Noda, M., Yoon, K., & Rodan, G. A. (1988) *J. Bone Miner. Res. 3 (Suppl. 1)*, S144.
Okazaki, T., Igarashi, T., & Kronenberg, H. M. (1988) *J. Biol. Chem. 263*, 2203-2208.
Otawara, Y., & Price, P. A. (1986) *J. Biol. Chem. 261*, 10828-10832.
Pan, L. C., & Price, P. A. (1984) *J. Biol. Chem. 259*, 5844-5847.
Pan, L. C., & Price, P. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6109-6113.
Partridge, N. C., Frampton, R. J., Eisman, V. P., Michelangeli, V. P., Elms, E., Bradley, T. R., & Martin, T. J. (1980) *FEBS Lett. 115*, 139-142.
Partridge, N. C., Alcorn, D., Michelangeli, V. P., Kemp, B. E., Ryan, G. B., & Martin, T. J. (1981) *Endocrinology (Baltimore) 108*, 213-219.
Perret, C., Lomri, N., Gouhier, N., Auffray, C., & Thomasser, M. (1988) *Eur. J. Biochem. 171*, 43-51.
Price, P. A. (1983) in *Bone and Mineral Research* (Peck, W. A., Ed.) Vol. 1, pp 157-190, Excerpta Medica, Amsterdam.
Price, P. A., & Baukol, S. A. (1980) *J. Biol. Chem. 255*, 11660-11665.
Sanger, F. S., Nicklen, A., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74* 5463-5467.
Simpson, R. U., Hsu, T., Beagley, D. A., Mitchell, B. S., & Alizadeh, S. N. (1987) *J. Biol. Chem. 262*, 4104-4108.
Spector, T. (1978) *Anal. Biochem. 58*, 541-548.
Spiess, Y. H., Price, P. A., Deftos, J. L., & Manolagas, S. C. (1986) *Endocrinology (Baltimore) 118*, 1340-1346.
Theofan, G., & Price, P. A. (1988) *J. Bone Miner. Res. 3 (Suppl. 1)*, S206.
Tsang, A. S., Mahbubani, H., & Williams, J. G. (1982) *Cell (Cambridge, Mass.) 31*, 375-380.
Weiher, H., Konig, M., & Gruss, P. (1983) *Science (Washington, D.C.) 219*, 626-631.
Yoon, K., Buenaga, R. F., & Rodan, G. A. (1987) *Biochem. Biophys. Res. Commun. 148*, 1129-1136.
Yoon, K., Thiede, M. A., & Rodan, G. A. (1988) *Gene 66*, 11-17.

Articles

Metabolism of D-Glucose in a Wall-less Mutant of *Neurospora crassa* Examined by ^{13}C and ^{31}P Nuclear Magnetic Resonances: Effects of Insulin†

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ABSTRACT: ^{13}C NMR and ^{31}P NMR have been used to investigate the metabolism of glucose by a wall-less strain of *Neurospora crassa* (slime), grown in a supplemented nutritionally defined medium and harvested in the early stationary stage of growth. With D-[1-^{13}C]- or D-[6-^{13}C]glucose as substrates, the major metabolic products identified from ^{13}C NMR spectra were [2-^{13}C]ethanol, [3-^{13}C]alanine, and C_1- and C_6-labeled trehalose. Several observations suggested the existence of a substantial hexose monophosphate (HMP) shunt: (i) a 70% greater yield of ethanol from C_6- than from C_1-labeled glucose; (ii) C_1-labeled glucose yielded 19% C_6-labeled trehalose, while C_6-labeled glucose yielded only 4% C_1-labeled trehalose; (iii) a substantial transfer of ^{13}C from C_2-labeled glucose to the C_2-position of ethanol. ^{31}P NMR spectra showed millimolar levels of intracellular inorganic phosphate (P_i), phosphodiesters, and diphosphates including sugar diphosphates and polyphosphate. Addition of glucose resulted in a decrease in cytoplasmic P_i and an increase in sugar monophosphates, which continued for at least 30 min. Phosphate resonances corresponding to metabolic intermediates of both the glycolytic and HMP pathways were identified in cell extracts. Addition of insulin (100 nM) with the glucose had the following effects relative to glucose alone: (i) a 24% increase ($P < 0.01$) in the rate of ethanol production; (ii) a 38% increase ($P < 0.05$) in the rate of alanine production; (iii) a 27% increase ($P < 0.05$) in the rate of glucose disappearance. Insulin thus increases the rates of production of ethanol and alanine in these cells, in addition to increasing production of CO_2 and glycogen, as previously shown.

Recently, the wall-less "slime" mutant of *Neurospora crassa* has been shown to respond to mammalian insulins. McKenzie et al. (1988) demonstrated that addition of modest concentrations (10-100 nM) of bovine, porcine, or human insulin to these cells, under defined conditions, resulted in growth stimulation and prolonged viability in a dose-dependent fashion. Treatment with insulin for as little as 30 min led to a 20% increase in the rate of carbon dioxide production from D-glucose (McKenzie et al., 1988), increased the cellular glycogen content, increased the rate of the enzyme glycogen synthesis, and caused specific activation of glycogen synthetase

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(Fawell et al., 1988). In light of these findings, a more extensive characterization of glucose metabolism in these cells was obviously of interest. The wall-less mutant has several properties that make it extremely useful in the study of fungal biochemistry. Plasma membranes (Scarborough, 1975) and vacuoles (Martinoia et al., 1979) are easily isolated from this organism in high yields without the problems associated with removal of the cell wall. The isolation of subcellular particles and enzymes from the mutant is also a great deal easier than from wild-type wall-bearing strains.

Nuclear magnetic resonance (NMR) is an excellent method for obtaining an overview of total metabolism in living organisms. The utility of NMR for metabolic studies has recently been reviewed (Avison et al., 1986). In this investigation, ^{13}C NMR has been employed to determine the end products of glucose oxidation and their in vivo rates of formation, and to estimate the steady-state concentration of several intermediates. In addition, ^{31}P NMR has been utilized to examine the phosphorylated metabolites in the cells. Acute and long-term effects of insulin on the metabolism of the slime variant have also been investigated. Several effects of mammalian insulin on glucose metabolism in *N. crassa* cells have been documented, in addition to those reported previously (McKenzie et al., 1988; Fawell et al., 1988).

