The use of tritiated water to measure absolute rates of hepatic glycogen synthesis

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Glycogen synthesis in rat liver *in vivo* was measured by the incorporation of ^3H from $^3\text{H}_2\text{O}$ into glycogen. In meal-fed rats incorporation of ^3H into glycogen was linear up to 100 min. Before feeding glycogen concentration and the incorporation of ^3H were both low; and both rose on feeding to give maximal values after 2–3 h. The glycogen concentration was maintained for a further 5 h but the incorporation of ^3H rapidly declined to pre-feeding values. This shows that glycogen turnover was low in the post-prandial rat. Streptozotocin diabetes decreased the rise in glycogen concentration on feeding and had a similar effect on $^3\text{H}_2\text{O}$ incorporation. Both effects were reversed by insulin administration. The number of ^3H atoms incorporated per glycogen glucose moiety formed in biosynthetic experiments (2.84 ± 0.47) was relatively constant and allowed absolute biosynthetic rates to be calculated. Degradation of glucose from glycogen labelled by $^3\text{H}_2\text{O}$ showed that most of the ^3H was located at C-2 and C-5. The incorporation would arise by rapid equilibration of hexose phosphates through phosphoglucose isomerase, transaldolase and triose phosphate isomerase.

^3H from $^3\text{H}_2\text{O}$ can be incorporated directly into the products of reactions that form new C–H bonds by protonation of carbanions. This has formed the basis for techniques for estimating the rates of cholesterol and fatty acid biosynthesis in a variety of tissues (Foster & Bloom, 1963; Windmueller & Spaeth, 1966; Jungas, 1968; Brunengraber *et al.*, 1973; Salmon *et al.*, 1974). These methods possess advantages over the incorporation of other radio-labelled compounds for measuring biosynthetic rates. For products with a variety of possible precursors, the incorporation of ^3H from $^3\text{H}_2\text{O}$ is independent of the particular precursor used. Also, absolute rates can be calculated as the specific radioactivity of tissue water can be readily measured.

^3H incorporation from $^3\text{H}_2\text{O}$ has been less extensively employed for measuring rates in carbohydrate metabolism. In an attempt to measure gluconeogenesis from $^3\text{H}_2\text{O}$ in isolated rat hepatocytes (Rognstad *et al.*, 1974) it was hoped to correlate the ^3H labelling pattern in glucose with the level of entry into the gluconeogenic pathway. The results were too complex for such a straightforward analysis due to the number of pathways where ^3H could be incorporated and to the relatively small amount of glucose formed.

For a number of reasons we considered incorporation of ^3H from $^3\text{H}_2\text{O}$ might be a suitable method for measuring absolute rates of hepatic glycogen synthesis *in vivo*. Glycogen synthesis in the liver *in vivo* is rapidly activated in response to meal feeding (Hopkins *et al.*, 1973). Once formed, glycogen is biochemically relatively inert and incorporated ^3H should be retained *in vivo*. High concentrations of glycogen are formed and it is easily isolated to provide adequate material for analysis.

Materials and methods

Animal experiments

Male albino Wistar rats (100–125 g) were trained to meal feed for 2 h per day only (10:00–12:00 h) for at least 14 days before the experiment. The lighting cycle was reversed, with the dark period from 04:00–16:00 h each day. Various diets were fed. These were standard diet (Labshure CRM-X) and diets containing 67% carbohydrate (Hopkirk & Bloxham, 1979), either glucose or fructose. Rats were fed high-carbohydrate diets for 7 days before experiment.

A group of rats were made diabetic with streptozotocin (55 mg/kg body wt. in 0.01 M-sodium citrate

buffer, pH 4.8) injected via the tail vein. Diabetes was confirmed two days later by a whole-blood glucose concentration greater than 10 mM. Control rats were injected with an equal volume of citrate buffer. Experiments were conducted on the third day after injection. The treated diabetic group received subcutaneous injections of protamine-zinc insulin (3 units/rat) at 08:00 h and the untreated diabetic group received an equivalent volume of 0.9% NaCl at the same time.

