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Nuclear Magnetic Resonance Studies of Carbohydrate Metabolism and Substrate Cycling in *Fasciola hepatica*

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SUMMARY

We have been interested in clarifying unique features of glycolytic metabolism in parasitic trematodes and in developing improved methods for monitoring the effects of pharmacologic agents that may alter functions of the pathway. In the present study metabolism of [1-^{13}C]glucose by the common liver fluke, *Fasciola hepatica*, was studied both directly with ^{13}C NMR and indirectly by observation of ^{13}C-induced multiplet splitting of the ^{1}H NMR resonances from the glycolytic end-products propionate and acetate. The extent of ^{13}C enrichment of the end-products demonstrated that exogenous glucose was the predominant source of glycolytic substrate under the incubation conditions used. Specific enrichments of propionate and acetate in ^{13}C were similar and enrichments at the acetate C-1 carboxyl and C-2 methyl were identical, demonstrating that acetate is generated preferentially from pyruvate formed by the malic enzyme reaction. End-product synthesized in substrate-free medium following incorporation of a small fraction of [1-^{13}C]glucose into endogenous glycogen demonstrates that glucose equivalents from the most recently synthesized polymeric chains, which have a specific activity in ^{13}C equal to that of the exogenous glucose, are preferentially used for glycogenolysis. Stimulation of flukes with 0.1 mM serotonin results in a reduction of the propionate/acetate ^{13}C enrichment ratio consistent with functional "compartmentation" of glycogen pools having different structures and/or specific enrichment in ^{13}C. Glucose equivalents were incorporated into glycogen in intact flukes with label at both the C-1 and C-6 positions during perfusion with [1-^{13}C]glucose as a consequence of "substrate cycling" at the phosphofructokinase/fructosebisphosphatase enzyme couple. The observed glycogen C-6/C-1 labeling ratio of 0.42 and the net glycolytic flux of 11 μmol/g wet weight/hr imply a total forward flux of about 29 μmol/g wet weight/hr through phosphofructokinase with a reverse flux of about 17 μmol/g wet weight/hr through fructosebisphosphatase. Net glycolytic flux is therefore a poor estimate of the true flux through phosphofructokinase in this preparation.

The chemotherapy of helminthic diseases is unique for the number of pharmacologic agents directed against fundamental bioenergetic processes of the parasites (1). However, a detailed understanding of the modes of action of these drugs is still limited by uncertain definitions of basic metabolic pathways and interactions between pathways in the intact organisms. Radioisotope tracer studies have done much to clarify directions of metabolic interconversion but are tedious to adapt for quantitative evaluation of substrate fluxes in competing pathways. Improved methodology for qualitative and quantitative evaluation of substrate interconversion in glycolysis that would be suitable for relatively rapid screening of the effects of pharmacologic interventions could speed development of more selective anthelminthic agents directed at key steps in the uniquely anaerobic metabolism of *Fasciola* and related trematodes.

^{13}C NMR has been used to follow the metabolic fates of site-specific ^{13}C labels in intact cells and tissues and in metabolic end-products released from them (2–4). This methodology has considerably lower sensitivity than radioisotope techniques, necessitating use of much higher degrees of label enrichment. However, this merely increases expense, for the nonradioactive ^{13}C isotope can be handled without special precautions. It offers a significant advantage in that enrichment at specific carbon sites in metabolites can be determined and relative enrichments at different sites in a molecule can be readily quantified. Analysis of enrichment patterns in a metabolic steady state can yield detailed kinetic descriptions of the pathways involved (5).

^{1}H NMR is more sensitive than ^{13}C NMR and can also be used to follow the distribution of ^{13}C labels at specific carbon sites using the appearance of ^{1}H-^{13}C multiplet splitting of attached protons as a probe (6, 7). Although analysis of the proton spectra may be more complex and resolution between protons bonded to different carbon sites is intrinsically lower, absolute quantitation of metabolic concentrations is easier in the proton spectrum. Furthermore, the *specific* enrichment in ^{13}C at each site can be directly measured from the ratio of ^{1}H-^{13}C spin-coupled to the non-spin-coupled proton resonances for a given site.

Both NMR methods may be used with intact biological

ABBREVIATIONS: DSS, 2,2-dimethyl-2-silapentane 5-sulfonic acid.

tissues. As they are inherently nondestructive, they are readily adapted to serial investigations of interconversions, with the ability to maintain a given preparation as its own control. The sensitivity of a chosen NMR-based metabolic screening assay may therefore be enhanced by eliminating the variability introduced by biological differences between preparations (8).

The liver fluke, *Fasciola hepatica*, is a useful model system for studying the predominantly anaerobic metabolism of the parasitic helminths (9). We have recently reported ^{31}P NMR studies defining intracellular phosphate metabolite compartmentation in the fluke and suggesting a role that this might play in glycolytic regulation (10). In the present communication we describe ^{13}C and ^{1}H NMR studies which follow the distribution of the carbon label of exogenous [1-^{13}C]glucose into glycolytic end-products and glycogen. These have allowed us to quantitatively examine substrate provision, relative fluxes through alternative pathways for glycolytic end-product synthesis, and rates of substrate cycling between fructose-6-phosphate and fructose-1,6-bisphosphate. Rates of substrate cycling are significant, making clear that net glycolytic flux is a poor measure of the activity of the "rate-limiting" enzyme phosphofructokinase. Measurement of rates of substrate cycling in this way should provide a more sensitive index of the effects of pharmacologic agents directed at this key regulatory step in glycolysis.

Materials and Methods

Fasciola hepatica were dissected from bile ducts of locally slaughtered cattle and immediately placed into warm substrate-free Hedon-Fleig solution (11) for transfer to the laboratory. Once in the laboratory they were washed and maintained at 37° in an identical medium containing 11 mM glucose prior to the onset of experiments. Flukes harvested more than 24 hr previously were discarded.

Incubations were performed in 25-ml Erlenmeyer flasks maintained at 37° in a constant-temperature room with three flukes (~0.3 gm wet wt) in 10 ml of incubation medium. The pH of the standard Hedon-Fleig solution was adjusted initially to 7.8 and either unenriched glucose of 99.8% 1-^{13}C-labeled enriched glucose (Merck, Sharp and Dohme, Inc., New York, NY) was added to a final concentration of 11 mM, depending on the experiment.

