner in which the level of the endogenous respiration is varied while keeping the substrate concentration constant (Clifton, 1937; Doudoroff, 1940; Syrett, 1951). With any form of the manometric technique, only an "either-or" decision can be made and, as noted by Barker (1936), values for the amount of assimilation are obtained that are just as constant when corrected for the endogenous respiration as when they are not so corrected. The conclusions reached with the indirect manometric have sometimes disagreed with results of the more direct isotopic techniques. Examples of this disagreement can be found in *Penicillium chrysogenum* (Blumenthal *et al.*, 1957), *Chlorella vulgaris* (Syrett, 1951; Moses and Syrett, 1955) and *Euglena gracilis* (Danforth and Wilson, 1961). With acetate-grown cells of *P. chrysogenum* the conclusions from both forms of the isotopic technique were in disagreement with the manometic results only when acetate was a substrate.

In the studies with *P. chrysogenum*, we determined the influence of glucose or acetate on the endogenous respiration of mycelium grown with either acetate or glucose as the carbon source. From the results of these experiments it was concluded that the endogenous respiration of glucose-

* This investigation was supported in part by research grants from the U. S. Public Health Service (E-1493) and the National Science Foundation (G-14331).

grown cells was not inhibited by the concurrent oxidation of either glucose or acetate. However, the endogenous respiration of acetate-grown cells while not affected by glucose was very markedly suppressed by acetate. These results demonstrated that the previous history of an organism may effect the manner in which the endogenous respiration subsequently behaves during the concomitant utilization of exogenous materials.

Using only the more convenient form of the isotopic technique, with unlabeled cells and uniformly-C^{14}-labeled substrates, differences between the behavior of glucose and acetate on the endogenous respiration have been extended to another filamentous fungus, *Neurospora crassa*. The results in the present study are now expressed quantitatively. In addition, the influence of factors such as the age of the cells and/or growth temperature, starvation, and the level of isocitritase or glutamate-glutamine in the free amino acid pool on the inhibition of the endogenous respiration by glucose and/or acetate have been studied.

Materials and Methods

Conidia of *Neurospora crassa* strain 5297a (wild-type) were grown on 25 ml. of solid N medium (Vogel, unpublished) contained in a 125 ml. Erlenmeyer flask. After 7 to 10 days incubation at room temperature the flasks were stored in the refrigerator for a period not exceeding 3 weeks. The conidia from one such flask were suspended in 10 ml. sterile water and used to inoculate 1,000 ml. of medium. This heavy conidial inoculum yielded small and uniform mycelial pellets.

Cells were grown in submerged culture in Fries' minimal medium (Beadle and Tatum, 1945) modified so that glucose or glucose and acetate were the sole carbon source. In this modified medium ammonium tartrate was replaced by 10 gm. NH_4Cl/l. and the concentration of the phosphate buffer was increased (9 gm. anhydrous Na_2HPO_4 and 1 gm. KH_2PO_4/l.). Unless mentioned otherwise, 1.5 per cent glucose was the sole carbon source. In the glucose-acetate medium, 1.42 per cent anhydrous sodium acetate and 0.08 per cent glucose were used. The cells were usually grown in 2,000 ml. Erlenmeyer flasks containing 1,000 ml. medium, on a rotary shaker at 37° C. Cells were grown at 25° C. on a reciprocating shaker.

The cells were harvested at appropriate times on several layers of cotton gauze, squeezed dry and washed twice with *M*/15 potassium phosphate buffer, *p*H 6.0. For each gm. (wet weight) of cells, 33 ml. of the same buffer was added. This yielded a relatively homogeneous suspension containing about 5 mg. (dry weight)/ml. When starved cell suspensions were employed, the suspensions just described were incubated on a shaker at the growth temperature. At the end of the starvation period, the cells were reharvested and resuspended in fresh buffer in the manner described previously. In several experiments, the mycelial suspension was blended in a Waring blendor for 10 seconds just prior to use.

In the combined manometric-isotopic experiments, the Warburg flasks

usually contained 2 ml. of cell suspension in the main compartment, 0.5 ml. of uniformly (U)-C^{14}-labeled substrate or water in one side arm and 0.5 ml. of 70 per cent perchloric acid in the other side arm. In experiments using two substrates, the substrates were contained together in one side arm in a final volume of 0.6 ml. The substrate or water was tipped into the main compartment at zero time and the respiration in duplicate flasks was measured at 30° C. in air. Duplicate flasks without alkali in the center well were also used so that the CO_2 released could be determined by the two-flask method. The experiment was terminated by the addition of perchloric acid and 10 min. later the final manometric readings were taken. The flasks, which contained no filter papers in the center well, were allowed to shake an additional 30 min. before the $C^{14}O_2$ in those flasks containing alkali was converted to $BaC^{14}O_3$, plated and counted by the techniques previously described (Blumenthal *et al.*, 1957).

The U-C^{14}- glucose and the 1- and 2-C^{14}-acetate were products of Volk Radiochemical Co. Approximately equal quantities of 1-C^{14}-, and 2-C^{14}-acetate were mixed and used as U-C^{14}-acetate. The specific activity of the solution was determined as $BaCO_3$ after persulfate combustion by the method of Katz *et al.* (1954). The specific activity of the acetate used was 698 cpm/μM C in experiments 1–12 and 682 cpm/μM C in the remaining experiments. The specific activity of the glucose was 149 cpm/μM C in all experiments.

In some experiments the level of L-glutamic acid plus L-glutamine in the free amino acid pool of the mycelium was measured in parallel on a separate sample of the same mycelial suspension used for the manometric-isotopic determinations. Usually 80 ml. of cell suspension was added to duplicate 250 ml. Erlenmeyer flasks and 20 ml. of water or substrate was added at zero time. Thus, the cell/substrate ratio was the same as that used in the isotopic-manometric experiment. The flasks were incubated on the 37° C. rotary shaker and at the desired time intervals, the cells in the flasks were filtered onto a Buchner funnel and washed with water. The cell mat was then added to 50 ml. of hot 75 per cent ethanol and blended for 2 min. The blended cells were then steamed for 20 min., filtered, and extracted with 40 ml. of chloroform, essentially as described by Fuerst and Wagner (1957).

The content of L-glutamine plus L-glutamate in this extract was determined manometrically by the procedure of Meister, Sober, and Tice (1951). L-Glutamate decarboxylase (Nutritional Biochemical Corp.), which also contains glutaminase, was used and cetyl trimethylammonium bromide was employed so that L-aspartate would not be assayed. The assays on duplicate extractions agreed within ± 0.5 μM/gm. dry weight.

Isocitritase assays were performed on the mycelial extracts using the spectrophotometric phenylhydrazine procedure of Dixon and Kornberg essentially as described by Turian (1961). The protein concentration in these extracts was determined spectrophotometrically by the procedure of Waddel (1956).