Materials and Methods

Materials. The mono- and diphosphates used as standards were all obtained from Sigma Biochemicals (St. Louis, MO) as were bovine insulin (crystalline, 0.5% Zn, approximately 25 IU/mg), sorbitol, and the buffer Bis-Tris (Sigma catalogue no. B9754). Methylene diphosphonate was obtained from Aldrich (Milwaukee, WI). D_2O was obtained from Thompson–Packard (Little Falls, NJ). D-[1-^{13}C]Glucose (99.5 atom % ^{13}C), D-[2-^{13}C]glucose (91.7%), and D-[6-^{13}C]glucose (99.2%) were all obtained from MSD Isotopes (St. Louis, MO) and used without further purification. All other materials were reagent grade.

Cell Culture. Stock cultures of *N. crassa*, strain FGSC 4761 (*fz;sg;os-1*(B135),A;V), were generously supplied by G. Scarborough (The University of North Carolina, Chapen Hill, NC) and were maintained routinely as previously described (McKenzie et al., 1988). For NMR experiments, cells (10^8) were inoculated into 50 mL of a supplemented defined medium (SDM)[1] and grown for 18 h as described (McKenzie et al., 1988), either without (control grown) or with (insulin grown) 100 nM added insulin. Total cell protein was determined by the method of Bradford (1976) using BSA as a standard.

NMR Measurements. ^{31}P NMR spectra were collected as described previously (Fawell et al., 1988). ^{13}C NMR measurements were collected on a Bruker WP-200 SY spectrometer operating in the pulse Fourier-transform mode at 50.3 MHz at 23 °C. The spectral width was 12 500 Hz; the pulse width was 10 μs with a 0.33-s cycle time. The pulse delay was 2 s. The field was locked on the deuterium resonance of D_2O, and chemical shifts were referenced to external tetramethylsilane (ppm = 0). To improve the signal to noise ratio in the spectra of the intact cells, broad-band noise decoupling of protons was employed, and an exponential multiplication of the free induction decay was performed with line broadenings of 3 Hz. No line broadening was used when the spectra of perchloric acid extracts of the cells were obtained.

Preparation of Samples for NMR Measurements. Cells were pooled and allowed to remain in their growth medium at 23 °C without shaking until the NMR experiments could be completed (1–5 h). Under these conditions, the cells did not continue to grow but maintained 90% viability for up to 12 h (McKenzie et al., 1988).

Cells were collected and resuspended in their conditioned medium, or in NS containing normal (37 mM) or reduced (0.075 mM) concentrations of phosphate, as indicated. Cells were oxygenated with a mixture of 95% O_2 + 5% CO_2 delivered to the bottom of the sample via tygon tubing at a rate of one bubble (2-mm diameter) per second, which was sufficient to prevent settling of the cells. Basal ^{31}P NMR spectra were collected for 20 min (360 scans, with a 2-s delay between pulses). Following the addition of D-glucose (100 mM), ^{31}P NMR spectra were collected for 10 periods of 3 min each (32 scans with a 4-s delay between pulses). For the ^{13}C NMR measurements, cells were administered ^{13}C-labeled D-glucose (50 mM). Spectra were collected for 10 periods of 6 min each (152 scans) starting 1 min after the addition of glucose.

The ^{31}P NMR measurements were made on 2-mL samples containing 55 mg/mL protein. The ^{13}C NMR measurements were determined on 2-mL samples containing 20–70 mg/mL protein. The intracellular pH value of the intact cells was estimated from the chemical shift of the cytoplasmic P_i resonance.

Cells remained viable throughout the NMR measurements as determined by exclusion of trypan blue, and by the ability of the cells to reinitiate vigorous growth when inoculated into fresh medium. The cells also consumed D-glucose at a rate comparable to that occurring at the lower cell densities characteristic of normal culture.

Preparation of Cell Extracts. For measurements of ^{31}P NMR spectra, cell extracts were prepared by two methods. (i) Cells were collected by centrifugation immediately after removal from the shaker, resuspended and incubated for 10–20 min in NS containing P_i (0.075 mM) plus or minus D-glucose (100 mM), and then lysed by rapid addition of perchloric acid to a final concentration of 7%. The extracts were neutralized to pH 6.5 with Bis-Tris within 15 s. (ii) The cells were pelleted in a clinical centrifuge for 1 min, resuspended in boiling distilled water to lyse the cells, and then boiled for 5 min to stop metabolism. To assign the resonances, the lysates were spiked with known standards, and their chemical shifts were determined as a function of pH. The positions of the phosphate resonances (including those in the monophosphate region) from the spectra of boiled lysates closely matched those of intact cells.

In order to study the effects of insulin on the metabolism of D-[^{13}C]glucose, cells were harvested in the early stationary stage of growth at 18 h and washed and resuspended in NS to a final concentration of approximately 25 mg/mL in a 4-mL volume. The cells were administered D-glucose, 100 μmol, and incubated at 30 °C with gyrotatory shaking in the presence or absence of insulin, 100 nM. The cells were collected by centrifugation for 2 min in a clinical centrifuge, and both the cells and supernatants were treated with perchloric acid to a final concentration of 7% and neutralized with an equimolar concentration of K_2HPO_4.

Resonances in the ^{13}C NMR spectra with intensities at least 2-fold above the noise level were identified by using computer

[1] Abbreviations: SDM, nutrient-defined supplemented medium (McKenzie et al., 1988); NS, salts of Vogel's minimal medium (KH_2PO_4, 37 mM; sodium citrate, 8.4 mM; NH_4NO_3, 24 mM; $MgSO_4$, 0.8 mM; and $CaCl_2$, 0.7 mM) (Vogel, 1956) containing sorbitol, 0.33 M; SeM, minimal medium supplemented with yeast extract and protein hydrolysates (Schulte & Scarborough, 1975); HMP, hexose monophosphate shunt pathway; P_i, inorganic orthophosphate; UDPG, uridinediphosphoglucose; UDPNAG, uridinediphospho-*N*-acetylglucosamine.

programs supplied by Bruker. The resonances were assigned to given metabolites if their chemical shifts were within 0.05 ppm of those predicted from the titration curves of the standards in the boiled extracts.

RESULTS

^{13}C NMR Spectra

Basal Natural-Abundance ^{13}C NMR Spectra. The ^{13}C NMR spectra of intact cells suspended in NS without added glucose ("basal" spectra) displayed low, but reproducible, characteristic resonances which arose from the ^{13}C present at natural abundance in compounds in the cells. The major resonances in the natural-abundance spectra corresponded to those of the fatty acids of triglycerides, suggesting that triglycerides are a major storage form of nutrients in the slime mutant of *N. crassa*, harvested at the early stationary stage of growth. There were prominent resonances at 129.5 and 127.9 ppm, corresponding to $-CH=$ groups, at 33.6, 32.0, 29.8, 29.3, 27.5, and 24.8 ppm, corresponding to various $-CH_2-$ groups, and at 22.6 and 13.9 ppm, corresponding to the $-CH_3$ groups. [The assignments are from Cohen (1987b).] The resonances of highest intensity corresponded to those of the fatty acyl $-CH_2-$ groups at 29.8 and 29.3 ppm, which were estimated to correspond to an intracellular concentration of approximately 2 μmol/mg of protein. In contrast, there were no observable resonances corresponding to glycogen.