At various times after the onset of the incubation, aliquots of 1 ml were withdrawn and immediately frozen. After withdrawal of each aliquot, 1 ml of substrate-free medium was added to the incubation medium to maintain a constant volume of 10 ml. Each aliquot was immediately frozen in 5-ml plastic vials. At the end of each experiment all vials were thawed, rapidly refrozen at the bottom of their containers with liquid N_2, and placed in a vacuum chamber until their contents were lyophilized. Care was taken to maintain samples frozen to prevent loss of material during lyophilization secondary to expansion of trapped gas.

After lyophilization, the samples were stored at −20° until NMR experiments could be performed. At this time 0.5 ml of D_2O containing 1.6 mM DSS was added to each tube and the contents were completely dissolved and transferred to the NMR sample tube.

^{1}H NMR measurements were made at 400 MHz or 300 MHz using Varian XL-400 or XL-300 spectrometers, respectively. In a few experiments the 360-MHz spectrometer at the Stanford Magnetic Resonance Laboratory was used. Free induction decays were accumulated using 16,000 data points, zero-filled to 32,000, and spectral widths of 6,500, 5,000, and 6,000 Hz, respectively. All data were accumulated under nonsaturating conditions with a 45° pulse angle and a recycle time of 8 sec, which is equal to the T_1 of the species with the longest relaxation time in the sample.

Metabolite concentrations in the incubation media were quantified by comparison of their resonance areas with that of the methyl resonance of the added DSS with correction for the relative numbers of protons. Corrections were made for dilution with addition of fresh buffer after withdrawal of each aliquot. Specific enrichment in ^{13}C at each site was calculated from the relative intensities of the central (I_C) and satellite (I_S) resonances for protons spin-coupled to directly bonded ^{13}C:

$$\% \text{ enrichment} = 100 \times 2I_s/(2I_S + I_C)$$

^{13}C NMR measurements were made at 75.45 MHz using a Varian XL-300 spectrometer. Data were accumulated with a 60° pulse angle and a recycle time of 1 sec, which did not significantly saturate resonances from intracellular glycogen. Chemical shifts are expressed relative to the DSS-methyl at 0 ppm.

Studies of intact flukes were performed using a perfusion apparatus previously described (10) with a total recirculating volume of 25 ml.

Mean values are expressed ± the standard error of the mean. Statistical significance was assessed using a two-tailed Student's t test.

Results

^{1}H NMR Studies of End-product Enrichment with ^{13}C

Substrate utilization and end-product production. *F. hepatica* harvested from bovine livers within the previous 24 hr were incubated at 37° with Hedon-Fleig solution containing an 11 mM concentration of 99.8% [1-^{13}C]glucose. Before addition of flukes to the incubation medium, 15 min after the start of the incubation and at various times thereafter up to 23 hr, 1-ml aliquots of medium were withdrawn and immediately frozen. They were later lyophilized and redissolved in 0.5 ml D_2O containing 1.6 mM DSS for ^{1}H NMR analysis.

F. hepatica utilize exogenous glucose, releasing primarily propionate and acetate as glycolytic end-products. Spectra from aliquots collected at progressively later times in the incubation period showed accumulation of propionate and acetate with increases in resonance intensity of the C-3 triplet and C-2 quartet of propionate and the C-2 singlet of acetate. A lactate C-3 resonance (which would arise at 1.32 ppm) was not observed, indicating a rate of lactate production less than about 5–10% of that for either acetate or propionate. Resonance intensities of glucose protons progressively decreased (Fig. 1). The spectra were accumulated under nonsaturating conditions so that resonance areas were directly proportional to the metabolite concentrations. Absolute metabolite concentrations were quantified by comparison of DDS-methyl and metabolite resonance areas (see Materials and Methods) (Fig. 2).

The propionate/acetate ratio of 2.2 ± 0.1 (n = 6) did not change between 3 and 23 hr of incubation and was not significantly different from 2. This is consistent with maintenance of cellular redox balance by direct utilization of the 2 mol of NADH generated per mol of acetate production in reduction of 2 mol of fumarate to 2 mol of succinate for synthesis of 2 mol of propionate (Fig. 3; Ref. 12). The absence of significant deviation from a 1:2 molar ratio of acetate/propionate suggests 1) that significant amounts of mitochondrial reducing equivalents are not generated by reactions other than the dismutation of malate, and 2) that reducing equivalents in the malic enzyme and pyruvate dehydrogenase reactions are primarily used for reduction of fumarate under these conditions.

The net rate of glycolysis as measured from net rate of product accumulation decreased over 23 hr of incubation by approximately 50%. The average rate of glycolysis over the first 3 hr was 18.2 ± 2.5 μmol glucose/g wet weight/hr (n = 3). It decreased to an average 15.2 ± 2.2 μmol glucose/g wet weight/

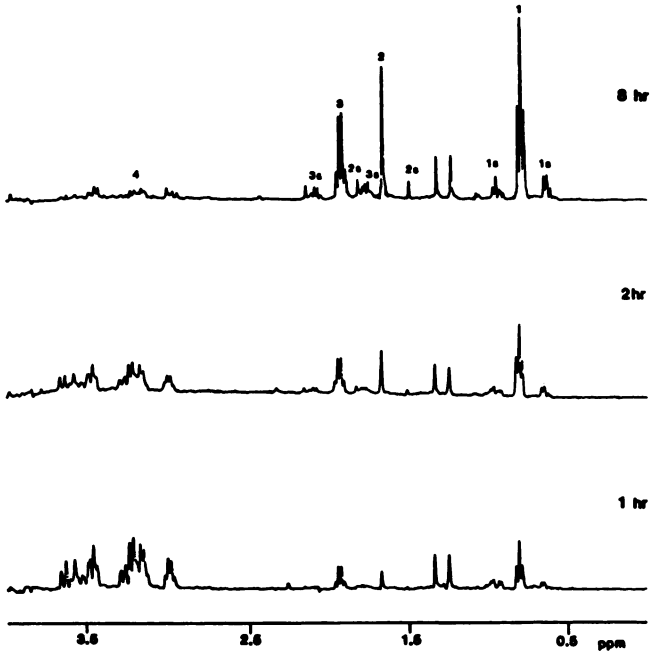

Fig. 1. ^{1}H NMR spectra at 400 MHz showing increases in [propionate] and [acetate] and decreases in [glucose] in the perfusion medium during a typical incubation. *1*, propionate methyl protons; *2*, acetate methyl; *3*, propionate methylene; *4*, glucose (mixed anomers) non-hydroxyl protons. ^{13}C-induced satellites in acetate and propionate are additionally marked with *s*. Other resonances present showed no ^{13}C label incorporation and were not assigned numbers. Samples were prepared from aliquots collected at the times shown as described in Materials and Methods. Each spectrum represents 16 scans with a total interpulse delay of 8 sec and a 45° pulse angle.