Results

More than 25 manometric-isotopic experiments were performed during the course of this study. The results of 4 experiments, using cells grown on glucose at 37° C., are presented in TABLE 1. The results of experiment 17 will be used to demonstrate how the inhibition of the endogenous respiration is calculated. In this experiment the specific activity of the initial glucose substrate was 149 cpm μM C and there was a total of 1177 cpm in the CO_2 when glucose alone was the substrate. Thus it can be calculated that $1177/149 = 7.9$ μM of carbon from the glucose were oxidized to CO_2 during the experiment. Since a total of 17.6 μM CO_2 was actually produced in the flasks containing glucose, the amount of endogenous CO_2 liberated in the presence of glucose can be calculated to be $17.6 - 7.9 = 9.7$ μM. The actual endogenous liberation of CO_2 (in the absence of substrate) was 9.8 μM so that the inhibition of endogenous CO_2 production during the oxidation of glucose was $(9.8 - 9.7)/9.8 \times 100 = 1.0\%$. By the same method it can be calculated that the oxidation of acetate alone caused a 76.5 per cent inhibition of the endogenous CO_2 production.

In addition to determining the effect on the endogenous respiration of these two substrates individually, the combined effect of the two substrates on the endogenous respiration can also be measured. Because instances are known where acetate and glucose oxidation are unequally competitive (Scott *et al.*, 1962) and where glucose interferes with the utilization of lactate in resting suspensions of *P. chrysogenum* (Hockenhull *et al.*, 1954), the actual oxidation of one C^{14}-labeled substrate in the presence of the other unlabeled substrate was determined separately. When U-C^{14}-glucose was oxidized by the mycelial suspension in the presence of unlabeled (C^{12}) acetate, the amount of glucose oxidized was inhibited by 25.4 per cent while the amount of U-C^{14}-acetate oxidized in the presence of C^{12}-glucose was inhibited 25.3 per cent. Thus the amount of glucose carbon oxidized in the presence of acetate was $878/149 = 5.9$ μM while the amount of acetate carbon oxidized in the presence of glucose was $8559/682 = 12.5$ μM. Therefore, the total amount of CO_2 produced from these two substrates in the presence of each other was $5.9 + 12.5 = 18.4$ μM. Since the total amount of CO_2 produced by the substrates together measured 20.8 μM, it can be calculated that the endogenous CO_2 production in the presence of both substrates was $20.8 - 18.4 = 2.4$ μM. Consequently the two substrates together inhibited the endogenous CO_2 production by $(9.8–2.4)/9.8 \times 100 = 75.5$ per cent. The use of this technique allows one to determine the complex interplay between two exogenous substrates competing for oxidation by the cell as well as the effect of the two substrates individually and together on the endogenous metabolism of the cell.

A summary of the results of 15 experiments concerned with the effects of glucose and/or acetate on the endogenous respiration of mycelium of two different ages are presented in TABLE 2. Under all the conditions thus far examined, the oxidation of acetate has always resulted in a much greater inhibition of the endogenous respiration than has the oxidation of

TABLE 1

INHIBITION OF ENDOGENOUS RESPIRATION OF GLUCOSE-GROWN *N. crassa* MYCELIUM DURING CONCOMITANT OXIDATION OF U-C^{14}-SUBSTRATES

Experiment	Age of mycelium (hours)	Mycelium starved, hours	Experimental time (minutes)	Substrate* (μM)	Cpm in CO_2	% activity in CO_2	μM CO_2 released		μM O_2 consumed		% inhibition of endogenous respiration
							exogenous	endogenous	exogenous	endogenous	
3	55	0†	540	glucose (10)	3934	44.0	80.1	58.4	91.2	74.2	8.0
				acetate (30)	29251	69.9	71.5	58.4	80.2	74.2	49.4
17	52	10	60	glucose (6)	1177	21.8	17.6	9.8	18.2	11.7	1.0
				acetate (18)	11462	46.6	19.1	9.8	20.1	11.7	76.5
				C^{14}-glucose (6) + C^{12}-acetate (18)	878	16.4	20.8	9.8	21.1	11.7	75.5
				C^{12}-glucose (6) + C^{14}-acetate (18)	8559	34.8					
23	56	0	90	glucose (6)	814	15.1	8.0	3.0	9.0	4.8	15.7
				glucose (12)	884	8.2	8.3	3.0	9.4	4.8	18.0
				acetate (18)	5387	21.9	9.0	3.0	9.7	4.8	63.0
				acetate (36)	5938	12.1	8.6	3.0	9.2	4.8	103.0
19	30	10	60	glucose (6)	1393	25.8	19.4	12.4	20.1	13.8	19.4
				acetate (18)	9945	40.5	20.7	12.4	21.1	13.8	50.8
				C^{14}-glucose (6) + C^{12}-acetate (18)	1011	18.7	21.3	12.4	21.3	13.8	60.5
				C^{12}-glucose (6) + C^{14}-acetate (18)	6553	26.7					

* Spec. Act. (cpm μM C): Glucose, 149; Acetate, experiment 3, 698; others, 682.

† Mycelium blended.

TABLE 2

INHIBITION BY GLUCOSE AND/OR ACETATE OF ENDOGENOUS METABOLISM OF MYCELIUM GROWN ON GLUCOSE AT 37° C.

Experiment	Mycelium* starved, 9-11 hr.	Experimental time (minutes)	% Inhibition		
			Glucose (μM; % oxid†)	Acetate (μM; % oxid†)	Glucose + acetate
			Mycelium grown 50-62 hours		
1	— B1	370	10.3 (10;43.0)	65.2 (30;60.0)	—
3	— B1	540	8.0 (10;44.0)	49.4 (30;69.9)	—
23	—	90	15.7 (6;15.1)	63.0 (18;21.9)	—
	—	90	18.0 (12;8.2)	103.0 (36;12.1)	—
29	—	60	54.0 (6;28.6)	74.3 (18;58.6)	66.8
5	+	370	9.1 (10;44.1)	33.0 (30;61.9)	—
	+ B1	370	—	37.0 (30;63.8)	—
9	+ ‡	240	1.1 (6;19.7)	32.2 (18;58.1)	62.8
10	+ ‡	120	—	80.0 (30;59.6)	—
17	+	60	1.0 (6;21.8)	76.5 (18;46.6)	75.5
27	+	60	0.6 (6;25.1)	83.8 (18;57.6)	72.5
			Mycelium grown 30-34 hours		
11	+	60	36.5 (10;16.4)	—	—
	+	120	36.6 (10;27.6)	—	—
	+	180	36.3 (10;31.1)	—	—
13	+	90	14.4 (10;31.1)	85.6 (30;53.9)	—
	+	150	—	75.0 (30;61.1)	—
15	+	60	40.0 (10;25.8)	83.6 (15;57.0)	—
	+	60	—	103.6 (30;32.3)	—
18	+	60	18.1 (6;23.5)	63.8 (18;43.9)	61.0
19	+	60	19.4 (6;25.8)	50.8 (18;40:5)	60.5
28	—	90	39.2 (6;41.4)	76.2 (18;58.3)	70.0

* Mycelium not blended except where marked B1.