Metabolic Products of D-*Glucose.* Spectra from perchloric acid extracts derived from cells treated with D-[1-^{13}C]- and D-[6-^{13}C]glucose in parallel incubations (30 °C for 30 min) are shown in Figure 1. The substrate was entirely consumed during the incubation period as measured by the loss of the peaks arising from 1-^{13}C-labeled α- and β-D-glucose. The major resonances in both spectra correspond to [2-^{13}C]ethanol, [3-^{13}C]alanine, and trehalose labeled at both C_1 and C_6. In the absence of insulin, the yields of ethanol and alanine from D-[1-^{13}C]glucose were approximately 22% and 6%, respectively, compared to 40% and 10% from D-[6-^{13}C]glucose. With D-[1-^{13}C]glucose as substrate, ca. 19% of the label appeared at the C_6-position of trehalose. In contrast, when D-[6-^{13}C]-glucose was used as substrate, only 4% of the total trehalose label appeared at the C_1-position.

A small resonance appeared at 63.3 ppm using either substrate. This corresponds to the resonance from both the C_1 and C_6 carbons of mannitol, which is a major metabolic product of fungi (Dijkema et al., 1985). When D-[1-^{13}C]-glucose was utilized as substrate, but not when D-[6-^{13}C]-glucose was used, a peak appeared at 178.5 ppm, corresponding to the carboxyl (C_1) group of gluconate and 6-phosphogluconate. When D-[6-^{13}C]glucose was the substrate, the cell extracts exhibited a resonance at 65.4 ppm corresponding to 6-phospho[6-^{13}C]gluconate. In addition to the resonances arising from D-glucose, the spectra showed six resonances arising from the natural abundance of ^{13}C in sorbitol (0.33 M) present in the incubation medium. Note that any resonances corresponding to [6-^{13}C]gluconate would not be observed because the chemical shift of the C_6 nucleus at 62.7 ppm overlaps the chemical shifts of the large sorbitol resonances at 62.6 and 62.9 ppm.

When cell supernatants were examined, resonances were found corresponding to [2-^{13}C]ethanol, and a very small amount of [1-^{13}C]ethanol. In addition, the supernatants of the cells incubated with D-[1-^{13}C]glucose exhibited a small [1-^{13}C]gluconate resonance at 178.5 ppm. The cell supernatants from the incubations with D-[6-^{13}C]glucose showed no peak corresponding to 6-phospho[6-^{13}C]gluconate.

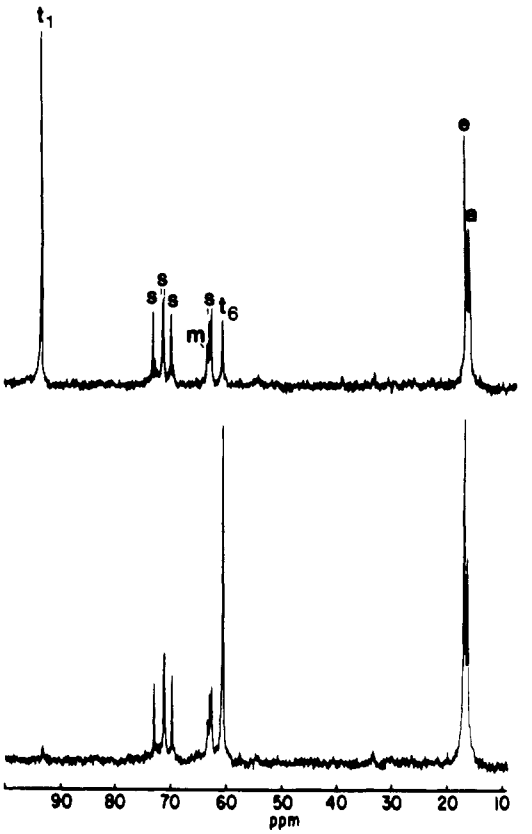

FIGURE 1: ^{13}C NMR spectra of perchloric acid extracts from cells incubated with D-[1-^{13}C]glucose (top) or D-[6-^{13}C]glucose (bottom), 50 mM, for 30 min at 30 °C with gyrotatory shaking in NS. The cells were pelleted in a clinical centrifuge for 3 min, and perchloric extracts of the cells were prepared. The spectra were accumulated for 1 h, 1520 FIDs. Abbreviations: t_1, [1-^{13}C]trehalose; t_6, [6-^{13}C]trehalose; e, [2-^{13}C]ethanol; a, [3-^{13}C]alanine; s, sorbitol; m, [1-^{13}C]- and [6-^{13}C]mannitol.

The total yield of measurable products arising from D-[6-^{13}C]glucose was much higher than that arising from D-[1-^{13}C]glucose. The ratios of recoveries of label (D-[6-^{13}C]-glucose/D-[1-^{13}C]glucose) in ethanol, alanine, and trehalose were 1.7 ± 0.1, 1.4 ± 0.2, and 1.1 ± 0.1, respectively, from cells incubated in the absence of insulin. The ratio of unrandomized label in trehalose (D-[6-^{13}C]glucose → [6-^{13}C]-trehalose/D-[1-^{13}C]glucose → [1-^{13}C]trehalose) was 1.3 ± 0.2.

When D-[2-^{13}C]glucose was employed as substrate, the major products were [1-^{13}C]ethanol, [2-^{13}C]alanine, and [2-^{13}C]trehalose. In addition, some of the label was rearranged to give small amounts of [2-^{13}C]ethanol and [3-^{13}C]alanine. Approximately 7% of the total label in ethanol was rearranged during the incubation.