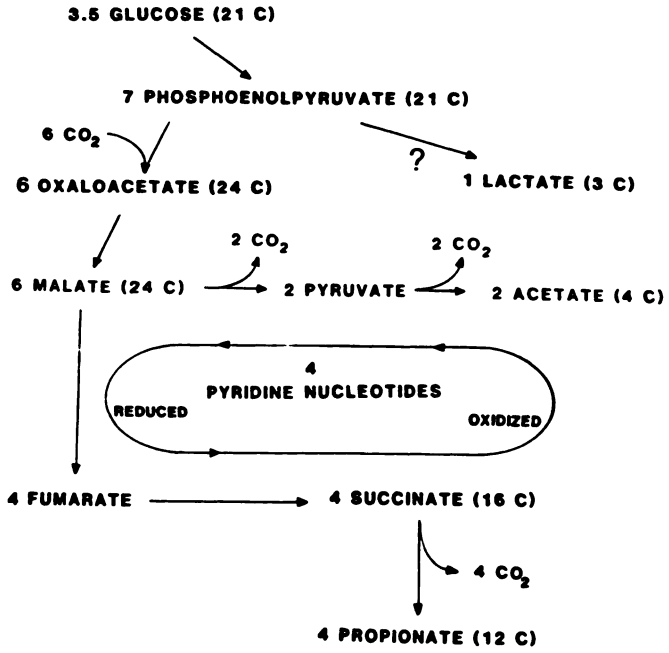

Fig. 3. Simplified scheme of metabolic pathways for formation of propionate and acetate illustrating a model (12) in which production of 2 mol of propionate is synthesized for each mol of acetate to maintain a net intracellular redox balance. The stoichiometry of the reactions is indicated by the numbers of molar equivalents given to the *left* of each compound name. *Numbers in parentheses* to the *right* show the number of carbon atoms in each step to illustrate overall carbon balance. The pathway from phospho*enol*pyruvate to lactate is marked *?*, as evidence from the current experiments and previous work cited in the Discussion suggests that this is not a significant pathway in *Fasciola*.

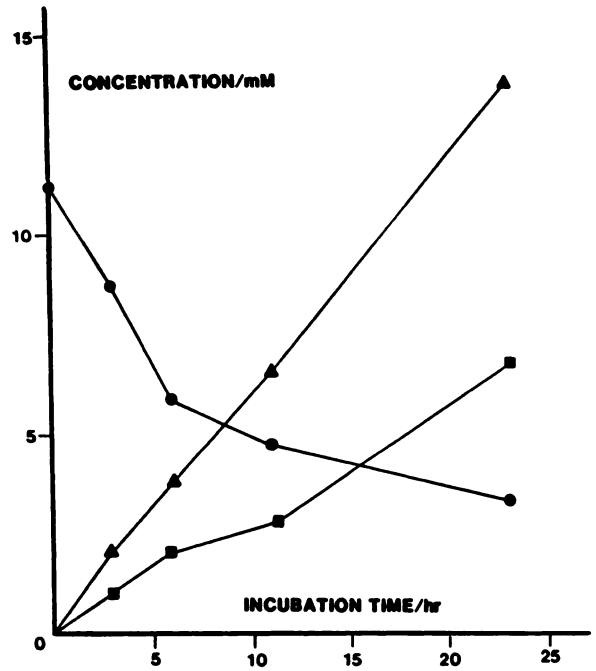

Fig. 2. Time course of [glucose], [propionate], and [acetate] in Hedon-Fleig incubation medium supplemented with 11 mM 50% [1-^{13}C]glucose. Values represent the average of three experiments with 0.33 ± 0.04-g net weight flukes in 10 ml of medium at 37°. ●, glucose; ▲, propionate; ■, acetate.

hr (n = 4) over 3–11 hr. The average rate of glucose utilization was not significantly greater than the net rate of glycolysis (11.4 ± 1.7 μmol glucose eq/hr/g wet weight). Although experiments described below demonstrate incorporation of ^{13}C-labeled glucose into glycogen, the net rate of incorporation is only ~2 μmol/g wet weight/hr, which is within the experimental error of these measurements.

^{13}C label distribution in end-products. Protons spin-coupled through a single bond to an adjacent ^{13}C will show multiplet splitting of 120–160 Hz (Fig. 1). The ratio of resonance intensities of such ^{1}H-^{13}C spin-coupled protons ("satellites") to the resonance areas of protons at the same site in the metabolite that show no carbon-proton spin-coupling as a consequence of being bonded to a ^{12}C nucleus is determined by the relative enrichment of the metabolite in ^{13}C at that specific position. The specific enrichments in ^{13}C at C-2 and C-3 of propionate and at C-2 of acetate can therefore be readily measured from the ^{1}H NMR spectrum.

The specific enrichments in ^{13}C at propionate C-2 and C-3 were equal within experimental error at 0.26 ± 0.01 and 0.22 ± 0.02 (n = 7), respectively. The specific enrichment did not decrease significantly over 23 hr of incubation (Table 1).

The maximum specific enrichment of propionate expected with the synthetic pathway shown in Fig. 3 is 0.25, assuming the percursor glucose-6-phosphate pool is fully labeled at either C-1 or C-6 (Fig. 4). The average enrichment observed at C-2 and C-3 (0.25 ± 0.01) (n = 7) is identical to this value, implying that glucose equivalents from unlabeled glycogen are not contributing under these conditions. In separate experiments in

which flukes were fasted for periods up to 24 hr to partially deplete endogenous glycogen stores, it was shown that the level of end-product enrichment in ^{13}C was independent of intracellular [glycogen] over a wide range of glycogen concentrations (data not shown).