† Percent of added C^{14}-substrate oxidized to $C^{14}O_2$.

‡ Mycelium grown on glucose-acetate carbon source.

glucose. In 6 experiments using cells grown for about 30 hours with glucose as the sole carbon source, glucose inhibited the endogenous respiration from 14 to 40 per cent while acetate inhibited the endogenous respiration from 51 to 104 per cent. The replication within a given experiment was usually quite good, certainly within ± 2 or 3 per cent, so that the range of about 25 per cent in the values for the inhibition by glucose obtained in different experiments must be due to some changes in experimental conditions as yet unknown.

When similar experiments were performed using cells grown for 50 to 62 hours, there was also some variation in the amount of inhibition observed in different experiments. The inhibition by acetate, however, was still always greater than the inhibition by glucose. At least some of the

variation in the degree of inhibition of the endogenous respiration caused by acetate can be traced to the effect of the acetate concentration. Thus, in experiment 23, when the amount of acetate added to the flasks was raised from 18 to 36 μM, the inhibition was raised from 63 to 103 per cent. A similar increase, raising the inhibition from about 84 to 104 per cent, was found in experiment 15 when the acetate concentration was doubled. In experiment 13 the same amount of acetate was added to two sets of flasks but one set was allowed to oxidize the acetate for 90 minutes while the other set oxidized the acetate for 150 minutes. The inhibition was about 10 per cent lower in the flask allowed to oxidize the acetate more completely. These results suggest that many of the values reported here for the per cent inhibition by acetate are minimal values. The effect of acetate concentration is similar in some aspects, to that observed with acetate-grown *P. chrysogenum* (Blumenthal *et al.*, 1957).

The concentration of glucose necessary to inhibit the endogenous respiration of *N. crassa* was apparently not as critical as the acetate concentration. Thus, either doubling the amount of glucose added (experiment 23) or allowing the same amount of glucose to be oxidized for longer periods of time (experiment 11) had little or no effect on the degree of inhibition of the endogenous respiration (TABLE 2).

TABLE 3

INHIBITION BY GLUCOSE OR ACETATE OF ENDOGENOUS METABOLISM OF MYCELIUM GROWN AT 25° C.

Experiment	Mycelial age (hours)	Carbon source	Mycelium* starved (hours)	Experimental time (minutes)	% Inhibition	
					Glucose (μM; % oxid.†)	Acetate (μM; % oxid.†)
4	56	glucose	12	280	24.6 (10;40.3)	49.6 (30;58.4)
7	50	glucose-acetate	13	280	25.1 (10;48.7)	40.4 (30;82.9)
8	50	glucose-acetate	12	210	—	44.6 (30;59.0)
12	30	glucose	9	50	34.8 (10;22.3)	—
	30	glucose	9	90	37.4 (10;28.4)	—
	30	glucose	9	120	32.2 (10;32.1)	—
16	30	glucose	9	60	32.3 (10;21.6)	68.4 (15;62.5)
	30	glucose	9	60	—	71.0 (30;36.7)

* Mycelium blended only in experiment 4.

† Percent of added C^{14}-substrate oxidized to $C^{14}O_2$.

Although the effect of prior starvation of the mycelium was not studied in detail, some comparison was made between unstarved mycelial suspensions and suspensions starved for a period of about 9 to 11 hours. The only

experiments to yield results showing essentially no inhibition of the endogenous respiration during the oxidation of glucose were those using the starved 50 to 60 hour old cells (TABLE 2). When the 30 to 34 hour old mycelial suspensions were used, the endogenous inhibition caused by glucose oxidation was greater with these cells than with older cells, even when the younger cells were starved. This reduced level of inhibition as a result of glucose oxidation by older starved cell suspensions was not observed in the few experiments using mycelium grown at 25° C. (TABLE 3). The explanation for this may be due to the fact that the growth rate of *N. crassa* is slower at 25° C. than at 37° C. Cells grown for an equivalent length of time at the lower temperature would then be physiologically "younger" than those grown at 37° C. for an equivalent length of time. The present results indicate that many different physiological conditions affect the status of the endogenous respiration of *N. crassa* during concurrent oxidation of an exogenous substrate.

In an attempt to determine the biochemical basis for the marked inhibition by acetate of the endogenous respiration, the level of isocitritase in the mycelium was analyzed. The endogenous respiration of acetate-grown *P. chrysogenum* was previously demonstrated to be inhibited by acetate, but not by glucose, while the endogenous respiration of glucose-grown mycelium was not inhibited during the oxidation of either glucose or acetate (Blumenthal *et al.*, 1957). Olson (1959) observed that acetate-grown *P. chrysogenum* had a seven fold increase in the level of isocitritase over that found in mycelium grown on a carbohydrate carbon source. This suggested the possibility that isocitritase might be involved in the acetate inhibition of the endogenous respiration. *N. crassa* was also shown to have a much higher level of isocitritase when grown on acetate than when grown on sucrose, although the mycelium also contained isocitritase when grown with sucrose as the main carbon source. Furthermore, with sucrose as the carbon source, more isocitritase was produced when grown at 37° C. than when grown at 25° C. (Turian, 1961).

The level of isocitritase in the *N. crassa* mycelium was measured using separate samples of the same mycelial suspension used in the individual manometric-isotopic experiments (TABLE 4). As expected, the mycelium grown in the glucose-acetate medium contained much more isocitritase than the glucose-grown cells. However, there was no apparent relation between the isocitritase level and the degree of inhibition of the endogenous respiration with either glucose or acetate as the substrate. This can be seen most clearly by comparing the results of experiments 10 and 17. Although acetate inhibited the endogenous respiration to about the same extent in the two experiments, the level of isocitritase was over six fold higher in the mycelium used in experiment 10 than in the mycelium used in experiment 17.