Incorporations of ^3H were measured in rats that had been injected intraperitoneally with $^3\text{H}_2\text{O}$ (2 mCi/rat in 0.9% NaCl) and killed 1 h later. Samples of liver were immediately freeze-clamped in liquid N_2 -cooled tongs and were stored under liquid N_2 until analysis. Groups of animals ($n = 4-8$) were killed at intervals from pre-feeding to up to 9 h after starting to feed. In some experiments rats were injected with $^3\text{H}_2\text{O}$ just before feeding and were killed some 3 h later to ensure that all newly synthesized glycogen in the liver became labelled.

Analytical

Hepatic glycogen and glucose concentrations. Frozen liver powder (1 g) was extracted with 3 ml of 6% (w/v) HClO_4 and centrifuged at 2000 g for 15 min. A sample (500 μl) of the supernatant was neutralized with 10% (w/v) KOH and the precipitated KClO_4 was removed by centrifugation at 2000 g for 10 min. The glycogen in 200 μl of neutral supernatant was hydrolysed by incubation at 37°C for 2 h with 20 μl (0.2 μkat) of α -amylglucosidase in 100 μl of 0.04 M-sodium acetate buffer, pH 4.8. Glycogen plus glucose and free glucose concentrations were measured on hydrolysed and unhydrolysed samples respectively by the hexokinase method (Keppler & Decker, 1974). The total hepatic glycogen content was calculated by difference.

Incorporation of ^3H from $^3\text{H}_2\text{O}$ into hepatic glycogen. The glycogen in 2 ml supernatant from the HClO_4 extract of liver was precipitated at 4°C with 4 ml of ethanol. The glycogen pellet obtained after centrifugation at 2000 g for 10 min was washed twice by dissolution in 1 ml of water and reprecipitation with 2 ml of ethanol. It was dried overnight under vacuum and then dissolved in 2 ml of 0.04 M-sodium acetate buffer, pH 4.8, containing 50 μl (50 μkat) of α -amylglucosidase. After hydrolysis at 37°C for 2 h the recovery of precipitated glycogen was estimated to be $96.9 \pm 1.6\%$ of the total measured glycogen. The solution was freeze dried, reconstituted with 2 ml of water and a sample was removed for determination of non-volatile ^3H incorporation into glycogen glucose by liquid-scintillation counting. The specific radioactivity of cell water was calculated from the ^3H content of 100 μl of HClO_4 supernatant, the liver water content being estimated at 70% (w/w). The incorporation of ^3H

into hepatic glycogen from $^3\text{H}_2\text{O}$ was then expressed as μg -atoms of H/h per g liver wet wt., ignoring ^3H isotope effects.

The quantity of glycogen precipitated from the livers of fed rats varied from 1 to 5 mg. Over this range the recovery was constant and so no carrier Na_2SO_4 was required. At lower glycogen concentrations (typically 10–100 μg) the use of a co-precipitant has previously been required to achieve quantitative recovery (Patterson *et al.* 1978).

Positional analysis of ^3H in glucose derived from glycogen. The ^3H content at specific glucose carbon atoms was measured in glycogen samples after precipitation and hydrolysis. The ^3H at C-1 and C-6 was determined by chemical methods and that at C-2, C-3, C-4 and C-5 by enzymic methods.

(i) C-1 and C-6. ^3H at C-1 was estimated from the loss of radioactivity during oxidation of glucose to gluconic acid (Andrews *et al.*, 1965). ^3H at C-6 was measured by periodate oxidation followed by the formation of a dimedone complex with the formaldehyde liberated from C-6 (Bloom, 1962).

(ii) C-2, C-3, C-4 and C-5. The ^3H at each position was estimated from ^3H exchange with water during specific enzyme reactions, the resulting $^3\text{H}_2\text{O}$ being removed by freeze drying. The percentage of ^3H at each position was calculated from that lost on freeze drying. The samples (200 μl) of ^3H glucose analysed contained typically 10–20 μmol of glucose.