Effect of Insulin on the Rate of Glucose Consumption and Product Formation. Figure 2 illustrates the rates of consumption of 1-^{13}C-labeled α- and β-D-glucose and the rates of production of ethanol, alanine, and the C_1 resonance of trehalose as a function of time, without added insulin. The rates observed in the presence and absence of insulin, 100 nM, are shown in Table I. Consumption of glucose was linear for at least 30 min. The rate of production of ethanol per milligram of cell protein from D-glucose did not vary with cell density from 20 to 70 mg/mL cell protein and did not vary during the 5-h period following cell harvest. Insulin caused a significant increase in the rate of production of ethanol (24%, $P < 0.01$). There was also an increase in the rate of alanine formation (38%, $P < 0.05$). The rate of glucose consumption increased in four of the seven experiments but was unchanged in the three remaining experiments. The average insulin-in-

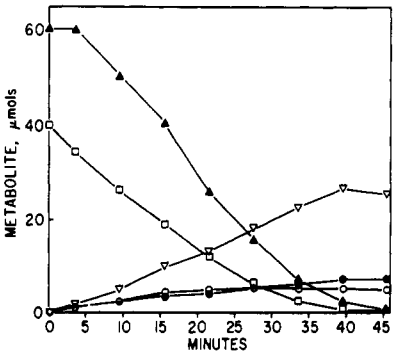

FIGURE 2: Loss of D-[1-^{13}C]glucose and formation of products as a function of time. Cells were suspended in NS and administered D-[1-^{13}C]glucose at time zero. Cells were incubated at 23 °C with oxygenation with 95% O_2 + 5% CO_2. Data were collected in 6-min periods starting 1 min after the addition of glucose. The time axis represents the middle of each collection period. The concentrations were estimated from the intensities of the ^{13}C resonances relative to an external standard of tetramethylsilane. (▲) β-D-[1-^{13}C]Glucose; (□) α-D-[1-^{13}C]glucose; (▽) [2-^{13}C]ethanol; (○) [1-^{13}C]trehalose; (●) [3-^{13}C]alanine.

Table I: Effect of Insulin on the Rate of Production of Metabolites from D-[1-^{13}C]Glucose

product or substrate	rate of appearance of loss[a] [nmol min^{-1} (mg of protein)$^{-1}$] −insulin	+insulin	significance of difference
ethanol	4.8 ± 0.4	6.0 ± 0.6	<0.01
alanine	1.3 ± 0.2	1.8 ± 0.2	<0.05
trehalose	1.6 ± 0.3	1.9 ± 0.3	NS[b]
D-glucose	−19.0 ± 1.4	−24.2 ± 1.8	<0.05

[a] The rates of loss of D-[1-^{13}C]glucose and of production of products were linear for 30 min at 23 °C. The data represent the mean and standard error of seven replicate experiments. [b] NS, not significant.

duced increase was 27% ($P < 0.05$).

Several resonances derived from D-[1-^{13}C]glucose did not change in intensity as a function of time, suggesting that they could be assigned to metabolic intermediates in steady state. These included one at 60.6–60.8 ppm, corresponding to the resonances from the C_6 carbons of D-glucose and trehalose, and the gluconate/6-phosphogluconate resonance at 178.5 ppm.

There were no significant insulin-dependent changes in the total yields of trehalose, alanine, or ethanol in the perchloric acid extracts measured after the glucose was consumed. There also was no change in the degree of randomization of the label in trehalose. The yield of the mannitol resonance at 63.3 ppm was 35% higher ($P < 0.03$, $N = 8$) in the cells treated with insulin as compared with controls. An increase in the degree of randomization of label from C_2 to C_1 in ethanol was also seen from 2.1 ± 0.5% in untreated cells to 4.2 ± 0.9% in insulin-treated cells ($P < 0.05$, $N = 10$).

No significant increases were found in the intensities of the fatty acid side chain resonances during the 60-min period following the administration of D-glucose, labeled with ^{13}C in either the C_1- or the C_6-position in either the absence or the presence of insulin.

^{31}P NMR

Basal ^{31}P NMR Spectra of Cells Grown in the Presence and Absence of Insulin. The gross features of the ^{31}P NMR spectra of cells cultured in the presence and absence of insulin were similar, although there were some insulin-dependent differences. Typical basal spectra are shown in Figure 3, and the classes of metabolites corresponding to each spectral region are identified in Table II.

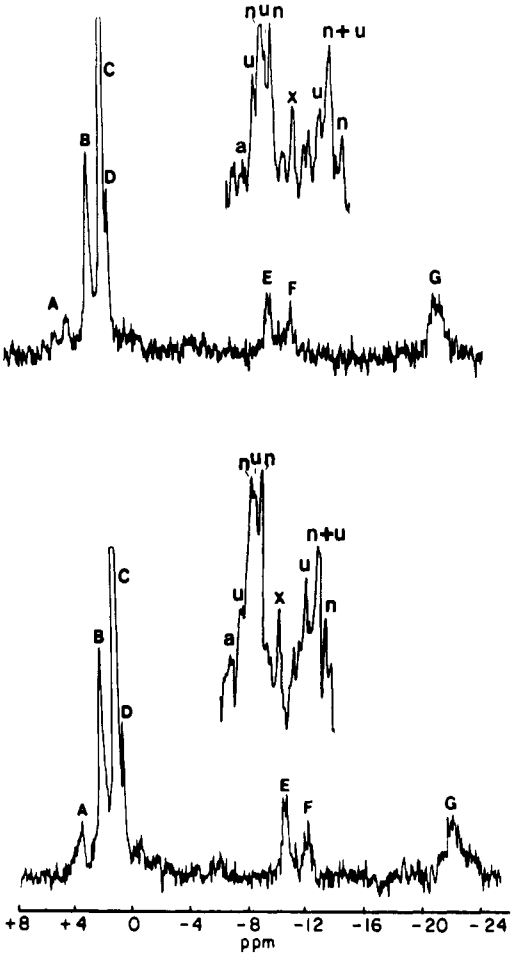

FIGURE 3: ^{31}P NMR spectra of cells grown to stationary stage in SDM without (top) or with (bottom) 100 nM bovine insulin. Spectra were collected for 20 min (32 FIDs) at 23 °C. Abbreviations: A, phosphomonoesters; B, cytoplasmic P_i; C, extracellular and vacuolar P_i and glycerophosphoethanolamine; D, glycerophosphocholine; E, sugar dinucleotides and other diphosphates; F, sugar dinucleotides; G, polyphosphate. Inserts: −10 to −13 ppm; a, ATP; u, UDPG; n, UDPNAG; x, unassigned.

Table II: Basal Levels of Phosphorylated Metabolites in Cells Grown in the Absence or Presence of Insulin

chemical shift (ppm)	metabolite class	nmol/mg of protein[a] control grown	insulin grown	significance of difference
2.5–5.0	monophosphates	204 ± 45	226 ± 24	NS[c]
1.7–2.1	P_i[b]	269 ± 35	345 ± 35	0.02
0.2–0.4	glycerophosphocholine	97 ± 11	78 ± 10	0.01
−10.0 to −13.0	diphosphates	169 ± 10	162 ± 01	NS
−20.0 to −25.0	polyphosphate	229 ± 09	250 ± 23	NS

[a] Mean and standard error of three data sets. Cells were suspended in growth medium, except for P_i measurement. [b] Measurements in NS containing 0.075 mM P_i. Data are the mean and standard error of six data sets. [c] NS, not significant.

