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The Effect of 2,4-Dinitrophenol on Adipose-Tissue Metabolism

By ROBERT ROGNSTAD and JOSEPH KATZ
Research Institute, Cedars-Sinai Medical Center, Los Angeles, Calif. 90029, *U.S.A.*

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1. The effect of dinitrophenol on the metabolism of glucose labelled with ^{14}C and tritium by epididymal fat-pad segments from fed rats was studied. Dinitrophenol at concentrations of 0·1–0·3mM: (*a*) had little effect on glucose utilization; (*b*) depressed synthesis of fatty acids and greatly increased that of lactate; (*c*) increased the T/^{14}C ratio in fatty acids synthesized from [U-^{14}C,3-T]glucose and decreased that in fatty acids synthesized from [U-^{14}C,4-T]glucose; (*d*) abolished randomization of ^{14}C from [6-^{14}C]glucose in lactate. 2. Dinitrophenol stimulated oxidation of pyruvate and greatly inhibited the oxidation of lactate. It inhibited lipogenesis from pyruvate and lactate. 3. From the isotope data it was calculated that: (*a*) dinitrophenol stimulates oxidation via the tricarboxylic acid cycle three- to six-fold; (*b*) dinitrophenol depresses markedly the operation of the pentose cycle; (*c*) in the presence of dinitrophenol, NADPH formed in the pentose cycle provides all the hydrogen equivalents for fatty acid reduction, whereas, in its absence, NADPH provides 50–70% of the hydrogen equivalents; (*d*) in the presence of dinitrophenol, there is an excess of ATP produced in the cytoplasm, which flows into the mitochondria. A reverse flow operates in the absence of dinitrophenol. 4. A balance of formation and utilization of reduced nicotinamide nucleotides in the cytoplasm was established. With dinitrophenol there is some excess of NADH. There are indications that this excess may be transferred into mitochondria in the form of malate. 5. Our results are interpreted to indicate the absence from adipose tissue of the α-glycerophosphate shuttle for transferring reducing equivalents from the cytoplasm to mitochondria. 6. The effects of dinitrophenol are accounted for in terms of decreased ATP concentrations in the cells, leading to marked decrease in pyruvate carboxylation in the mitochondria and depression of fatty acid synthesis in the cytoplasm.

Previous studies in this Laboratory have attempted to establish a balance of utilization and formation of reduced nicotinamide nucleotides and ATP in adipose-tissue slices (Rognstad & Katz, 1966; Katz & Rognstad, 1966). Under conditions of high lipogenesis we found a near balance between the total NADH and NADPH formed in the cytoplasm and that required for synthesis of fatty acids, glycerol and lactate in the cytoplasm. The pentose cycle contributed about half the NADPH required for fatty acid synthesis, and the rest was supplied by transhydrogenation from cytoplasmic NADH, catalysed by malate dehydrogenase (EC 1.1.1.37) and 'malic' enzyme (EC 1.1.1.40) (Katz, Landau & Bartsch, 1966; Flatt & Ball, 1964). We also found that under these conditions the total ATP production did not greatly exceed the ATP requirements for fatty acid synthesis and esterification. Most of the ATP formation in this tissue occurred in the mitochondria, but most of it was utilized in the cytoplasm.

A number of studies (Young, Shrago & Lardy, 1964; Kornacker & Ball, 1965) support the concept that conversion of pyruvate into cytoplasmic acetyl-CoA proceeds via the 'pyruvate cycle' (Rognstad & Katz, 1966), i.e. via conversion into oxaloacetate and citrate in the mitochondria, with citrate passing into the cytoplasm where it is cleaved to acetyl-CoA and oxaloacetate. Accordingly three molecules of ATP are required in the synthesis from pyruvate of each C_2 moiety of a fatty acid, one molecule of ATP each in the reactions catalysed by pyruvate carboxylase (EC 6.4.1.1), citrate-cleavage enzyme (EC 4.1.3.8) and acetyl-CoA carboxylase (EC 6.4.1.2). (For the terminal C_2 unit only two ATP molecules are required.) It was decided to study the metabolism of adipose tissue under conditions when ATP synthesis is decreased. With limited ATP production, alternative pathways for fatty acid synthesis requiring less ATP, such as the carnitine pathway (Fritz, 1963), may become apparent. In the present experiments we

used 2,4-dinitrophenol to decrease the ATP supply in rat epididymal fat-pad tissue.