For each glucose solution, four samples were separately diluted in 2.0 ml of 0.1 M-Tris/HCl buffer, pH 8.0, and quantitatively converted to glucose-6-phosphate in the presence of yeast hexokinase (0.25 μkat), 0.1 M- MgCl_2 and a 1.2-fold molar excess of ATP. ^3H at C-2 was removed from all samples by a subsequent 2 h incubation at 37°C with phosphoglucose isomerase (0.1 μkat) (Rose & O'Connell, 1960). The three samples for further analysis were then incubated for 2 h at 37°C with phosphofructokinase (0.1 μkat), fructose 1,6-bisphosphate aldolase (0.05 μkat) and a 1.2-fold molar excess of ATP. Completion of conversion was monitored by measuring the disappearance of glucose-6-phosphate in control samples by using glucose-6-phosphate dehydrogenase and monitoring the production of NADPH. The samples were then heated at 80°C for 5 min to inactivate the enzymes. This prevented removal of C-4 in all subsequent incubations, which has been shown to proceed by reversal of aldolase in the presence of triose-phosphate isomerase (Rose & Warms, 1969).

For C-5 analysis, α -glycerophosphate dehydrogenase (0.02 μkat) and 1.0 μmol of NADH were added to trap dihydroxyacetone phosphate containing the ^3H originally at C-3 of glucose. ^3H at C-5 was then removed by a subsequent incubation with triose-phosphate isomerase (1.7 μkat) for 2 h at 37°C

(Rieder & Rose, 1959). The ^3H at both C-3 and C-5 was removed by incubation with triosephosphate isomerase before treatment with α -glycerophosphate dehydrogenase. ^3H at C-3 was calculated by difference.

^3H at C-4 was transferred to (4*S*)-[^3H]NADH by glyceraldehyde 3-phosphate dehydrogenase and then to ethanol by alcohol dehydrogenase. Because alcohol dehydrogenase has the opposite stereospecificity to that of glyceraldehyde 3-phosphate dehydrogenase (Loewus *et al.*, 1956; Vennesland, 1956) and would remove ^3H only from (4*R*)-[^3H]NADH, a recycling assay with catalytic quantities only of NAD^+ was employed. The sample mixture, incubated at 37°C for 2 h, contained glyceraldehyde 3-phosphate dehydrogenase (0.15 μkat), NAD^+ (0.2 μmol), 50 mM-sodium arsenate, 50 mM-acetaldehyde and alcohol dehydrogenase (1.5 μkat). The [^3H]ethanol was removed when the sample was freeze-dried.

The specificities of the positional analyses were determined by using [^3H]glucose standards. These were first purified by paper chromatography (solvent: butan-1-ol/ethanol/water, 4:1:5 by vol.) and then processed by the analytical procedures in the presence of 10 μmol of unlabelled glucose.

Hepatic lipogenesis

The incorporation of ^3H into liver fatty acid during 1 h incubation with $^3\text{H}_2\text{O}$ was measured as described by Windmueller & Spaeth (1966). Results were expressed as μg -atoms of H/h per g wet wt. of liver.

Chemicals

All general chemicals were obtained from BDH; enzymes and coenzymes were from Sigma; radioisotopes were from The Radiochemical Centre and protamine-zinc insulin was from Duncan, Flockhart and Co. (London, U.K.). Streptozotocin was a gift from Upjohn (Kalamazoo, MI, U.S.A.).

Results

Time course for incorporation of ^3H from $^3\text{H}_2\text{O}$ into glycogen

To calculate a biosynthetic rate from the incorporation of a substrate over a fixed time period requires both the substrate concentration to be constant and the incorporation to be linear during that period. The constant concentration and complete equilibration of $^3\text{H}_2\text{O}$ in the rat over the 1 h incubation were demonstrated in two ways. In one group of four rats, the femoral arteries were cannulated and sequential blood samples were taken after injection of $^3\text{H}_2\text{O}$. The specific radioactivity of these samples was constant from 10 min to 2 h after injection. In another group of 13 rats, both skeletal

muscle and liver were sampled after 1 h incubation with $^3\text{H}_2\text{O}$. The specific radioactivities of the tissue water were identical; the ratio specific radioactivity in muscle: specific radioactivity in liver was 0.97 with a correlation coefficient of 0.97.

The incorporation of ^3H into hepatic glycogen over different time intervals was measured *in vivo* after feeding (Fig. 1). Rats were injected with $^3\text{H}_2\text{O}$ 1 h after starting to feed and were killed up to 100 min later. The incorporation was reasonably linear with time.

Hepatic glycogen concentration was low before feeding (22 h fasted) in rats from all groups. After an intraperitoneal injection of 2 mCi of $^3\text{H}_2\text{O}$ there was a low accompanying ^3H incorporation during incubation for 1 h *in vivo* (Figs. 2, 3 and 4). This result served as a control for the subsequent experiments in fed rats.