The average specific activity of acetate C-2 was 0.20 ± 0.01 (n = 7). Throughout the incubations the specific enrichment in ^{13}C of the acetate C-2 was lower than that of the propionate C-3. The average ratio of enrichment (propionate C-3/acetate C-2) was 1.22 ± 0.08 (n = 7), which is significantly greater than unity ($p < 0.025$).

The specific enrichment of acetate would be 0.5 with 100% enrichment of the precursor glucose pool at C-1 (if it was derived directly from pyruvate produced by direct hydration and dephosphorylation of phospho*enol*pyruvate). However, if the pyruvate precursor is derived solely from pyruvate generated from oxaloacetoacetate *via* the malic enzyme reaction, then the maximum specific enrichment would be similar to that of propionate (Fig. 4), as is observed.

TABLE 1

Time course of ^{13}C-specific enrichment in propionate C-2/C-3 and acetate C-2 during incubation of *F. hepatica* with [1-^{13}C]glucose

Time of incubation	Specific enrichment	
	Average of propionate C-2/C-3	Acetate C-2
hr		
3	0.26 ± 0.04	0.14 ± 0.02
6	0.24 ± 0.04	0.20 ± 0.02
11	0.26 ± 0.05	0.21 ± 0.02
23	0.26 ± 0.02	0.18 ± 0.01

The latter pathway gives rise to symmetric ^{13}C enrichment at acetate C-1 and C-2, whereas the former pathway incorporates ^{13}C from [1-^{13}C]glucose only at C-2. ^{13}C NMR spectra of aliquots of incubation medium clearly show ^{13}C incorporation into the acetate C-1 position at 182 ppm. The assignment of the carboxyl was confirmed by demonstrating that it is a quartet with spin-coupling J_{CH} ~5.8 Hz on the undecoupled spectrum and that the multiplet splitting collapses to a singlet with selective irradiation at the acetate methyl proton frequency (data not shown). Integration of spectra accumulated under nonsaturating conditions during which protons were decoupled but any nuclear Overhauser enhancements were suppressed shows an acetate C-1/C-2 intensity ratio of 0.8. These results in conjunction with those above are most consistent with a model in which acetate is predominantly derived *via* the malic enzyme reaction from oxaloacetate in which the ^{13}C label distribution reflects that of its symmetric mitochondrial precursors, fumarate and succinate (Fig. 4).

Enrichment of end-products derived from glycogen. A propionate C-3/acetate C-2 ratio of enrichment greater than unity may arise if the specific ^{13}C enrichment of the precursor glucose for acetate is lower as a consequence of an increased

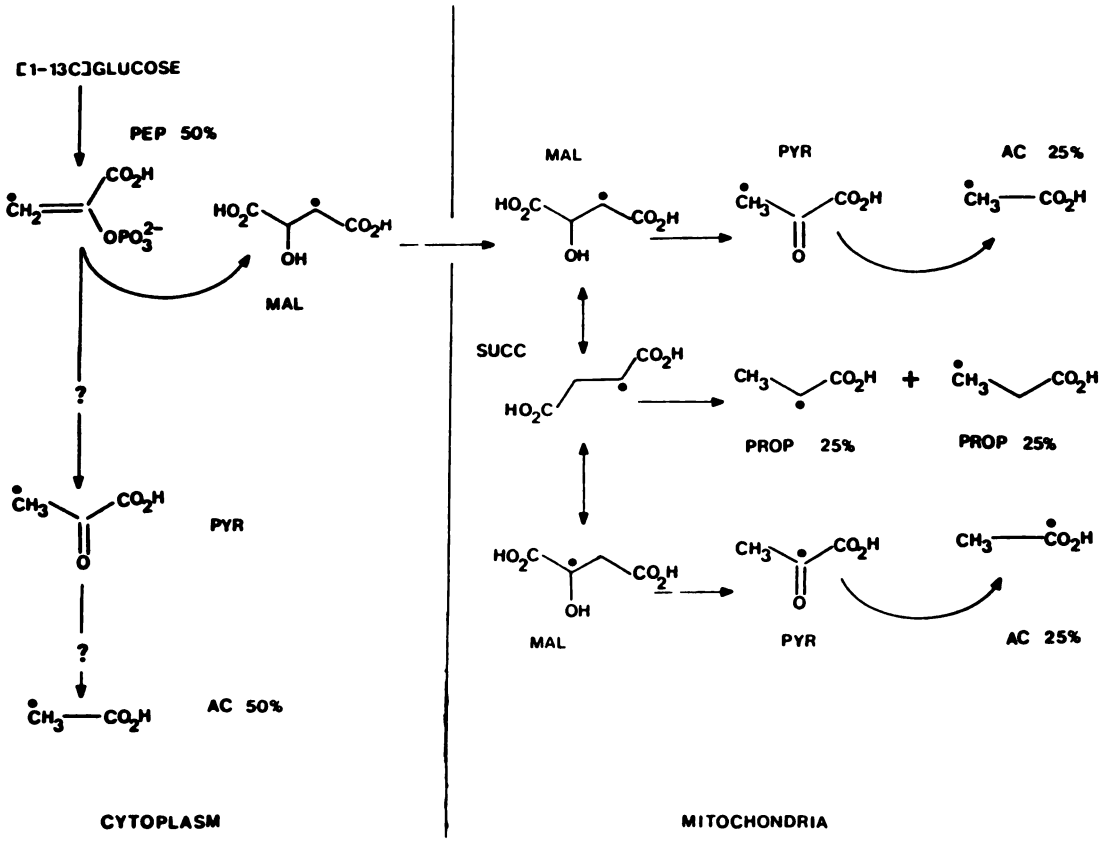

Fig. 4. Possible metabolic pathways for synthesis of acetate and propionate in *F. hepatica*. Sites of ^{13}C enrichment during incubation with [1-^{13}C]glucose are shown (●) and the maximum specific enrichment in ^{13}C possible (assuming utilization of 100% C-1-labeled glucose) is given. The direct pathway from phospho*enol*pyruvate to pyruvate is marked ? as evidence presented in the text suggests this is a very minor or inoperative pathway. Intramitochondrial malate and succinate pools are joined by *double-headed arrows* to suggest an equilibration rate rapid with respect to the rate of acetate synthesis. Note that if cytoplasmic oxaloacetate, a precursor of malate, exchanges sufficiently rapidly with the mitochondrial pool, the maximum specific enrichment of cytoplasmic malate may be lowered to 0.25 at any site with symmetric labeling at C-2 and C-3. *PEP*, phospho*enol*pyruvate; *PYR*, pyruvate; *AC*, acetate; *MAL*, malate; *SUCC*, succinate; *PROP*, propionate.

relative contribution of unlabeled glucose equivalents from endogenous unlabeled (or relatively poorly enriched) glycogen. The *overall* enrichment of glycogen is low as approximately 200 μmol/g wet weight of unlabeled glucose equivalents (9) are present at the onset of the experiment and labeled glucose is incorporated at a rate of only about 2 μmol/ wet weight/hr. However, the polymeric structure of glycogen may necessitate utilization of the most recently added glucose equivalents during glycogenolysis. This would give rise to an effective specific enrichment of glycogen approximating that of the exogenous labeled glucose.