The ^{31}P NMR spectra collected on the intact cells proved to have high resolution. All four resonances in the UDPG spectrum (at −10.6, −10.9, −12.25, and −12.5 ppm) arising from phosphate–phosphate coupling were observed (see expanded regions, Figure 3). It was also possible to resolve the four resonances of UDPNAG (at −10.8, −11.0, −12.5, and −12.75 ppm) although the peaks at −10.8 and −11.0 ppm appeared to overlap those of other compounds. The small peak at −10.48 ppm was tentatively assigned to half of the doublet arising from the α-phosphate of ATP. This peak is more prominent in spectra of perchloric acid extracts (Fawell et al.,

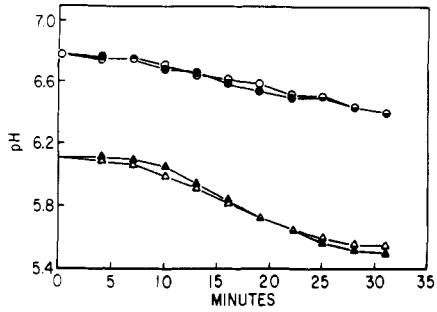

FIGURE 4: Typical changes in the cytoplasmic and extracellular pH after the addition of D-glucose. Cells were cultured in the absence of insulin and harvested in the early stationary stage of growth. They were resuspended in NS. D-Glucose, 100 mM, ± bovine insulin, 100 nM, were added at time zero, and spectra were collected in 3-min periods starting 1 min after the addition of D-glucose. The time axis portrays the end of the acquisition period. (●) Cytoplasm − insulin; (○) cytoplasm + insulin; (▲) external − insulin; (△) external + insulin.

1988) and in spectra of cells grown in medium containing reduced trace elements (unpublished results).

Cells grown in the presence of insulin had somewhat higher levels of P_i than cells cultured in its absence. In addition, the intracellular (cytoplasmic) pH of cells grown in the presence of insulin was 0.05 unit higher than cells cultured in its absence (6.84 ± 0.05 and 6.79 ± 0.05, respectively, $P = 0.05$). The vacuolar pH of cells suspended in spent medium could not be determined because of overlap of the vacuolar and the external P_i peaks. When the cells were washed and resuspended in NS containing a reduced concentration of P_i (0.075 mM) at pH 5.8, the apparent cytoplasmic pH was unchanged. A small previously buried resonance was revealed, however, which presumably corresponds to vacuolar P_i. The vacuolar pH value, determined from this peak, was 6.2 ± 0.1 in cells cultured both in the presence and in the absence of insulin.

Cells cultured in the presence of insulin displayed several other differences when compared to cells cultured in the absence of insulin. The resonance corresponding to glycerophosphocholine, a membrane catabolite, was lower in the cells cultured in the presence of insulin. The most striking difference was that the areas of the resonances assigned to UDPG were significantly higher ($P < 0.01$, $N = 15$) in the cells grown in the presence of insulin (29 ± 3 nmol/mg of protein) compared to controls (20 ± 3 nmol/mg of protein). The same average concentration of UDPG was calculated in the cells by using the proportional area of the resonance at −10.9 ppm alone, or by using the proportional area of the resonance at −12.25 ppm alone, or by adding all four of the resonances assigned to UDPG and correcting for the contribution from UDPNAG at −12.5 ppm. The ratio of UDPG to UDPNAG was also higher in insulin-grown cells. Short-term effects of insulin administration on UDPG levels in these cells have been reported previously (Fawell et al., 1988).

The monophosphate region of the basal ^{31}P NMR spectra also showed compounds associated with lipid turnover and compounds characteristic of unenergized cells. The major resonances of the basal ^{31}P NMR spectra had chemical shifts which were consistent with α-glycerophosphate [which is a further catabolite of glycerophosphocholine (Unkefer et al., (1982)], AMP, phosphocholine, and 3-phosphoglycerate. There were small insulin-dependent differences in the areas of these peaks; however, interpretation of the changes was precluded by resonance overlaps.

Acute Effects of Insulin on the Levels of Phosphorylated Metabolites following the Administration of D-*Glucose.* Upon addition of D-glucose to the cells, the intensity of the phosphomonoester resonance region rapidly increased while the cytoplasmic P_i peak decreased. The resonances due to both cytoplasmic and external P_i peaks shifted rapidly upfield, reflecting a drop in both the cytoplasmic and external pH, from 6.8 to 6.5 and from 6.1 to 5.5, respectively (Figure 4). No insulin-dependent effects on the rate of pH change were observed. The profile of the sugar monophosphate region also changed rapidly with time, reflecting changes in intracellular pH and in the levels of specific metabolites (Figure 5).

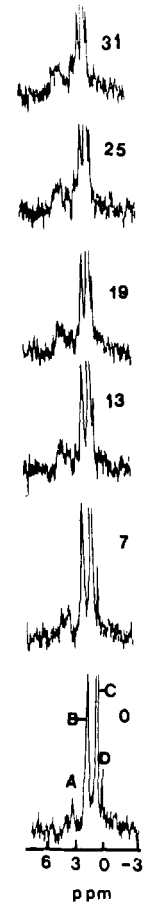

FIGURE 5: Typical changes in the monophosphate, P_i, and phosphodiester regions following the addition of D-glucose, 100 mM, to the slime variant of *N. crassa*. Cells, 55 mg of protein/mL, were suspended in NS. Spectra were collected in 3-min intervals starting 1 min after the addition of D-glucose. The times listed represent the end of each collection period. Abbreviations: A, monophosphates; B, cytoplasmic P_i; C, vacuolar and external P_i and glycerophosphoethanolamine (truncated); D, glycerophosphocholine.

A typical spectrum of the monophosphate region of an aqueous extract of *N. crassa* cells, prepared after addition of D-glucose, is shown in Figure 6. Prominent spectral resonances could be assigned to metabolites of the HMP shunt, including 6-phosphogluconate, as well as to some intermediates of the glycolytic pathway. When cells were treated with insulin before the addition of glucose, there appeared to be some insulin-dependent differences in the spectra of the monophosphate region, both in intact cells (not shown) and in extracts. Unfortunately, the extracts could not be used for determining the intracellular concentrations of the monophosphates because of variable losses of several metabolites during the preparation of the extracts.