The hepatic glycogen concentration of control rats increased rapidly in response to feeding (Fig. 2). The rate of ^3H incorporation from $^3\text{H}_2\text{O}$ into glycogen increased at the same time, to a maximum value of about 70 000 d.p.m./h per g liver wet wt. The rate of ^3H incorporation was greatest 2–3 h after feeding. It then declined to a rate, from 5 h onwards, comparable with that of pre-fed rats. By contrast, the hepatic glycogen concentration remained elevated at the maximum concentration up to 8 h after feeding. The combination of low ^3H incorporation into glycogen and high glycogen concentration demonstrated that the rate of glycogen turnover in fed rats was low. It also showed that

Fig. 1. Time course of incorporation of ^3H from $^3\text{H}_2\text{O}$ into hepatic glycogen

Rats were injected intraperitoneally with $^3\text{H}_2\text{O}$ at about 11:00 h, 1 h after feeding. Groups of four rats were killed at the times indicated and the ^3H in liver glycogen was determined. Results are expressed as means $\pm$ s.e.m. The line was calculated by the least squares method; the correlation coefficient $r = 0.75$.

Fig. 2. Hepatic glycogen synthesis in meal-fed normal rats

The open bars of the histogram represent the total ^3H incorporation into glycogen over the 1 h period indicated. The hatched bars represent that proportion found at C-2 of glucose from glycogen. The graph denotes the glycogen concentration at the end of the incubation period. The hatched area on the abscissa indicates the limited period of meal feeding. These rats were fed on standard laboratory diet. Results are expressed as means $\pm$ S.E.M. of between four and eight rats.

Fig. 3. Hepatic glycogen synthesis in meal-fed diabetic rats

Experimental conditions, diet and symbols are the same as in Fig. 2. Rats were made diabetic 3 days before the experiment by intravenous injection of streptozotocin (55 mg/kg body wt.). All rats displayed fasting hyperglycaemia in excess of 10 mmol/litre.

^3H was not incorporated from $^3\text{H}_2\text{O}$ by any exchange reaction with pre-formed glycogen.

Glycogen synthesis in diabetic rats

The response of hepatic glycogen synthesis to meal feeding was impaired, but not abolished, by streptozotocin diabetes (Fig. 3). This provided further evidence *in vivo* for the validity of the $^3\text{H}_2\text{O}$ incorporation method. The increase in glycogen concentration after feeding was both decreased and delayed by diabetes (compare Fig. 2 with Fig. 3). The incorporation of ^3H from $^3\text{H}_2\text{O}$ into glycogen was also decreased, the maximum rate reaching only 60% of the control value. This demonstrated that incorporation of ^3H truly reflected glycogen synthesis and not merely, for instance, the rate of sugar phosphate labelling *in vivo*. Treatment of diabetic rats with 3 units of protamine-zinc insulin 2 h before feeding (Fig. 4) confirmed this observation. The feeding response of glycogen synthesis was restored to normal. Both glycogen concentration and ^3H incorporation into glycogen were within the range for control rats by 2 h after feeding (compare Fig. 2 with Fig. 4).

Correlation of glycogen accumulation and the incorporation of ^3H from $^3\text{H}_2\text{O}$

Data accumulated in the experiments described so far (Figs. 2, 3 and 4) were used to construct a plot of

Fig. 4. Hepatic glycogen synthesis in meal-fed diabetic rats after insulin pretreatment

Experimental conditions were the same as in Fig. 2. Diabetic rats were injected with protamine-zinc insulin (3 units) intraperitoneally at 08:00 h, 2 h before feeding.