To differentiate between these possibilities, fluke glycogen was enriched with ^{13}C glucose during a 12-hr incubation of flukes in Hedon-Fleig solution containing 11 mM 99.8% [1-^{13}C] glucose. They were subsequently washed and transferred to a substrate-free medium. The time courses of the specific enrichments of the end-products acetate and propionate released into the incubation medium were then followed over 12 hr. The specific enrichments of acetate and propionate were 0.20 and 0.23, respectively, over the first 3 hr and, given the error of the measurements, did not significantly change at 0.23 and 0.26, respectively, after 12 hr. This indicates that the most recently synthesized portions of the glycogen polymer are preferentially hydrolyzed in glycogenolysis: the *effective* specific activity of glycogen reflects that of the exogenous glucose substrate. If the specific enrichment of the products in ^{13}C reflected the *average* specific enrichment of all glucose equivalents in glycogen, then the enrichments measured would be considerably lower and would decrease with time. Thus, the results cited in the preceding section cannot differentiate between product formation from glucose equivalents directly from the exogenous glucose pool and those that have been transiently incorporated into endogenous glycogen.

Incubations with serotonin. Serotonin stimulates motility of the liver fluke (13, 14) and increases energy utilization (15, 16), probably secondary to an increase in intracellular [cAMP] (17). Increased energy production may be regulated in part directly by the increased intracellular [cAMP] which stimulates both glycogen breakdown and glycolysis in many tissues (18). We investigated the effect of incubation with 0.1 mM serotonin on the fluxes and enrichment patterns described above.

In experiments including matched control and serotonin-stimulated experimental incubations, the average rate of [1-^{13}C]glucose utilization over the first 3 hr was greater (140%) in the serotonin-treated group (24.8 versus 10.2 μmol glucose/ hr/g wet weight) (n = 2). The average glycolytic flux also increased from 17.9 to 19.8 μmol/hr/g wet weight (n = 2) with serotonin stimulation. This implies that a larger proportion of the glucose carbon taken up was incorporated into intracellular components such as glycogen in the serotonin-related flukes.

The specific enrichments of the end-products acetate and propionate did not change significantly over 23 hr of incubation (Table 2). As in control incubations, although the mean enrichment of propionate C-3 was consistently less than at C-1 (0.23 ± 0.02 at C-2 and 0.18 ± 0.01 at C-3, n = 3), at no sampling time were the differences statistically significant ($p < 0.05$). No significant difference was noted in the extent of label incorporation into acetate relative to control incubations (0.20 ± 0.02, n = 3). Although the main propionate enrichment was consistently lower with serotonin than in control incubations, samples taken after identical incubation periods did not demonstrate statistically significant differences in enrichment between the two groups. However, in contrast to control incubations, with serotonin the ratio of relative ^{13}C enrichment of propionate/acetate was only 1.06 ± 0.05, which is not significantly different from unity.

TABLE 2

Time course of ^{13}C-specific enrichment in propionate C-2/C-3 and acetate C-2 during incubation of *F. hepatica* with [1-^{13}C] glucose and 0.1 mM serotonin

Time of incubation	Specific enrichment	
	Average of propionate C-2/C-3	Acetate C-2
hr		
3	0.20 ± 0.02	0.17 ± 0.01
6	0.21 ± 0.01	0.19 ± 0.02
11	0.21 ± 0.02	0.21 ± 0.02
23	0.21 (n = 2)	0.22 (n = 2)

The average relative concentrations of propionate/acetate of 1.9 ± 0.1 (n = 4) were not significantly different from 2, as in the serotonin-free incubations.

^{13}C NMR Studies of Intact *F. hepatica*

^{13}C label distribution and time course of incorporation. We have previously demonstrated that *F. hepatica* can be maintained in a physiologically viable state for prolonged periods by perfusion in a specially designed sample chamber within the spectrometer magnet (10). Six flukes (approximately 0.6 g wet weight) were perfused with a recirculating 50-ml volume of Hedon-Fleig buffer, initially at pH 7.8, containing 11 mM [1-^{13}C]glucose after preliminary perfusion with a 250-ml volume of an identical buffer containing an 11 mM concentration of unlabeled glucose. During perfusion with unlabeled buffer, three broad-band ^{1}H-decoupled natural abundance ^{13}C NMR spectra (600 scans each with a total accumulation time of 10 min each) were accumulated. Perfusion was then begun with [1-^{13}C]glucose containing buffer, and sequential ^{13}C NMR spectra were accumulated using identical operating parameters. In addition to resonances arising from the ^{13}C-labeled C-1 of exogenous glucose and unenriched carbons of endogenous fatty acyl carbons, resonances from C-1- and C-6-labeled glucose equivalents in glycogen were clearly observed. The intensity of resonances from glucose equivalents in glycogen increased with time of perfusion (Fig. 5). Smaller resonances arising from the C-1 and C-6 of glucose-6-phosphate, the C-2 of acetate, and the C-2 and C-3 of propionate were also observed but are not noted in Fig. 5. ^{13}C NMR spectra of the incubation medium alone accumulated later do not show a resonance in the glucose C-6 region, confirming assignment of the observed resonance in the intact flukes to C-6 in glucose equivalents of endogenous glycogen. A number of other smaller resonances appeared but were not further characterized.