DISCUSSION

General Considerations. The NMR studies reported herein had several objectives: (i) to characterize the overall metabolism of the *N. crassa* slime variant when harvested at the early

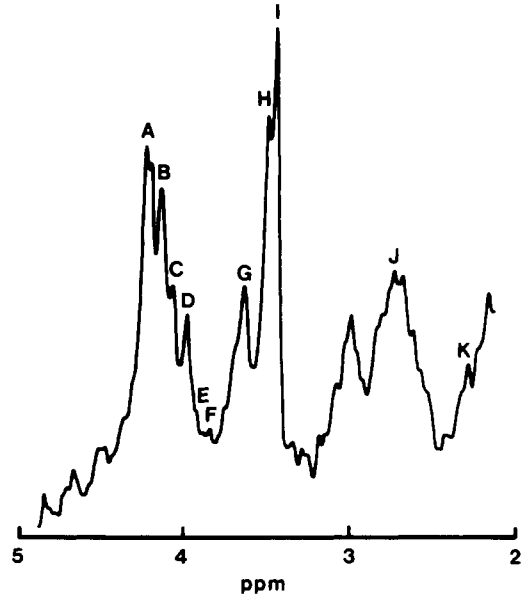

FIGURE 6: Phosphomonoester region from the ^{31}P NMR spectra of an aqueous boiled extract of the slime variant of *N. crassa* which was administered D-glucose. Cells were resuspended in NS containing reduced phosphate, 0.075 mM, and sorbitol, 90 g/L. D-Glucose, 100 mM, was added at time zero, and the cells were incubated with aeration for a total of 16 min. The cells were collected, lysed with boiling water, and boiled to stop metabolism. The extract was adjusted to pH 7.0. Abbreviations: A, α- and β-glucose 6-phosphate and 6-phosphogluconate; B, ribulose 5-phosphate and xylulose 5-phosphate; C, glyceraldehyde 3-phosphate and α-glycerol phosphate; D, phosphoethanolamine; E, dihydroxyacetone phosphate (keto form); F, fructose 1,6-diphosphate; G, ribose 5-phosphate, fructose 6-phosphate, and β-glycerol phosphate; H, 5'AMP, fructose 1,6-diphosphate, and 3-phosphoglycerate; I, phosphocholine; J, anomeric sugar phosphates; K, glucose 1-phosphate.

stationary stage of growth; (ii) to determine whether growth in the presence of insulin had any detectable effects on the basal metabolites present; (iii) to measure any acute effects of insulin addition on D-glucose metabolism by the cells.

Although NMR studies provide an excellent means of gaining an overview of metabolism in intact cells, spectra must be obtained at high cell densities. In these studies, the cells remained viable throughout the NMR measurements and consumed D-glucose at a similar rate to cells in more dilute suspension.

The intracellular pH of the resting cells as determined from the ^{31}P NMR spectra (6.84 ± 0.05) was in reasonable agreement with other reports. Sanders and Slayman (1982) used microelectrodes to measure the cytoplasmic pH of individual mycelia of wild-type *N. crassa* and obtained values of 6.85–7.62. They suggested that the variation could be a function of age of the cells.

The pH of the vacuolar peak of the slime variant, determined by ^{31}P NMR in low-phosphate media, was 6.2 ± 0.1. Legerton et al. (1983) obtained cytoplasmic and vacuolar pH values of 7.15 ± 0.10 and 6.1 ± 0.4, respectively, in exponentially growing wild-type *N. crassa* cells, using ^{15}N NMR, in reasonable agreement with our results.

Effects of Insulin on the Basal Spectra. The basal ^{31}P NMR spectra, like the basal ^{13}C spectra, showed the presence of compounds associated with nutrient storage and lipid turnover. Large peaks were seen due to polyphosphate (a storage form of phosphate) and phosphodiesters, including glycerophosphocholine and glycerophosphoethanolamine. The glycerophosphodiesters are normally found in conidia of wild-type *N. crassa*, but not in mycelia (Ribolow & Burt, 1981; Burt & Ribolow, 1984). Glycerophosphocholine was present in significantly lower amounts in cells cultured in the presence of insulin than those cultured in its absence (Table II).

The major resonances in the diphosphonucleotide region arose from UDPG and UDPNAG. These are the most common nucleotides in wild-type *N. crassa* cells (Slayman, 1973; Smith & Wheat, 1960; Kulaev & Mel'gunov, 1967). Slayman (1973) has reported intracellular concentrations of UDPG and UDPNAG in mycelia of wild-type *N. crassa* cells, harvested during exponential growth, of 2.60 and 1.45 mM, respectively. In our studies, the concentrations of UDPG and UDPNAG in cells cultured in the absence of insulin were each 20 nmol/mg of protein. The water content in the wall-less mutant was estimated as 0.006 mL/mg of protein by assuming that the intracellular volume of the cells was approximately equal to the excluded volume as determined from the extracellular concentration of sorbitol. This led to calculated concentrations of the sugar dinucleotides of ca. 3 mM each, in reasonable agreement with Slayman's values. Somewhat lower values than either ours or Slayman's have been reported by Leal-Morales and Ruiz-Herrera (1985) for wild-type *N. crassa* and for a different strain of slime, grown under different nutrient conditions.

The cells grown in the presence of insulin had a significantly higher concentration of UDPG (29 nmol/mg of protein) and a lower concentration of UDPNAG (14 nmol/mg of protein) than cells grown in its absence. Sabina et al. (1981) have shown that levels of UDPG are higher than levels of UDPNAG in germinated cells of *N. crassa*. However, in conidia, the concentrations of UDPNAG exceed those of UDPG. The insulin-dependent differences in concentrations of sugar dinucleotides and glycerophosphodiesters suggest that insulin may be affecting the rate of development, possibly by regulating nutrient utilization.

Metabolism of D-*Glucose.* The major products of glucose metabolism are ethanol and alanine, which are end products of both the glycolytic and HMP pathways. Both the ^{31}P NMR and ^{13}C NMR studies provide evidence that *N. crassa* cells utilize both pathways. ^{31}P NMR spectra of the cell extracts displayed prominent resonances arising from metabolites of the HMP shunt, including 6-phosphogluconate, ribulose, and xylulose 5-phosphate (which overlapped the spectrum of glyceraldehyde 3-phosphate) and ribose 5-phosphate (Figure 6).