Fig. 5. Correlation of changes in glycogen concentration with ^3H incorporation from $^3\text{H}_2\text{O}$

The dashed line indicates the rate of ^3H incorporation from $^3\text{H}_2\text{O}$ into glycogen in pre-fed control rats. Other data points were taken from experiments of the type shown in Figs. 2, 3 and 4. The symbols are grouped as follows; (○) decrease in glycogen concentration; (△) period after feeding in control (13:00–14:00 h) and diabetic (14:00–15:00 h) rats; (●) all other experimental times. The correlation coefficient for the last group 0.922.

glycogen accumulation versus the incorporation of ^3H from $^3\text{H}_2\text{O}$ (Fig. 5). For a reasonable number of experimental times (●) in both control and diabetic animals there is a good correlation between the two parameters ($r = 0.922$). There are two types of clear exception. Firstly, when glycogen synthesis decreased (○) then ^3H incorporation from $^3\text{H}_2\text{O}$ was at the same value as the incorporation in pre-fed rats. We regarded this as the basal rate of incorporation. Secondly, there was a group (△) that showed a much higher incorporation than was anticipated from glycogen accumulation. These measurements were obtained in the period just following the end of feeding. The high incorporation may reflect that glycogen recycling is active during this phase or, alternatively, this may reflect an error in the estimation of glycogen accumulation during this period.

Number of atoms of ^3H incorporated per glycogen glucose moiety synthesized

Knowledge of the ratio of ^3H incorporated to glycosyl units formed is required to calculate absolute glycogen synthetic rates from the rate of $^3\text{H}_2\text{O}$ incorporation. This was obtained by dividing

the incorporated ^3H (μg -atoms of H/h per g) by the accompanying increment of glycogen ($\mu\text{mol/h}$ per g). For control and diabetic animals this ratio was 2.84 ± 0.47 (Table 1). The ratio calculated for rats fed the high-glucose diet was similar, but was slightly increased in those rats fed the high-fructose diet.

Positional analysis of [^3H]glucose from glycogen

The efficiencies of the glucose degradation procedures were estimated by using standard glucose samples (Table 2). These standards were purified by paper chromatography before analysis. Each positional analysis accounted for at least 78% of the ^3H content of the corresponding standard. The discrimination between positions was good, with little cross-contamination.

The positional distribution of ^3H in hepatic glycogen from rats in all experimental groups is summarized in Table 1. A feature common to all groups was the substantial ^3H content at C-2, which ranged from 26 to 34% and was equivalent to 0.77–0.92 ^3H atoms at C-2 per glucose moiety of glycogen synthesized. As this value approached the incorporation of 1 g-atom of ^3H at C-2 it enabled the pattern of ^3H incorporation into liver glycogen after feeding to be checked. In control animals throughout the period of increased glycogen synthesis, the ^3H incorporation at C-2 of the glucose moieties of glycogen accurately reflected the pattern of total incorporation with a mean of 34% of that total (Fig. 2).

In normal rats fed the standard diet, the high-glucose diet or the high-fructose diet, ^3H was also incorporated into C-5 of the glucose moieties. This analysis was not performed for diabetic rats. This incorporation represented 0.6–1.0 ^3H atoms at C-5 per glucose molecule. The distribution of the remaining ^3H between the other glucose carbon atoms varied with the diet eaten. For rats eating the standard diet this ^3H was evenly divided between C-1, C-3 and C-6; on the high-glucose diet ^3H was distributed between C-1 and C-6; on the high-fructose diet ^3H was located largely at C-3. The increased incorporation at C-3 observed in rats fed the fructose diet is significant as fructose is obligatorily metabolized via triosephosphates (Hers, 1957).

Muscle glycogen synthesis

An attempt was made to apply the $^3\text{H}_2\text{O}$ technique to measure the rate of glycogen synthesis in striated muscle *in vivo*. Muscle glycogen content increased after feeding from 12.5 to 23.4 $\mu\text{mol/g}$. This increase is only 2–4% of that observed for liver glycogen under identical conditions. The associated rates of ^3H incorporation were correspondingly low and so no significant analysis was possible. We estimate that the body load of $^3\text{H}_2\text{O}$ would have to be increased to 100–225 mCi per rat to achieve

Table 1. *Pattern of ^3H in glucose derived from glycogen*

Rats were injected with 2mCi of $^3\text{H}_2\text{O}$ at 10:00h; normal rats were killed at 14:00h; diabetic rats were killed at 12:00h. The number of ^3H atoms incorporated per glucose molecule from glycogen was calculated by dividing the ^3H incorporation by the corresponding increase in glycogen concentration. Results are expressed as means $\pm$ S.E.M. where appropriate. The values in parentheses indicate the number of rats per group. Each liver sample was analysed on at least three separate occasions.