Estimation of substrate cycling rates. Incorporation of ^{13}C label from glucose C-1 into the C-6 of glycogen is of particular interest. A C-1 label in glucose labels the C-1 in fructose-1,6-bisphosphate, which is scrambled to the C-6 position by triose phosphate isomerase and aldolase activities (Fig. 6). The presence of a high activity of these enzymes in the cytoplasm relative to glycolytic flux gives rise to equalization of specific enrichments at the C-1 and C-6 sites of fructose-1,6-bisphosphate (19). Dephosphorylation of fructose-1,6-bisphosphate by fructose bisphosphatase introduces C-6 label into the fructose-6-phosphate and glucose-6-phosphate pools, from which it is incorporated into glycogen.

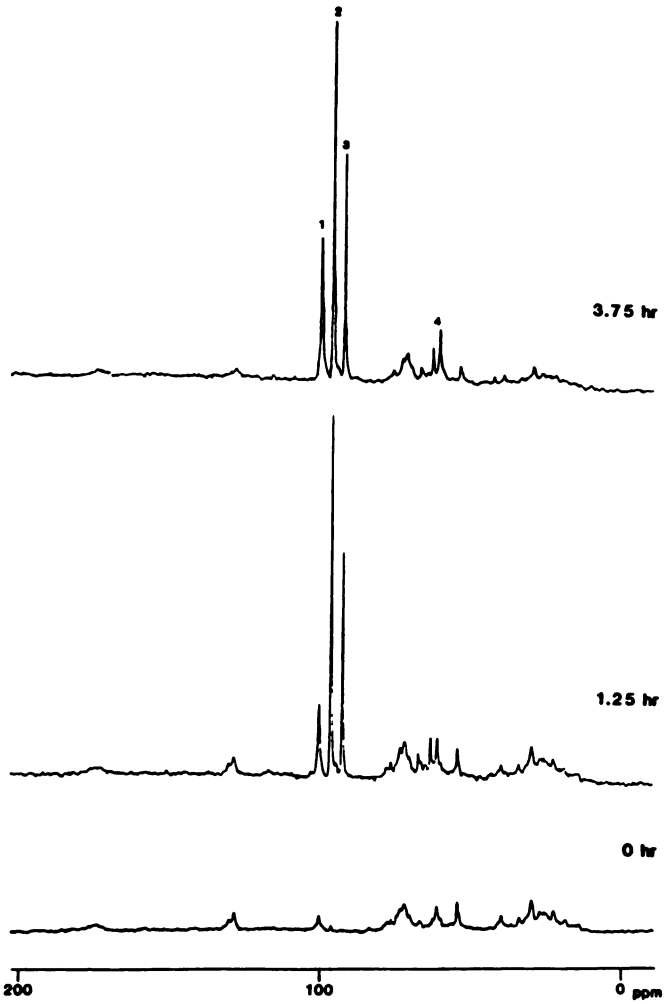

Fig. 5. ^{13}C NMR spectrum at 75.45 MHz of intact *F. hepatica* (~0.6 g wet weight) before and after 3.75 hr of perfusion with Hedon-Fleig solution that initially contained 11 mM 50% [1-^{13}C] glucose. Each spectrum represents 3000 scans with a total interpulse delay of 1 sec and a pulse angle of 60°. Chemical shifts are referenced to the methyl carbon of DSS at 0 ppm. *1*, glycogen glucose, C-1; *2*, glucose β-anomer, C-1; *3*, glucose α-anomer, C-1; *4*, glycogen glucose, C-6.

In a metabolic steady state, the relative extents of C-1 and C-6 label in glucose equivalents of glycogen with [1-^{13}C]glucose as the sole exogenous glycolytic substrate can be used to estimate the relative flux through phosphofructokinase and fructose-1,6-diphosphatase. In this calculation it is assumed that: 1) the rate of net glycogen synthesis is significant relative to the net rate of glycolysis; 2) rates of glucose-6-phosphate and fructose-6-phosphate interconversion are rapid relative to the net glycolytic flux; 3) the ^{13}C label distribution in the glucose equivalents of glycogen accurately reflects label enrichment of the glucose-6-phosphate pool; and 4) the aldolase and triose phosphate isomerase activities are high enough relative to glycolytic fluxes to fully equilibrate label between the C-1 and C-6 positions of fructose-1,6-bisphosphate. Given these conditions, the steady state fraction of ^{13}C-labeled glucose equivalents in glycogen with ^{13}C at C-6, $X_{C\text{-}6}$, is such that

$$X_{C\text{-}6} = 0.5 - \left(\frac{F_F - F_R}{F_F}\right)0.5$$

where F_F is the forward flux through phosphofructokinase (fructose-6-phosphate → fructose-1,6-bisphosphate) and F_R is the reverse flux through fructose-1,6-biphosphatase. Note that the *net* glycolytic flux equals $F_F - F_R$.

A steady state of relative glycogen C-1, C-6 labeling was reached after the first hour following the onset of perfusion with [1-^{13}C]glucose. In these experiments, the average C-6/C-1 labeling ratio of 0.42 ($n = 2$) implies that $X_{C\text{-}6} = 0.30$. The net glycolytic flux, measured by the rate of accumulation of the end-products, acetate and propionate, was about 11 μmol glucose eq/hr/g wet weight as described above. Solution of the equation above for these conditions gives a total forward flux through phosphofructokinase (fructose-6-phosphate → fructose-1,6-bisphosphate) of ~29 μmol/hr/g wet weight and a total reverse flux through fructose-1,6-biphosphatase of ~17 μmol/g/wet weight/hr. This demonstrates significant "futile cycling" through the phosphofructokinase/fructose-1,6-diphosphatase enzyme couple. Measured changes in *net* glycolytic flux therefore may be a poor estimate of true changes in flux through