The ^{13}C NMR spectra of the cells and media of the fungi showed the presence of the ^{13}C-labeled carboxylate resonance of gluconate and 6-phosphogluconate when D-[1-^{13}C]glucose was used as the substrate. Since fungi have high levels of glucose oxidase (Blumental, 1965), and the compound appeared in the medium, its resonance probably corresponds to the unphosphorylated sugar. Gluconate is phosphorylated by fungi and enters the HMP pathway before it is further metabolized (Blumental, 1965). The HMP pathway normally appears in wild-type *N. crassa* when nutrients are depleted and the cells begin to conidiate (Turian, 1975; Garraway & Evans, 1984), and thus the appearance of shunt metabolites in stationary-stage cells is not surprising. Cha and Fawell have confirmed the presence of an active shunt by measuring a much higher (ca. 8-fold) yield of carbon dioxide from D-[1-^{14}C]glucose than from D-[6-^{14}C]glucose under normal culture conditions (personal communication). CO_2 is lost from the C_1-position of glucose in the first step (6-phosphogluconate → ribulose 5-phosphate) of the pathway.

When D-[6-^{13}C]glucose was incubated with the cells, 70% of the label was recovered in spectrally visible products, without correction for any possible losses due to nuclear Overhauser effects. Cha and Fawell (personal communication) found that

20% of radiolabeled D-[6-^{14}C]glucose was incorporated into TCA-precipitable material by the cells. If a similar amount of NMR-invisible ^{13}C-labeled protein is assumed, then almost all of the D-[6-^{13}C]glucose label is accounted for. On the other hand, only 35% of the original label from D-[1-^{13}C]glucose was recovered in NMR-visible products. This result is consistent with the finding that as much as 35% of the C_1 label is lost as carbon dioxide in the HMP pathway.

Another indication of the presence of the HMP shunt in the cells comes from an examination of the rearrangement of label from C_1 of glucose into C_6 of trehalose and from C_6 of glucose into C_1 of trehalose. When scrambling occurs at the level of fructose 1,6-diphosphate, from the action of aldolase, triosephosphate isomerase, and fructose-1,6-bisphosphatase, this implies the existence of futile cycling (den Hollander et al., 1986). Scrambling by futile cycling will result in equal conversion of C_6 to C_1 and of C_1 to C_6. On the other hand, if scrambling occurs by equilibration of glyceraldehyde 3-phosphate with the aldols produced by the HMP pathway via the transketolase and transaldolase reactions, then C_1 of glucose will appear at C_6, but C_6 cannot migrate to C_1. Conversion of C_1-labeled D-glucose to C_6-labeled trehalose was approximately 19% while the reverse conversion was only 4%. This finding suggests that there is little futile cycling through fructose 1,6-diphosphate in the *N. crassa* cells but provides additional evidence of a substantial HMP pathway.

A long-standing problem in metabolic studies is the determination of the amount of D-glucose that actually proceeds though the HMP pathway. Several equations have been formulated to calculate this value by utilizing the specific yields of glycogen, carbon dioxide, and trioses from C_1- and C_6-labeled D-glucose (Katz & Wood, 1963) and by utilizing the randomization of label from D-[2-^{13}C]glucose to the C_1- and C_3-positions (Wood et al., 1963). Unfortunately, these methods of estimating the proportion of the HMP pathway gave variable values, ranging from 7% based on the randomization of label in ethanol from D-[2-^{13}C]glucose to 40% based on the specific yields of glycogen from D-[1-^{14}C]- and D-[6-^{14}C]glucose (Cha and Fawell, unpublished results).

In summary, when the *N. crassa* slime mutant was grown in submerged culture and harvested at the early stationary stage of growth, ca. 7–40% of added labeled glucose was converted to gluconate and the C_1 label subsequently lost as CO_2. In addition to the glycolytic pathways, 7–10% of the glucose was converted to trehalose and 0.5% to glycogen (Fawell et al., 1988). Very little of the added glucose appeared to be oxidized through the citric acid cycle as long as glucose was present in the medium. Moreover, very little was converted to storage triglycerides in the first hour following the addition of D-glucose to stationary-stage cells.

Acute Effects of Insulin on the Metabolism of D-*Glucose.* The administration of insulin to the *N. crassa* cells before the addition of D-glucose resulted in a significant ($P < 0.01$) 25% increase in the rate of ethanol production from D-[1-^{13}C]-glucose. This increase might have been expected, since previously McKenzie et al. (1988) demonstrated that the acute administration of insulin caused a 20% increase in the rate of CO_2 production from uniformly labeled D-[^{14}C]glucose. Since the bulk of the carbon dioxide derives from the C_3- and C_4-positions when ethanol is produced, the concomitant increase in the rate of ethanol production is consistent with the increase in CO_2 production.

The effect of insulin on trehalose production in these cells could not be measured, since production and consumption were apparently occurring simultaneously. Trehalose was produced rapidly when glucose was added; however, its level dropped as the glucose was consumed and the stored trehalose was further metabolized.

Addition of insulin to the cells resulted in a small but significant increase in the randomization of the label in ethanol, produced from either D-[6-^{13}C]- or D-[1-^{13}C]glucose, from the expected C_2-position of ethanol to C_1. The randomization of label may occur when pyruvate formed from the trioses is carboxylated in the first steps of gluconeogenesis. Cohen (1987a) demonstrated that the administration of insulin to diabetic rats caused an analogous randomization of label from the C_3-position in alanine to C_2. She attributed the increase in randomization to an insulin-dependent increase in the flux of substrate through pyruvate kinase.

There were no insulin-dependent changes in the level of gluconate in the cells, no changes in the degree of randomization from C_1 of glucose into C_6 of trehalose, and no changes in the degree of incorporation of label from D-[2-^{13}C]glucose into the C_2-position of ethanol. These results suggest that insulin had little effect on the HMP shunt in *N. crassa*.

The effect of insulin on intracellular UDPG levels, as determined by ^{31}P NMR, has previously been reported (Fawell et al., 1988). The ^{31}P NMR spectra of the intact cells, collected in 3-min time intervals at 81 MHz, proved to have surprisingly high resolution. For example, although the signal to noise ratios were low, ca. 2, all four resonances assigned to UDPG were resolved. Thus, it was possible to determine the effects of insulin on the concentration of UDPG within the intact cells following the addition of glucose (Fawell et al., 1988). In the present study, a similar attempt was made to analyze the effects of insulin on the monophosphate region in the intact cells following the addition of D-glucose (Figure 5). The signal to noise ratio was very low, and the region contained many overlapping peaks, preventing a convincing quantitative analysis of concentrations of the phosphorylated metabolites. However, the major resonances in this spectral region were quite reproducible and consistently appeared in positions corresponding to those expected from HMP and glycolytic pathway intermediates. We suggest that the information content of the monophosphate region in the 81-MHz spectra of these cells is higher than is commonly encountered and might be useful for quantitatively following the intracellular concentrations of these metabolites.