	Intact rats			Diabetic rats (standard diet)	
	Standard diet (4)	Glucose diet (3)	Fructose diet (3)	Untreated (6)	Insulin-treated (6)
Hepatic glycogen concentration ($\mu\text{mol/g}$)					
Fasting	109 $\pm$ 28	73 $\pm$ 7	74 $\pm$ 8	35 $\pm$ 12	17 $\pm$ 2
Fed	446 $\pm$ 43	375 $\pm$ 30	532 $\pm$ 11	147 $\pm$ 29	234 $\pm$ 47
Increase on feeding	337	302	458	112	217
Incorporation of ^3H from $^3\text{H}_2\text{O}$ into glycogen (μg -atoms of H/g)	1016 $\pm$ 54	783 $\pm$ 136	1650 $\pm$ 85	280 $\pm$ 49	542 $\pm$ 64
Number of ^3H atoms per glucose molecule in glycogen	3.0	2.6	3.6	2.5	2.5
Percentage of total ^3H at glucose carbon atom number					
1	10.4 $\pm$ 1.4	14.3 $\pm$ 1.3	7.8 $\pm$ 1.3	—	—
2	30.4 $\pm$ 2.7	29.7 $\pm$ 2.1	25.7 $\pm$ 0.8	34.4 $\pm$ 2.7	31.9 $\pm$ 1.5
3	12.7 $\pm$ 2.2	8.6 $\pm$ 2.0	19.2 $\pm$ 1.4	—	—
4	3.2 $\pm$ 1.9	7.8 $\pm$ 0.7	9.5 $\pm$ 2.7	—	—
5	22.7 $\pm$ 3.2	21.8 $\pm$ 0.5	29.0 $\pm$ 3.1	—	—
6	13.6 $\pm$ 1.6	24.2 $\pm$ 2.8	10.3 $\pm$ 0.4	—	—
Number of atoms of ^3H incorporated at glucose carbon atom number					
1	0.31	0.37	0.27	—	—
2	0.91	0.77	0.92	0.86	0.80
3	0.38	0.22	0.69	—	—
4	0.10	0.20	0.34	—	—
5	0.68	0.57	1.04	—	—
6	0.41	0.60	0.37	—	—

satisfactory incorporation *in vivo* into muscle glycogen.

Integration of hepatic glycogen and fatty acid biosynthesis

Incorporation of ^3H from $^3\text{H}_2\text{O}$ was used to measure fatty acid biosynthesis on samples previously analysed for glycogen synthesis (Fig. 6). In control rats fed the standard diet, the hepatic lipogenic rate was low before feeding ($3.8 \pm 0.6 \mu\text{g}$ -atoms of H/h per g) and increased by the end of feeding to $44.4 \pm 2.6 \mu\text{g}$ -atoms of H/h per g. It remained increased for the following 7h. Comparison of the pattern of fatty acid biosynthesis after feeding (Fig. 6) with that of glycogen biosynthesis (Fig. 2) enables a variety of deductions to be made. Immediately after feeding, when ^3H is being rapidly incorporated into both fatty acid and glycogen, both synthetic pathways were simultaneously active. Under these conditions, glycogen could not be a net precursor for fatty acid synthesis *de novo*. In the post-prandial state 5–9h after feeding, glycogen synthesis was low whereas hepatic

Fig. 6. *Hepatic fatty acid synthesis in meal-fed rats*
Incorporation of ^3H from $^3\text{H}_2\text{O}$ into fatty acids was determined on the samples of liver used for glycogen synthesis (Fig. 2).

Table 2. *Positional analysis of standard [^3H]glucose samples*

The analyses were performed in the presence of 10 μmol of unlabelled glucose. Results are the means of duplicate determinations.

Glucose carbon atom number	Nominal position of ^3H in glucose standard	^3H found (%)				
		[1- ^3H]	[2- ^3H]	[3- ^3H]	[5- ^3H]	[6- ^3H]
1		83.9	12.6	0.6	10.0	3.5
2		3.6	78.7	1.3	1.0	0
3		3.1	1.5	87.0	1.1	0.6
4		3.8	3.1	3.5	4.3	9.2
5		5.6	0.8	6.5	77.6	0
6		0.1	3.3	1.4	6.8	85.8

lipogenesis remained increased. During this period the conversion of glycogen to fatty acid (Salmon *et al.*, 1974) would have been possible and so hepatic glycogen is a potential fatty acid precursor during this period.