Dawes and Holms (1958) demonstrated that the free amino acid pool was responsible for the high endogenous respiratory activity of *Sarcina lutea* and when this pool was decreased to approximately one half, the

TABLE 4

COMPARISON OF ISOCITRITASE LEVELS IN MYCELIUM AND INHIBITION OF ENDOGENOUS RESPIRATION BY SUBSTRATES

Mycelium growth conditions				% Endogenous inhibition		Isocitritase, Spec. Act.*
Experiment	Carbon Source(s)	Temperature	Age (hours)	Glucose	Acetate	
7	glucose-acetate	25° C.	50	25	40	>700
8	glucose-acetate	25° C.	50	—	45	720
10	glucose-acetate	37° C.	52	—	80	774
17	glucose	37° C.	52	1	76	124
5	glucose	37° C.	51	9	33–37	40–106
12	glucose	25° C.	30	35	—	66
16	glucose	25° C.	30	32	68–71	64
11	glucose	37° C.	30	36	—	51
13	glucose	37° C.	30	14	75–86	131
15	glucose	37° C.	30	40	84–100	110
18	glucose	37° C.	30	18	64	101

* Net change in absorbancy at 324 mμ × 1,000/mg. protein/7 min.

endogenous respiration fell to a negligible value. Nitrogenous substances participate in the endogenous metabolism of a number of microorganisms (Gronlund and Campbell, 1961) as indicated by the endogenous liberation of NH_3. There is evidence in *Escherichia coli* that the level of free amino acids is always maintained and that NH_3 is not released until after the glycogen is first consumed (Ribbons and Dawes, 1963). There is some evidence that proteins, through the free amino acid pool, are involved in the endogenous respiration of *Nocardia rugosa* (Bardi and Boretti, 1958) *P. chrysogenum* (Goldschmidt, 1952) and *Blastomyces dermatitidis* (Bernheim, 1942).

Halvorson *et al.* (1955) demonstrated the depletion of the free amino acid pool of resting yeast suspensions in the presence of glucose, although endogenously or in the presence of pyruvate or lactate, the pool levels actually increased. *N. crassa* 5297a has a complete amino acid pool and the combined levels of L-glutamate and L-glutamine in the cells are high and relatively stable after growth in a number of different media (Fuerst and Wagner, 1957). Consequently, the combined level of glutamate and glutamine were taken to be representative of the total amino acid pool. The results of some exploratory studies on the levels of these amino acids in the free amino acid pool, along with the results of the parallel manometric-isotopic estimation of the effect of glucose or acetate on the endogenous

respiration, are presented in TABLE 5. In experiment 27 there was about 15 μM of glutamate-glutamine/gm. mycelium even after a period of starvation for 10 hours. Furthermore there were no major changes in the pool levels after the oxidation of glucose or acetate for 1 hour although acetate inhibited the endogenous respiration over 80 per cent and glucose had essentially no inhibitory effect in this instance. Thus, we cannot come to any conclusion from this experiment or the following one. It would be necessary to determine the specific activity of the free amino acid pool, after using U-C^{14} substrates, to determine the extent to which the free amino acid pool serves as an endogenous substrate, and the extent to which it is refilled from the exogenous carbon source.

The results of experiment 29 suggest that endogenous sources other than the free amino acids must be contributing significantly to the endogenous respiration. After 90 minutes the level of glutamate and glutamine in the mycelium was reduced by approximately one half either in the absence of substrate or in the presence of glucose or acetate. Since the manometric-isotopic estimations indicated that glucose and acetate had inhibited the endogenous respiration by 54.0 per cent and 74.3 per cent, respectively, then something other than the free amino acid pool must have served as the endogenous carbon source.

TABLE 5

THE EFFECT OF GLUCOSE OR ACETATE ON THE LEVEL OF GLUTAMATE PLUS GLUTAMINE IN THE FREE AMINO ACID POOL OF MYCELIUM GROWN ON GLUCOSE AT 37°C.

Experiment	Cells	Experimental time (minutes)	Substrate*			% Inhibition of endogenous respiration†	
			Endogenous	Glucose	Acetate	Glucose	Acetate
27	grown 50 hr., starved 10 hr.	0	14.9	—	—	—	—
		60	16.4	13.9	15.9	0.6	83.8
28	grown 32 hr., unstarved	0	22.9	—	—	—	—
		90	18.2	25.2	16.2	39.2	76.2
29	grown 50 hr., unstarved	0	30.6	—	—	—	—
		60	—	—	—	54.0	74.3
		90	15.1	16.2	16.2	—	—
		180	14.2	13.0	—	—	—

* The numbers are the μmoles of combined L-glutamate and L-glutamine/g. dry weight. The substrate/cell ratios were exactly the same as those used in the isotopic experiments *i.e.*, 6 μM glucose or 18 μM acetate 3 ml. In experiment 29 the initial substrate concentration in the flasks used for the determination of the free amino acid pools was 20 per cent higher than in the Warburg flasks.

† Data from TABLE 2.

Discussion

The status of endogenous metabolism during the concurrent metabolism of exogenous substrates has been studied only to a limited extent. As mentioned at the outset of this paper, the validity of one of the two main approaches which have been used to study this problem, the indirect manometric method (Barker, 1936), is subject to question, especially when applied as the sole manner of reaching a decision as to the course of the endogenous metabolism.

Two forms of the second major approach, the more direct isotopic technique, have been used to study the effect of exogenous metabolism on endogenous metabolism. In the first form of the isotopic technique cells are uniformly labeled with C^{14} and unlabeled substrates are used (Burris, 1949). The release of $C^{14}O_2$ from such cells is then a measure of the endogenous respiration. Examples of the use of this method with *P. chrysogenum* uniformly labeled by growth on U-C^{14}-glucose or U-C^{14}-acetate are presented in FIGURES 1 and 2, respectively (Blumenthal *et al.*, 1957). There was no inhibition of the endogenous respiration during the first 3 hours of glucose oxidation by the glucose-grown cells while acetate actually stimulated the endogenous respiration during that same time interval. Glucose also had no effect on the endogenous release of $C^{14}O_2$ from acetate-grown cells during the same time period although the oxidation of acetate markedly inhibited the release of $C^{14}O_2$.

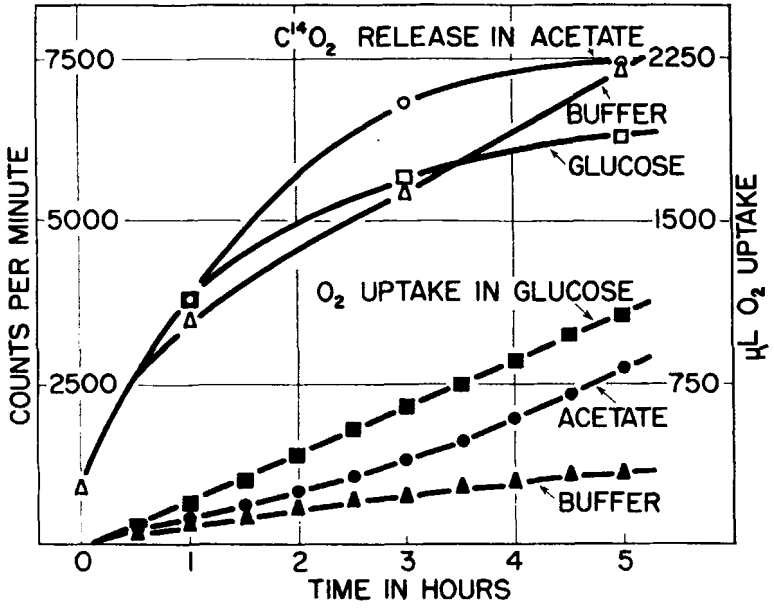

FIGURE 1. The effect of 0.1 *M* glucose or acetate on the time course of O_2 uptake and $C^{14}O_2$ release from *P. chrysogenum* cells labeled by growth for 45 hours in a medium containing U-C^{14}-glucose. Reproduced by permission of the *Journal of Cellular and Comparative Physiology*.