The ^{13}C NMR results suggest that insulin may modulate enzymes in the glycolytic pathway of *N. crassa*. A direct effect of insulin on the enzyme glycogen synthetase has previously been shown (Fawell et al., 1988). Glycolytic enzymes that might be modified by insulin include phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase, and pyruvate kinase. These enzymes are all modified by the action of insulin in mammalian tissues (Pilkis et al., 1986; Goldfine, 1981; Krahl, 1974).

A protein closely resembling mammalian insulin has previously been detected in wild-type *N. crassa* (LeRoith et al., 1980). This protein has been shown to have physical properties very similar to insulin, to cross-react with insulin in radioimmunoassays, and to have insulin-like activity in bioassays (LeRoith et al., 1980, 1981, 1985). Saturable binding of insulin by these cells has been demonstrated (McKenzie et al., 1988; Fawell & Lenard, 1988). There have also been reports that insulin inhibited the activity of adenylate cyclase when added to isolated plasma membranes of *N. crassa* (Flawia & Torres, 1973a,b). The present NMR study of glucose metabolism provides an additional indication that the role of insulin-like molecules in regulating metabolism is very ancient

and predates the evolution of specific endocrine glands.

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Registry No. Glucose, 50-99-7; ethanol, 64-17-5; alanine, 56-41-7; trehalose, 99-20-7; insulin, 9004-10-8.

REFERENCES

Avison, M. J., Hetherington, H. P., & Shulman, R. G. (1986) *Annu. Rev. Biophys. Bioeng. 15*, 377–402.

Blumental, H. J. (1965) in *The Fungi, an Advanced Treatise* (Ainsworth, G. C., & Sussman, A. S., Eds.) pp 229–268, Academic, New York.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Burt, C. T., & Ribolow, H. J. (1984) *Biochem. Med. 31*, 21–30.

Campbell, I. D., Jones, R. B., Kiener, P. A., & Waley, S. G. (1979) *Biochem. J. 179*, 607–621.

Cohen, P., Parker, P. J., & Woodgett, J. R. (1985) in *The Molecular Basis of Insulin Action* (Czech, M. P., Ed.) pp 213–233, Plenum, New York.

Cohen, S. M. (1987a) *Biochemistry 26*, 573–580.

Cohen, S. M. (1987b) *Biochemistry 26*, 581–589.

den Hollander, J. A., Ugurbil, K., Brown, T. R., & Shulman, R. G. (1981) *Biochemistry 20*, 5871–5880.

den Hollander, J. A., Ugurbil, K., & Shulman, R. G. (1986) *Biochemistry 25*, 212–219.

Dijkema, C., Kester, H. C. M., & Visser, J. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 14–18.

Fawell, S. E., & Lenard, J. (1988) *Biochem. Biophys. Res. Commun. 155*, 59–65.

Fawell, S. E., McKenzie, M. A., Cha, M., Greenfield, N. J., Adebodun, F., Jordan, F., & Lenard, J. (1988) *Endocrinology (Baltimore) 122*, 518–523.

Flawia, M. M., & Torres, H. N. (1973a) *J. Biol. Chem. 248*, 4517–4520.

Flawia, M. M., & Torres, H. N. (1973b) *FEBS Lett. 30*, 74–78.

Garraway, M. O., & Evans, R. C. (1984) *Fungal Nutrition and Physiology*, Wiley, New York.

Goldfine, I. D. (1981) in *Biochemical Actions of Hormones* (Litwack, G., Ed.) Vol. VIII, pp 273–305, Academic, New York.

Katz, J., & Wood, H. G. (1963) *J. Biol. Chem. 238*, 517–523.

Krahl, M. E. (1974) *Annu. Rev. Physiol. 36*, 331–360.

Kulaev, I. S., & Mel'gunov, V. I. (1967) *Biochemistry 6*, 757–767.

Leal-Morales, C. A., & Ruiz-Herrera, J. (1985) *Exp. Mycol. 9*, 28–38.

Legerton, T. L., Kanamori, K., Weiss, R. L., & Roberts, J. D. (1983) *Biochemistry 22*, 899–903.

LeRoith, D., Shiloach, J., Roth, J., & Lesniak, M. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6184–6188.

LeRoith, D., Shiloach, J., Roth, J., & Lesniak, M. A. (1981) *J. Biol. Chem. 256*, 6533–6536.

LeRoith, D., Shiloach, J., Heffron, R., Rubinovitz, C., Tanenbaum, R., & Roth, J. (1985) *Can. J. Biochem. Cell Biol. 63*, 839–849.

Martinoia, E., Heck, U., Boller, Th., Wiemken, A., & Matile, Ph. (1979) *Arch. Microbiol. 120*, 31–34.

McKenzie, M. A., Fawell, S. E., Cha, M., & Lenard, J. (1988) *Endocrinology (Baltimore) 122*, 511–517.

Pilkis, S. J., Fox, E., Wolfe, L., Rothbarth, L., Colosia, A., Stewart, H. B., & El-Maghrabi, M. R. (1986) *Ann. N.Y. Acad. Sci. 478*, 1–19.

Ribolow, H. J., & Burt, C. T. (1981) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 40*, 1632.

Sabina, R. L., Dalke, P., Hanks, A. R., MaGill, J. M., & MaGill, C. W. (1981) *Can. J. Biochem. 59*, 899–905.

Sanders, D., & Slayman, C. L. (1982) *J. Gen. Physiol. 80*, 377–402.

Scarborough, G. A. (1975) *J. Biol. Chem. 250*, 1106–1111.

Schulte, T. H., & Scarborough, G. A. (1975) *J. Bacteriol. 122*, 1076–1080.

Scott, A. I., & Baxter, R. L. (1981) *Annu. Rev. Biophys. Bioeng. 10*, 151–174.

Slayman, C. L. (1973) *J. Bacteriol. 114*, 752–766.

Slayman, C. L., & Slayman, C. W. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1935–1939.

Smith, E. J., & Wheat, R. W. (1960) *Arch. Biochem. Biophys. 86*, 267–269.

Turian, G. (1975) *Trans. Br. Mycol. Soc. 64*, 367–380.

Unkefer, C. J., Jackson, C., & Gander, J. E. (1982) *J. Biol. Chem. 257*, 2491–2497.

Vogel, H. J. (1956) *Microb. Genet. Bull. No. 13*, 42–43.

Wood, H. G., Katz, J., & Landau, B. R. (1963) *Biochem. Z. 338*, 809–847.