Discussion

The incorporation of ^3H from $^3\text{H}_2\text{O}$ is a well-established technique for estimating rates of fatty acid and cholesterol synthesis (Brunengraber *et al.*, 1973; Foster & Bloom, 1963; Jungas, 1968; Windmueller & Spaeth, 1966). The principle of these methods has now been extended to the measurement *in vivo* of rates of hepatic glycogen synthesis. Alternative methods to quantify glycogen synthetic rates, such as measuring either the incorporation of ^{14}C -labelled precursors or glycogen concentration changes, possess inherent limitations that are not present in the $^3\text{H}_2\text{O}$ incorporation method.

The radioactivity incorporated into glycogen from precursors such as [^{14}C]glucose, [^{14}C]fructose or [^{14}C]lactate is a function not only of the rate of glycogen synthesis but also of the rate of exchange of label between various metabolite pools. The problems involved in measuring glycogen synthesis by incorporation of [^{14}C]glucose were discussed recently (Katz *et al.*, 1979).

The extent of ^{14}C incorporation *in vivo* would be further affected by the operation of the glucose/lactate cycle (Cori cycle) between muscle and liver (Dunn *et al.*, 1967). The resultant retention of label in glycogen precursors would lead to increased radioactivity incorporated into glycogen. Even under conditions when ^{14}C -labelled substrate incorporation does reflect the glycogen synthetic rates, calculation of an absolute synthetic rate is not possible. Unknown factors include competition by other unlabelled substrates, the degree of intracellular compartmentation and the extent of dilution with endogenous substrate. Glycogen concentration changes represent the net sum of the rates of

glycogen synthesis and degradation. Thus a rapid rate of glycogen synthesis would not be reflected by any change of glycogen concentration if it was opposed by an equally rapid rate of degradation. Such glycogen recycling has been thought unlikely due to the reciprocal hepatic activities of phosphorylase and glycogen synthase (Stalmans *et al.*, 1974) but has been described for foetal rat glycogen synthesis (Gilbert & Bourbon, 1978).

None of the problems discussed above greatly affected the measurement of glycogen synthesis by ^3H incorporation from $^3\text{H}_2\text{O}$. The complete equilibrium of body water, shown by the identical specific radioactivity of muscle and liver tissue water after injection of $^3\text{H}_2\text{O}$, meant that dilution with endogenous unlabelled substrate could not occur. The lack of compartmentation of intracellular water enabled rates of ^3H incorporation to be converted, by using liver specific radioactivity, into total rates of hydrogen atoms incorporated. The pattern of ^3H incorporation into glycogen in livers of control rats 5 h after feeding indicated that the rate of hepatic glycogen turnover in post-absorptive rats was indeed slight (Fig. 2). The rapid return of the increased rate of ^3H incorporation to the pre-fed rate by 5 h after feeding would not have been expected in the presence of continued substantial glycogen recycling. This is direct evidence *in vivo* that glycogen synthesis and degradation are not simultaneously active in the liver of fed rats. The only time interval when glycogen recycling could be substantial is in the period immediately following feeding (Fig. 5).

The extent of ^3H incorporation into glycogen will be affected by any ^3H isotope effect. The overall discrimination against ^3H compared with hydrogen incorporation into the α -D-glucose 1-phosphate pool used for glycogen synthesis is not known. Indirect evidence suggests that it may be slight. Large ^3H isotope effects occur in unidirectional and rate-limiting reactions, but where a rapid equilibrium occurs these effects are masked (Katz & Rognstad,

1976). Isotope effects have been classically calculated for removal of ^3H from substrate (Rose & O'Connell, 1961; Rose, 1970; Katz & Rognstad, 1976) and may not apply equally to incorporation of ^3H from $^3\text{H}_2\text{O}$. This conclusion was supported by measurement of glucose synthesis by hepatocytes in $^3\text{H}_2\text{O}$ (Rognstad *et al.*, 1974). By making no corrections for ^3H isotope effects, 80–90% of the maximum number of seven atoms of ^3H incorporated per glucose molecule were observed during gluconeogenesis from lactate. In our experiments there were 0.77–0.92 ^3H atoms incorporated at C-2 per glucose molecule in the glycogen formed. With a large ^3H isotope effect, values so close to 1 would not have been expected. For these reasons we have ignored isotope effects in all calculations.