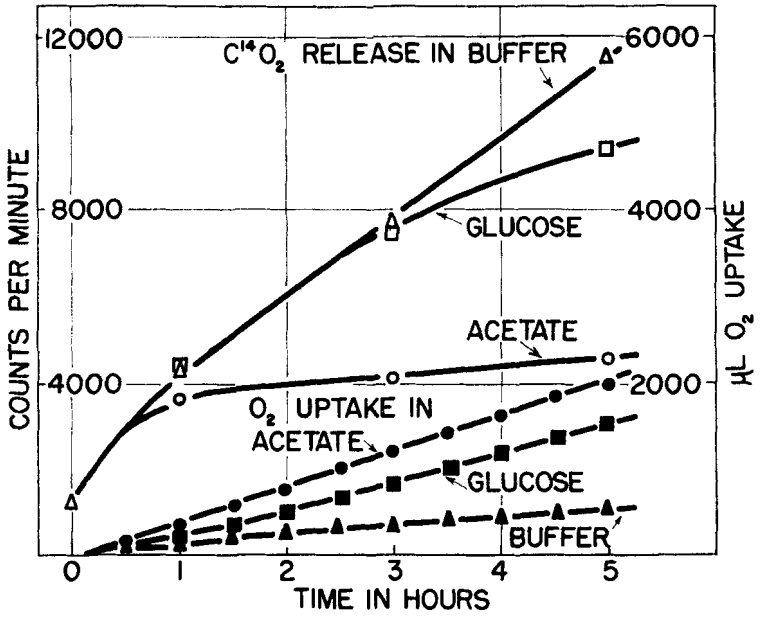

FIGURE 2. The effect of 0.1 *M* glucose or acetate on the time course of O_2 uptake and $C^{14}O_2$ release from *P. chrysogenum* cells labeled by growth for 45 hours in a medium containing U-C^{14}-acetate. Reproduced by permission of the *Journal of Cellular and Comparative Physiology.*

In experiments using C^{14}-labeled cells, the cells have to be uniformly labeled for the $C^{14}O_2$ to be representative of the total endogenous CO_2, at least at the start of an experiment. If the cells are not uniformly labeled, the results may lead to improper conclusions. When *P. chrysogenum* was labeled by growth on U-C^{14}-glucose only during the last 6 hours of a 45 hour growth period, rather than by growth on the U-C^{14}-glucose for the whole 45 hour period, it was found that glucose inhibited the release of $C^{14}O_2$ from these incompletely-C^{14}-labeled cells by about 50 per cent whereas glucose caused essentially no inhibition of the release of $C^{14}O_2$ (FIGURE 1) during the same time interval from the corresponding uniformly-C^{14}-labeled cells (Blumenthal *et al.*, 1957).

In a number of instances, cells have been labeled by oxidative assimilation of a C^{14} substrate. The $C^{14}O_2$ released from cells labeled in this manner may be even less representative of the total endogenous CO_2 than cells incompletely labeled during growth. Thus, when acetate-grown *P. chrysogenum* cells were labeled by oxidative assimilation of C^{14}-acetate, the rate of release of $C^{14}O_2$ from such cells in the presence of acetate was found to be markedly different than the rate of $C^{14}O_2$ release from similar cells labeled by growth for 6 hours on C^{14}-acetate (Blumenthal *et al.*, 1952; Blumenthal, 1952). Gibbs and Wood (1952) also recognized differences

in the results obtained with *Pseudomonas fluorescens* labeled either by growth or by oxidative assimilation. Consequently, it is difficult to assess the results of experiments with incompletely labeled cells, especially when labeled by oxidative assimilation, such as those performed with *Saccharomyces cerevisiae* (Reiner *et al.*, 1949; Moses and Syrett, 1955), *Pseudomonas saccharophila* (Wiame and Doudoroff, 1951), *Zygorhyncus moelleri* (Moses and Syrett, 1955), and *Nocardia corallina* (Midwinter and Batt, 1960).

In studies with essentially uniformly-labeled cells, Cochrane and Gibbs (1951) found that the endogenous respiration of *Streptomyces coelicolor* was not inhibited during the oxidation of glucose or pyruvate and glucose was reported not to inhibit the endogenous respiration of *Staphylococcus aureus* (Ramsey, 1962). Glucose stimulated the release of $C^{14}O_2$ from C^{14}-glucose-grown *S. griseus* during the first 6 hours of the experiment (Wang *et al.*, 1958), while both sucrose and acetate stimulated the endogenous respiration of a surface mat of *N. crassa* labeled by growth on U-C^{14}-sucrose and unlabeled tartrate (Heplar and Tatum, 1954). In this study, though, the levels of $C^{14}O_2$ evolved were all quite low. In a non-isotopic experiment, Hockenhull *et al.* (1954) observed that acetate stimulated the endogenous metabolism of starved, resting cells of carbohydrate-grown *P. chrysogenum*. Instances where there was an inhibition in the release of $C^{14}O_2$ from essentially uniformly-labeled cells during the oxidation of glucose have been reported in *Penicillium digitatum* (Reed and Wang, 1959) and *Bacillus cereus* (Clifton and Sobek, 1961). In *P. digitatum* the release of $C^{14}O_2$ from the glucose-grown mycelium was inhibited 25 per cent while in *B. cereus* the inhibition by glucose was between 20 and 40 per cent. As mentioned previously, acetate inhibited the endogenous release of $C^{14}O_2$ from acetate-grown *P. chrysogenum* but not from glucose-grown cells (Blumenthal *et al.*, 1957). Acetate was also found to inhibit the release of $C^{14}O_2$ from *Euglena gracilis* (Danforth and Wilson, 1961).