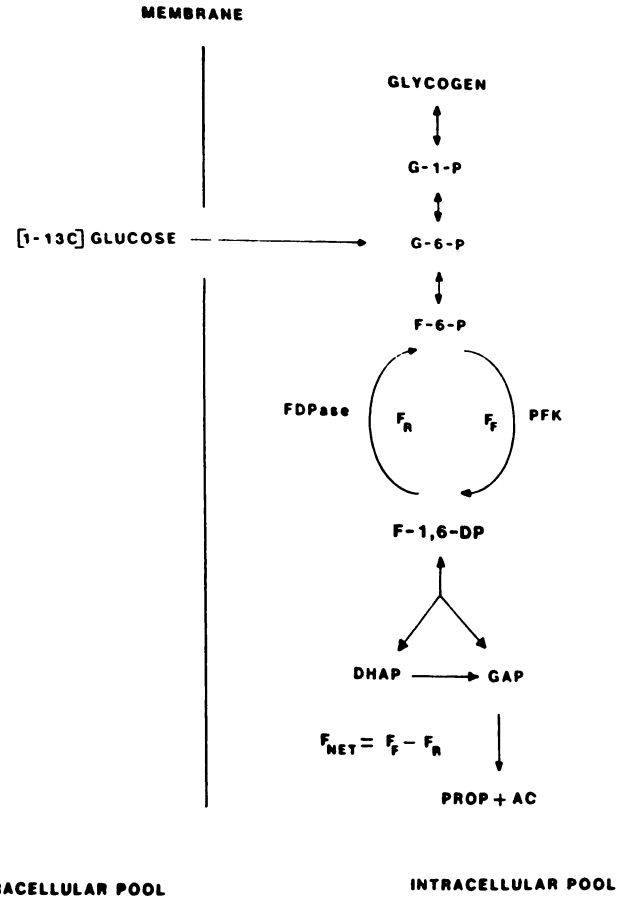

Fig. 6. Abbreviated metabolic pathway model used for estimation of rates of substrate cycling by the phosphofructokinase/fructose-1,6-bisphosphatase couple. Interconversions indicated with *double-headed arrows* are assumed to occur rapidly with respect to overall flux through glycolysis. Other assumptions used in the model are described in the text. *G-1-P*, glucose-1-phosphate; *G-6-P*, glucose-6-phosphate; *F-6-P*, fructose-6-phosphate; *F-1,6-P$_2$*, fructose-1,6-bisphosphate; *GAP*, D-glyceraldehyde 3-phosphate; *DHAP*, dihydroxyacetone phosphate; *FDPase*, fructose-1,6-bisphosphatase; *PFK*, phosphofructokinase; *PROP*, propionate; *AC*, acetate; F_F, true forward flux through PFK; F_R, true reverse flux through FDPase; F_{net}, net glycolytic rate.

phosphofructokinase between different metabolic states or after pharmacologic interventions.

Discussion

There has been uncertainty regarding the predominant metabolic pathway for generation of acetate in *F. hepatica*. Evidence from ^{14}C tracer studies has been interpreted to imply that acetate is largely produced from pyruvate generated by the action of pyruvate kinase on cytosolic phospho*enol*pyruvate (19). Observation of propionate and acetate production in the proportion of 2:1 has led other workers to favor a model in which the precursor pyruvate is generated predominantly from malate (11). This latter model is supported by the studies of Prichard and Schofield (20, 21), who found little pyruvate kinase activity and only relatively low lactate dehydrogenase activity in *Fasciola* extracts. However, one cannot rule out such apparent low activities as artifacts of extraction and assay.

Three observations in the present experiments are important with respect to this question: 1) the observed specific enrichments of acetate and pyruvate are similar after incubation with [1-^{13}C]glucose; 2) the specific enrichment of acetate is lower than would be expected if acetate were derived from pyruvate generated directly from phospho*enol*pyruvate by pyruvate kinase; and 3) the acetate produced is enriched in ^{13}C approximately equally at the C-1 and C-2 positions. These results suggest that both acetate and pyruvate are derived from symmetric precursors, such as fumarate and succinate. This is most consistent with the latter model in which the primary synthetic pathway for both end-products proceeds *via* malate, which is in equilibrium with the mitochondrial fumarate (and perhaps succinate) pool (Figs. 4 and 5). As this would predict, high activities of phospho*enol*pyruvate carboxykinase and malate dehydrogenase have been demonstrated in *Fasciola* extracts (22). Thus, this parasite may possess the same pathway described by Saz and his associates for the nematode *Ascaris lumbricoides* (23) and the cestode *Hymenolepis diminuta* (24), worms also apparently deficient in pyruvate kinase activity.

The specific enrichment in ^{13}C at propionate C-2 and C-3 during control incubations is at the theoretical maximum (0.25) within experimental error. However, the acetate C-2 enrichment was found to be lower, about 20% below the predicted value (also 0.25) if all acetate carbons were derived from the same glucose precursor pool and increased after the first 3-hr period. There are at least two possible explanations for this observation: 1) an unlabeled or structurally different endogenous glycogen pool may preferentially provide substrate for generation of acetate by a minor pathway, or 2) a fraction of acetate synthesized is aerobically oxidized and ^{13}C label lost as CO_2. The contribution of either could change with time and be subject to regulatory factors independent of those for propionate synthesis. Although a Pasteur effect has not been documented (9), it is possible that a fraction of the acetate generated was oxidized by tricarboxylic acid enzymes as ambient O_2 was available. However, in preliminary experiments no significant differences in labeling were noted in glycolytic end-products from flukes incubated in 100% O_2 and those incubated under 100% N_2.[1]

Experiments described above demonstrate that ^{13}C label is readily incorporated into glycogen and can subsequently be utilized for glycolysis, apparently with the most recently incorporated glucose equivalents used first. However, all cellular glycogen may not participate equally in these reactions: previous histological and biochemical studies in *Fasciola* have defined two populations of intracellular glycogen with different metabolic characteristics (25). It is possible that a fraction of the total acetate is preferentially formed via the intimately associated glycolytic enzymes of the "glycogen particle" (26) from a glycogen pool functionally "compartmentalized" (in a single cell type or between different cells in the worm) (27) to have a low incorporation of ^{13}C label or to have a structure from which a relatively smaller proportion of newly added, labeled glucose equivalents is hydrolyzed. Evidence for compartmentation of substrate leading to acetate and propionate makes it tempting to speculate that independent catalytic "units" may be present, functionally integrated by changes in cellular redox state (Fig. 3).

It is interesting that, during incubations with serotonin, an agent previously demonstrated to increase intracellular [cAMP] and stimulate glycogen breakdown (15–17), the enrichment pattern shows no evidence for functionally distinct glycogen substrate for the two end-products. Although a change in the absolute enrichment of propionate did not reach statistical significance, the decrease in the propionate/acetate ratio toward unity suggests that the relatively ^{13}C-enriched glycogen pool(s) preferentially catabolized to propionate in control incubations makes a proportionally smaller contribution during serotonin stimulation in the fluke. This may reflect differences in the relative kinetics of glycogen synthesis from labeled exogenous ^{13}C-glucose and breakdown during serotonin stimulation. It is also consistent with altered catabolism from different functional compartments as speculated above.