There are a number of reactions where ^3H from $^3\text{H}_2\text{O}$ can be incorporated into glycogen precursors. In theory, analysis of the ^3H labelling pattern in the glucose from glycogen should indicate the relative significance of the different glycogen precursors. In practice, the ^3H labelling pattern was too similar under all conditions for any significant conclusions to be drawn (Table 1). The features common to all analyses were the incorporations of approx. 1 atom of ^3H at C-2 and C-5. These could occur at C-2 by the action of phosphoglucose isomerase (Rose & O'Connell, 1960) and at C-5 by the combined actions of transaldolase and triosephosphate isomerase (Hue & Hers, 1974). The rapid equilibrium of the hexose monophosphate pool through these reactions would cause ^3H incorporation at C-2 and C-5 irrespective of the source of substrate. The additional feature of rats fed the diet high in fructose was the increased ^3H at C-3. This was probably due to the obligatory metabolism of fructose via triose phosphates (Hers, 1957) and the subsequent ^3H incorporation at triosephosphate isomerase (Rieder & Rose, 1959). The low ^3H content at C-3 for rats fed standard diet or high-glucose diet argues against substantial fructose 6-phosphate/fructose 1,6-bisphosphate 'futile' cycling in the liver (Clark *et al.*, 1973). ^3H can be incorporated at C-4 by the equilibrium of aldolase and triosephosphate isomerase (Rose & Warms, 1969) and at C-1 and C-6 by equilibration between pyruvate and dicarboxylic acids (Katz & Rognstad, 1976).

The decreased incorporation of ^3H into glycogen in diabetic rats provided further evidence *in vivo* for the validity of this method as a measure of the rate of glycogen synthesis (Fig. 3). This was also supported by the restoration of the impaired incorporation of ^3H into glycogen in diabetes by insulin (Fig. 4). Streptozotocin diabetes is a condition where hepatic glycogen synthesis is known to be defective, and which responds to insulin treatment (Whitton & Hems, 1975).

Under all these hormonal and nutritional

conditions, the ratio of ^3H atoms incorporated to glucose moieties of glycogen synthesized was relatively constant and equal to approx. 3 (range 2.5–3.6; Table 2). This enabled division by 3 of the rate of ^3H incorporation (μg -atoms of H/h per g) to give a reasonable estimate of the absolute rate of hepatic glycogen synthesis *in vivo* (μmol /h per g).

The comparison of the rates of hepatic lipogenesis and glycogen synthesis after feeding demonstrated a further application of the $^3\text{H}_2\text{O}$ incorporation method. During the initial 5 h after feeding the rates of both glycogen synthesis and fatty acid synthesis were increased in livers from control rats (Figs. 2 and 6). It follows that, in this phase, glycogen could not have been a precursor for the production *de novo* of fatty acid. A similar conclusion was drawn for hepatic fatty acid biosynthesis from glycogen in mice (Hems *et al.*, 1975). A greater lipogenic role for glycogen has been described in the post-prandial state (Wood & Krebs, 1971; Salmon *et al.*, 1974; Hems *et al.*, 1975) and a phase where this would have been possible can be seen in our results. From 5 to 9 h after feeding the rate of lipogenesis remained high whereas that of glycogen synthesis was low. This division of the feeding response of hepatic lipogenesis into two distinct periods when glycogen cannot, and then can, be a lipogenic precursor has implications for the regulation of fatty acid biosynthesis. For instance, inhibition of lipogenesis by glucagon has been suggested to be secondary to the associated glycogen depletion (Hems, 1977). It is evident, however, that in the initial phase of feeding when glycogen synthesis and fatty acid synthesis are both active that the action of glucagon on lipogenesis cannot be explained by this mechanism.

In conclusion, the incorporation of ^3H from $^3\text{H}_2\text{O}$ into glycogen is a convenient method for analysing absolute rates of glycogen synthesis. Information can be obtained about glycogen recycling and about the role of glycogen as a fatty acid precursor. Unfortunately, the interpretation of the analysis of the positional distribution of ^3H in glucose from glycogen is too complex to provide estimates *in vivo* of the relative significance of different potential glycogen precursors.

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