Technically it is troublesome to grow uniformly-C^{14}-labeled cells in the laboratory and relatively large amounts of C^{14} must be used in order to obtain cells with a sufficiently high specific activity. For this reason, the use of unlabeled cells and U-C^{14}-substrate is particularly convenient to ascertain the status of the endogenous metabolism during substrate oxidation. This approach is based upon the fact that endogenously produced CO_2 from unlabeled cells results in the dilution of the $C^{14}O_2$ derived from the radioactive substrate. The extent of the participation of the endogenous metabolism in the total respiration can be calculated from the specific activities of the C^{14}-substrate and the $C^{14}O_2$ released, and the total amounts of CO_2 liberated endogenously in the presence of the substrate during the experimental period.

This method, first used with *P. chrysogenum*, revealed that glucose or acetate did not inhibit the endogenous respiration of glucose-grown cells. However, the endogenous respiration of acetate-grown cells, while not affected by glucose was suppressed by acetate. In the main, then, these

results agreed with those obtained using uniformly-C^{14}-labeled cells (Blumenthal *et al.*, 1957).

In the present use of this approach with *N. crassa*, some slight modifications of the technique have been made and the results of the inhibition are now expressed quantitatively. By not allowing the substrate to always be oxidized to completion, the time course of the reaction can be followed. In the case of glucose oxidation (TABLES 2 and 3) the constancy of the specific activity of the $C^{14}O_2$ during a 2 or 3 hour period demonstrated that the system was in equilibrium. The importance of ensuring equilibration might be of significance if mycelial mats or larger mycelial pellets are used. With animal tissue slices (Allweis *et al.*, 1961) or even whole organs (Geiger *et al.*, 1960), the period until the CO_2 reaches a constant specific activity may vary from 30 to 180 minutes. Scott *et al.* (1962) recently used the dilution of the specific activity of glucose and acetate to measure the interplay of these substrates with the endogenous metabolism of spermatozoa.

The results of the present studies with *N. crassa* emphasize the fact that in our present state of knowledge, we must be very careful not to extend the results obtained under one set of conditions to a different set of conditions, even if the same organism is used.The results with glucose as a substrate attest to this (TABLE 2). Factors such as the age of the cells and whether or not they were starved, the growth temperature and the substrate in question can all affect the response of the endogenous respiration during the course of the substrate oxidation.

The observed effect of starvation on the absence of glucose inhibition of the endogenous respiration of 50-hour-old cells is an interesting one because fungal cells are often starved before respirometric studies and the differences in the properties between starved and fresh cells are not fully appreciated by all investigators. The use of starvation or prolonged aeration, as first introduced for bacteria (Quastel and Whetham, 1924) has led to variable results with fungi (see Cochrane, 1958). The endogenous respiration of *Fusarium*, for example, is successfully reduced by starvation (Gould and Tytell, 1941) although attempts to reduce the endogenous respiration of many fungi have failed in that the reduction in the endogenous rate was achieved only by a similar or greater reduction of the respiratory activity in the presence of substrate. In some instances, this loss of activity can be traced to losses of vitamins, anions, *etc.* (see Blumenthal, 1952).

A few filamentous fungi that could not be starved successfully under the conditions tested are *S. coelicolor* (Cochrane and Peck, 1953), *P. chrysogenum* (Stout and Koffler, 1951) and *Scopulariopsis brevicaulis* (MacMillan, 1956). The biochemical results of starvation on *Z. moelleri* have been well documented (Moses, 1958). The possibility should always be considered that absence of an increase in the rate of respiration upon addition of a substrate, or even an inhibition of the respiration, may not mean that the substrate is not being oxidized. The Crabtree effect (Ibsen, 1961),

a respiratory inhibition upon addition of hexoses, is not uncommon in animal tissues, and in these instances the substrate is oxidized. Perhaps this effect may also be found in microorganisms, especially those with a high rate of endogenous respiration. The use of C^{14}-labeled substrates can easily be used to determine if a substrate is being oxidized in the presence of only limited respiratory stimulation or even inhibition.

In the present study with *N. crassa,* the age at which the cells were starved affected the response of the endogenous respiration to glucose (TABLE 2). Other investigators have noted an effect of age and/or the level of endogenous respiration. Clifton and Sobek (1961) suggested that the variations of the glucose inhibition of the endogenous respiration might be due to the age of the cells because of the different levels of endogenous respiration. Dawes and Holms (1958) demonstrated that high levels of endogenous metabolism in *Sarcina lutea* partially suppressed the oxidation of glucose, while Eaton and Klein (1957) noted differences in the endogenous metabolism of "young" and "old" suspensions of *S. cerevisiae.* The young cell, with a relatively low endogenous respiration, produced almost all of its endogenous CO_2, in the presence of glucose as a substrate, via the tricarboxylic acid cycle. By contrast, "old" cells, with a considerably higher endogenous respiration, produced substantial amounts of CO_2 from endogenous sources, while oxidizing either glucose or acetate, by some pathway not involving the tricarboxylic acid cycle. As far as the differences in the results with temperature are concerned, such factors as the content of isocitritase (Turian, 1961) and the level of an amino acid in the free amino acid pool (Matchett and DeMoss, 1962) are known to be affected to a considerable degree by the growth temperature of *N. crassa.* These, and similar changes, might contribute to the differences between the cells grown at 25° C. and 37°C.

Microorganisms can have more than a single endogenous reserve and not all of these endogenous substrates are used equally under all conditions. For example, this appears to be true for the endogenous reserves in yeast (Eaton, 1960), *Neurospora* ascospores (Sussman, 1961) and *E. coli* (Ribbons and Dawes, 1963).

An explanation for the greater inhibition of the endogenous respiration by acetate than by glucose is not known. It is apparently unrelated to the level of isocitritase in the cell (TABLE 4). Stone (1962) believes that during growth *N. crassa* mycelium has at least two metabolic pools of "acetate," one used, among other metabolic processes, for the biosynthesis of ergosterol and fatty acids, and the other required for particular processes involved in growth. The differences observed in the present study between the effects of glucose and acetate, both individually and together, on the endogenous respiration of *N. crassa* suggest that these two substrates affect two different endogenous substrate "pools" and that these separate endogenous substrates interact in the cell. Obviously this is a complex interplay not only with different endogenous substrates but with the two different exogenous substrates competing (TABLE 1). However, the meth-

odology used here can be employed to study such complex interrelationships.