Alternatively, ^{13}C incorporation in different glycogen compartments may be constant and the glycogen pools may be structurally different so that relatively more of the unlabeled glucose equivalents from the glycogen core are catabolized as suggested above. For example, assume that hydrolysis of the more exposed glucose equivalents occurs in chain sequence. Smaller glycogen particles, which have a relatively greater surface area/volume ratio, would be expected to give rise to a lower specific enrichment of end-products as a greater proportion of previously polymerized unlabeled glucose equivalents are exposed for hydrolysis.

Analysis of the C-1/C-6 scrambling of ^{13}C label in glucose equivalents incorporated into glycogen rests on four assumptions. The first is that the rate of glycogen synthesis is low relative to the rate of glycolysis. The *maximum* rate of net glycogen synthesis is given by the rate of label incorporation into glycogen, which was determined to be 11% of the total glycolytic rate over the first 3 hr of incubation. Earlier data suggest that *net* glycogen synthesis is even slower (0.8 μmol glucose eq/g wet weight/hr or 4% of the glycolytic rate) during incubation under these conditions (12).

The remaining three assumptions outlined are that the reactions catalyzed by phosphoglucoisomerase, by triosephosphate isomerase and aldolase acting in concert, and by phosphoglucomutase are "near-equilibrium," i.e., that the unidirectional flux is small relative to the total flux through them. This demands that the ratio of forward to reverse velocities is approximately unity and that the observed mass action ratios

[1] P. M. Matthews and D. L. Foxall, unpublished observations.

and apparent equilibrium constants are of similar magnitude (less than 20-fold different in magnitude) (28). The phosphoglucoisomerase mass action ratio is ~0.3 [using data from Mansour (16)], which is approximately equal to the apparent equilibrium constant of 0.36–0.47 (29, 30). This has been independently confirmed (12). With a measured combined mass action ratio of 19.4 and an apparent equilibrium constant of 2.45, data collected by Cornish and Bryant (12) suggest that the coupled triosephosphate isomerase-aldolase reactions are also near equilibrium. Accurate measurement of triosephosphate concentrations is difficult, however. A different approach is offered by den Hollander *et al.* (19), who analyzed ^{13}C label scrambling in fructose bisphosphate from both 1- and 6-[^{13}C] glucose to calculate forward and reverse fluxes through aldolase and triosephosphate isomerase in *Saccharomyces cerevisiae*. The velocity ratios were close to unity for both reactions, as predicted from mass action ratios. Similar data for the phosphoglucomutase reaction in *F. hepatica* are lacking. However, in none of the mammalian glycogenolytic systems studied (reviewed in Ref. 28) does the reaction appear to be a non-equilibrium, regulatory step.

The C-1/C-6 label scrambling in the hexose pool analyzed as described above suggests significant fructose-6-phosphate/fructose-1,6-bisphosphate substrate cycle activity in the liver fluke. As reviewed elsewhere (31), substrate cycles provide a mechanism by which the sensitivity of non-equilibrium reactions to changes in concentration of metabolic regulators may be enhanced, although at the cost of increased rates of ATP turnover. Evidence has accumulated for operation of fructose-6-phosphate/fructose-1,6-bisphosphate substrate cycles in mammalian liver, kidney cortex, and skeletal muscle (31–36). Hormonal modulation of net glycolytic flux can occur through effects on this cycle. A preliminary report based on an experiment similar to that described here has demonstrated that changes in both forward flux through phosphofructokinase and reverse flux through fructose bisphosphatase are responsible for the enhanced glycolytic fluxes of the Pasteur effect (36). The rate of substrate cycling calculated in the present experiments suggests that an approximately 3-fold stimulation of net glycolytic flux will occur by reduction of the reverse fructose-1,6-bisphosphatase flux alone, without increasing phosphofructokinase activity. The regulatory sensitivity conferred by this mechanism is even more apparent if the effects of regulators act simultaneously at both phosphofructokinase and fructose-1-,6-bisphosphatase. For example, changes in cytoplasmic effector concentrations that can directly stimulate phosphofructokinase activity by 3-fold and simultaneously reduce fructose-1,6-bisphosphatase activity by the same proportion will give rise to a 9-fold increase in net glycolytic flux. The energy cost of this control is not insignificant, however. According to the results above, approximately 25% of the total ATP turnover can occur in the fructose-6-phosphate/fructose-1,6-bisphosphate substrate cycle; if 6 mol of ATP are generated per mol of glucose catabolized (37), then this substrate cycle alone consumes about 17 μmol of ATP/g wet weight/hr with an overall rate of ATP production of only about 68 μmol of ATP/g wet weight/hr.

The approach to investigation of carbohydrate metabolism used in the present study is quite generally applicable to studies of the intermediary metabolism of parasites and of the effects of pharmacologic agents that may modify it. Direct ^{13}C NMR studies of the intact tissue are limited to applications in which at least several hundred mg of tissue can be maintained physiologically viable in an NMR spectrometer. However, the same information on C-1/C-6 label scrambling can also be obtained with much smaller amounts of extracted glycogen, which can be maintained stable in a spectrometer for considerably more prolonged periods to increase sensitivity. Participation of different intracellular glycogen compartments in carbohydrate metabolism can be assessed in principle.

Following ^{13}C label distribution indirectly by observation of ^{1}H NMR signals offers a tremendous increase in sensitivity as a consequence of the higher intrinsic sensitivity of the proton nucleus. In addition, ^{1}H relaxation times (T_1) are, in general, shorter, allowing for more efficient data collection in the Fourier transform NMR experiment. Quantitation of the ^{1}H NMR spectrum for measurement of absolute metabolite concentrations is also considerably easier than in ^{13}C NMR where one must be concerned with relative resonance intensity differences due to differential nuclear Overhauser enhancements. A further advantage of great importance in elucidation of metabolic pathways is that the specific enrichment at each proton-bearing carbon is readily measured.

We believe these techniques will have a particular utility in affording a relatively rapid simultaneous assessment of changes in a number of key metabolic pathways following physiological changes induced by pharmacologic interventions. In addition, the ability to measure parameters such as rates of substrate cycling should provide an assay of enhanced sensitivity for agents directed at the catalytic sites involved.

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