Endogenous metabolism means different things to different people (Lamanna and Mallette, 1959). When part of an exogenous substrate is assimilated by a cell, some of it may be converted to materials that the cell uses endogenously. Most likely there is an interplay between the two, and the question as to when the substrate ceases to be exogenous and becomes endogenous involves an exercise in semantics. However, the nature of the isotopic techniques requires an operational definition. Thus, with U-C^{14}-labeled exogenous substrates, any $C^{14}O_2$ found in the CO_2 at the time of sampling is considered as exogenous substrate while the unlabeled CO_2 is endogenous. The reverse is true for experiments with uniformly-C^{14}-labeled cells and unlabeled substrates. The longer the experiment proceeds, the less the cell resembles the cell at the start. The decision that the investigator has to make is the length of time the experiment should be conducted. The results in FIGURES 1 and 2 were considered to be representative of the endogenous metabolism only during the first 3 hours. Some investigators have chosen to put special interpretations on the rate of release of $C^{14}O_2$ from C^{14}-labeled cells (Moses and Syrett, 1955; Danforth and Wilson, 1961). Unless the details of the endogenous-exogenous interactions are known, and they are certainly not known now, it would be best for all investigators to use an operational definition of endogenous metabolism.

In some metabolic experiments, it is not necessary to know the fate of the total endogenous metabolism in the presence of an exogenous substrate, but only the proportion of a particular metabolic product, such as ethanol, that is being formed from the endogenous reserves, and the proportion being formed from the added substrate. This can be done by comparing the specific activity of the isolated product after utilization of the U-C^{14}-substrate by unlabeled cells (Blumenthal *et al.*, 1954).

It is clear that the response of the endogenous metabolism of a cell during the concurrent metabolism of an exogenous substrate is complex. The previous history of an organism, perhaps by regulating the quantitative distribution of enzymes and/or endogenous reserves within the cell, does affect the way in which the endogenous respiration behaves subsequently in the presence of an added substrate. Although the data in this report are descriptive, and do not offer support for any specific explanation of the observations made, they do suggest future experiments that may lead to a better understanding of this complex interplay between endogenous and exogenous substrates.

Summary

The status of the endogenous respiration of *Neurospora crassa* was determined quantitatively during the concurrent utilization of glucose and/or acetate employing a manometric-isotopic technique with unlabeled cells and uniformly-C^{14}-labeled substrates. The degree of inhibition of the

endogenous respiration was influenced by many factors, such as the nature and concentration of the exogenous substrate, the age of the cells, the growth temperature and whether or not the cells were starved.

The endogenous respiration of cells grown on glucose as the sole carbon source at 37° C. for 30 hours was inhibited *ca.* 15 to 40 per cent during glucose oxidation and *ca.* 50 to 100 per cent during acetate oxidation. With cells 50– to 60-hours-old, the degree of inhibition by these substrates was the same. However, when the older cells were starved, the inhibition by glucose was eliminated. The concentration of acetate, but not glucose, influenced the degree of inhibition of the endogenous respiration. When glucose and acetate were added as cosubstrates, the resulting inhibition of the endogenous respiration was not equivalent to the sum of the inhibitions caused by each substrate separately. These results suggested that glucose and acetate inhibited the utilization of different endogenous substrates. There was competition between the endogenous, as well as exogenous, substrates when acetate and glucose were used as cosubstrates.

The concentration of isocitritase in the cells was found to be unrelated to the degree that glucose or acetate inhibited the endogenous respiration. The levels of L-glutamate plus L-glutamine in the free amino acid pool were determined. No conclusion was drawn concerning the quantitative contribution of the free amino acid pool to the total endogenous respiration.

Acknowledgment

I wish to acknowledge the valuable assistance of Y.-L. Pan and T. Jepson during the course of the work. I also wish to acknowledge a gift of DL-isocitric acid lactone from J. F. Hogg.

References

ALLEN, A., B. FRIEDMANN & S. WEINHOUSE. 1955. Tissue preferences for fatty acid and glucose oxidation. J. Biol. Chem. **212**: 921–933.

ALLWEIS, C. L., H. GAINER & I. L. CHAIKOFF. 1961. A micromethod for studying kinetics of conversion of C^{14} substrate to CO_2 by tissue slices, and its application to brain. Arch. Biochem. Biophys. **92**: 159–167.

BARDI, U. & G. BORETTI. 1958. Observazioni sul metabolismo endogeno di un *Proactinomyces*: *Nocardia rugosa*. Giorn. Microbiol. **6**: 91–102.

BARKER, H. A. 1936. The oxidative metabolism of the colorless alga, *Prototheca zopfii*. J. Cellular Comp. Physiol. **8**: 231–250.

BEADLE, G. W. & E. L. TATUM. 1945. Neurospora. II. Methods of producing and detecting mutations concerned with nutritional requirements. Am. J. Botany, **32**: 678–686.

BERNHEIM, F. 1942. The effect of various substances on the oxygen uptake of *Blastomyces dermatitidis*. J. Bacteriol. **44**: 533–539.

BLUMENTHAL, H. J. 1952. Studies on the endogenous metabolism of *Penicillium chrysogenum* Q-176 during oxidative assimilation. Ph.D. Thesis. Purdue University. Lafayette, Indiana.

BLUMENTHAL, H. J., H. KOFFLER & E. P. GOLDSCHMIDT. 1952. The rate of endogenous respiration as affected by the oxidation of exogenous substrates. Science. **116**: 475–477.

BLUMENTHAL, H. J., H. KOFFLER & E. HEATH. 1957. Biochemistry of filamentous fungi. V. Endogenous respiration during concurrent metabolism of exogenous substrates. J. Cellular Comp. Physiol. **50**: 471–497.

BLUMENTHAL, H. J., K. F. LEWIS & S. WEINHOUSE. 1954. An estimation of pathways of glucose catabolism in yeast. J. Am. Chem. Soc. **76**: 6093–6097.

BURRIS, R. H. 1949. *In* Umbreit, W. W., R. H. Burris & J. F. Stauffer, Eds. Manometric Techniques and Tissue Metabolism. 2nd Ed. Burgess Publishing Co. Minneapolis, Minn.

CLIFTON, C. E. 1937. On the possibility of preventing assimilation in respiring cells. Enzymologia. **4**: 246–253.

CLIFTON, C. E. & J. M. SOBEK. 1961. Oxidative assimilation of glucose and endogenous respiration of *Bacillus cereus*. J. Bacteriol. **81**: 284–287.

COCHRANE, V. W. 1958. Physiology of Fungi. John Wiley and Sons. New York, N. Y. Pp. 210–211.

COCHRANE, V. W. & M. GIBBS. 1951. The metabolism of species of *Streptomyces*. IV. The effect of substrate on the endogenous respiration of *Streptomyces coelicolor*. J. Bacteriol. **61**: 305–307.

COCHRANE, V. W. & H. D. PECK, JR. 1953. The metabolism of species of *Streptomyces*. VI. Tricarboxylic acid cycle reactions in *Streptomyces coelicolor*. J. Bacteriol. **65**: 37–44.

DANFORTH, W. F. & B. W. WILSON. 1961. The endogenous metabolism of *Euglena gracilis*. J. Gen. Microbiol. **24**: 95–105.

DAWES, E. A. & W. H. HOLMS. 1958. Metabolism of *Sarcina lutea*. III. Endogenous metabolism. Biochim. et Biophys. Acta. **30**: 278–293.

DOUDOROFF, M. 1940. The oxidative assimilation of sugars and related substances by *Pseudomonas saccharophila*. Enzymologia. **9**: 59–72.

EATON, N. R. & H. P. KLEIN. 1957. Studies on the aerobic degradation of glucose by *Saccharomyces cerevisiae*. Biochem. J. **67**: 373–381.

EATON, N. R. 1960. Endogenous respiration of yeast. I. The endogenous substrate. Arch. Biochem. Biophys. **88**: 17–25.

FUERST, R. & R. P. WAGNER. 1957. Analysis of the free intracellular amino acids of certain strains of *Neurospora*. Arch. Biochem. Biophys. **70**: 311–326.

GEIGER, A., Y. KAWAKITA & S. S. BARKULIS. 1960. Major pathways of glucose utilization in the brain in brain perfusion experiments *in vivo* and *in situ*. J. Neurochem. **5**: 323–338.

GOLDSCHMIDT, E. P. 1952. The oxidative metabolism of acetate by *Penicillium chrysogenum* Q-176. Ph.D. Thesis. Purdue University. Lafayette, Ind.

GOULD, B. S. & A. A. TYTELL. 1941. Studies in the physiology of Fusaria. The respiratory and fermentative mechanisms. J. Gen. Physiol. **24**: 655–667.

GRONLUND, A. F. & J. J. R. CAMPBELL. 1961. Nitrogenous compounds as substrates for endogenous respiration in microorganisms. J. Bacteriol. **81**: 721–724.

HALVORSON, H., W. FRY & D. SCHWEMMIN. 1955. A study of the properties of the free amino acid pool and enzyme synthesis in yeast. J. Gen. Physiol. **38**: 549–573.

HEPLAR, J. Q. & E. L. TATUM. 1954. Some factors affecting carbon dioxide metabolism in *Neurospora crassa*. J. Biol. Chem. **208**: 489-494.

HOCKENHULL, D. J. D., M. HERBERT, A. D. WALKER, G. D. WILKIN & F. G. WINDER. 1954. Organic acid metabolism of *Penicillium chrysogenum*. 1. Lactate and acetate. Biochem. J. **56**: 73–82.

IBSEN, K. H. 1961. The Crabtree effect: a review. Cancer Research. **21**: 829–841.

KATZ, J., S. ABRAHAM & N. BAKER. 1954. Analytical procedures using a combined combustion diffusion vessel. Improved method for combustion of organic compounds in aqueous solution. Anal. Chem. **26**: 1503–1504.

LAMANNA, C. & M. F. MALLETTE. 1959. Basic Bacteriology, 2nd Edition. The Williams and Wilkins Co. Baltimore, Md. Pp. 612–624.

MACMILLAN, A. 1956. The relation between nitrogen assimilation and respiration in *Scopulariopsis brevicaulis*. Physiol. Plantarum. **9**: 533–545.

MATCHETT, W. H. & J. A. DEMOSS. 1962. Factors affecting increased production of tryptophan synthetase by a TD mutant of *Neurospora crassa*. J. Bacteriol. **83**: 1294–1300.

MEISTER, A., H. A. SOBER & S. V. TICE. 1951. Determination of aspartic and glutamic acids by enzymatic decarboxylation. J. Biol. Chem. **189**: 591–595.

MIDWINTER, G. G. & R. D. BATT. 1960. Endogenous respiration and oxidative assimilation in *Nocardia corallina*. J. Bacteriol. **79**: 9–17.

MOSES, V. 1958. (^{14}C)-Glucose metabolism in fungal cells. J. Gen. Microbiol. **20**: 184–196.

MOSES, V. & P. J. SYRETT. 1955. The endogenous respiration of microorganisms. J. Bacteriol. **70**: 201–204.

OLSON, J. A. 1959. The purification and properties of yeast isocitric lyase. J. Biol. Chem. **234**: 5–10.

QUASTEL, J. H. & M. D. WHETHAM. 1924. The equilibria existing between succinic, fumaric and malic acids in the presence of resting bacteria. Biochem. J. **18**: 519–534.

RAMSEY, H. H. 1962. Endogenous respiration of *Staphylococcus aureus*. J. Bacteriol. **83**: 507–514.

REED, D. J. & C. H. WANG. 1959. Glucose metabolism in *Penicillium digitatum*. Can. J. Microbiol. **5**: 59–66.

REINER, J. M., H. GEST & M. D. KAMEN. 1949. The effect of substrates on the endogenous metabolism of living yeast. Arch. Biochem. **20**: 175–177.

RIBBONS, D. W. & E. A. DAWES. 1963. Environmental and growth conditions affecting the endogenous metabolism of bacteria. Ann. N. Y. Acad. Sci. **102**(3): 564–585.

SCOTT, T. W., I. G. WHITE & E. F. ANNISON. 1962. Glucose and acetate metabolism by ram, bull, dog and fowl spermatozoa .Biochem. J. **82**: 398–404.

STONE, D. 1962. The formation and utilization of acetate in an *acetateless* strain of *Neurospora crassa*. Arch. Biochem. Biophys. **97**: 199–207.

STOUT, H. A. & H. KOFFLER. 1951. Biochemistry of filamentous fungi. I. Oxidative metabolism of glucose by *Penicillium chrysogenum*. J. Bacteriol. **62**: 253–268.

SUSSMAN, A. S. 1961. The role of endogenous substrates in the dormancy of *Neurospora* ascospores. *In* Spores II. Burgess Publishing Co. Minneapolis, Minn. Pp. 198–213.

SYRETT, P. J. 1951. The effect of cyanide on the respiration and oxidative assimilation of glucose by *Chlorella vulgaris*. Ann. Botany (London) **15**: 473–492.

TURIAN, G. 1961. Cycle glyoxylique, transaminase-glyoxylate et différenciation sexuelle chez *Allomyces* et *Neurospora*. Pathol. Microbiol. **24**: 819–839.

WADDEL, W. J. 1956. A simple ultraviolet spectrophotometric method for the determination of protein.. J. Lab. Clin. Med. **48**: 311–314.

WANG, C. H., J. J. BIALY, S. KLUNGSOVYR & C. M. GILMOUR. 1958. Studies on the biochemistry of Streptomyces. III. Glucose catabolism in *Streptomyces griseus*. J. Bacteriol. **75**: 31–37.

WIAME, J. M. & M. DOUDOROFF. 1951. Oxidative assimilation by *Pseudomonas saccharophila* with C^{14}-labeled substrates. J. Bacteriol. **62**: 187–193.