METHODS

Male Wistar rats were starved for 24 hr. and then returned to their previous fat-free high-carbohydrate diet (Nutritional Biochemical Co., Cleveland, Ohio, U.S.A.) for 48 hr. ('starved–re-fed' animals). Epididymal fat-pad segments (approx. 300 mg.) were incubated for 3–4 hr. at 37° in Krebs–Henseleit buffer (Krebs & Henseleit, 1932) in an atmosphere of CO_2+O_2 (5:95) with 25 μmoles of glucose plus labelled substrates as indicated. Duplicate flasks contained dinitrophenol (0·15–0·5 mM). The major metabolic products were isolated as described previously (Katz *et al.* 1966; Rognstad & Katz, 1966). [2-T]Glucose was synthesized as described by Katz & Dunn (1967). [3-T]-Glucose was synthesized by the method of Gabriel & Ashwell (1965). [4-T]Glucose was synthesized by an enzymic method (Rognstad, Kemp & Katz, 1965). Other ^{14}C- and T-labelled substrates were purchased from New England Nuclear Corp. (Boston, Mass., U.S.A.).

Lactate degradations were carried out essentially as described by Katz, Abraham & Chaikoff (1955). The first step (lactate → acetate) was modified by carrying out the $KMnO_4$ oxidation in 0·2 M-H_3PO_4 (final concn.) at 4° for 16 hr.

RESULTS

Effect of dinitrophenol on glucose utilization. Table 1 presents results of experiments in which epididymal fat-pad segments from starved–re-fed rats were incubated in replicate with various concentrations of dinitrophenol. Glucose utilization and specific ^{14}C yields from glucose labelled with ^{14}C uniformly and in C-1 and C-6 are shown. Specific ^{14}C yield is defined as the fraction or percentage of utilized ^{14}C-labelled substrate incorporated into a product. In the absence of dinitrophenol, the results with [U-^{14}C]glucose showed that incorporation into fatty acids accounted for 35–52% of the glucose carbon utilized. The pentose cycle contribution was in the range 22–26% (Table 2).

A dinitrophenol concentration that would give partial uncoupling of oxidative phosphorylation, without completely depressing fatty acid synthesis, was needed. This condition was obtained only within a narrow range of concentrations, which was not closely reproducible. Dinitrophenol concentrations below 0·1 mM showed little or no effect on adipose-tissue metabolism, and concentrations of 0·5 mM caused a depression of glucose utilization and nearly complete cessation of lipogenesis. Within the range 0·1–0·3 mM, glucose utilization was not changed or even somewhat increased, but fatty acid synthesis was considerably depressed. Apparently the response to dinitrophenol is modified by metabolic conditions of the tissue.

It has been previously stressed (Katz *et al.* 1966) that yields of ^{14}C from [1-^{14}C]- and [6-^{14}C]-glucose into lactate, carbon dioxide, fatty acids, glycerol and other products do not measure quantitatively the synthesis of these compounds, since the specific radioactivity of the hexose 6-phosphates and triose phosphates depends markedly on the pentose cycle and the extent of isotope equilibration. ^{14}C yields from [U-^{14}C]glucose are not affected by these factors, and provide a true measure of incorporation of glucose carbon, but do not permit establishment of a complete carbon balance. However, such a balance can be calculated (Katz *et al.* 1966) from the ^{14}C yields of the three labelled glucoses used.

The Appendix describes some of the methods used to calculate the activity of the pentose cycle and of the tricarboxylic acid cycle. The metabolism of glucose via the pentose cycle was calculated from the data of Table 1 by four methods, with the results presented in Table 2. The agreement between independent methods is good.

Table 3 presents estimations of metabolic flows to the major products formed from glucose in the experiments of Table 1. We have previously reported (Katz *et al.* 1966; Rognstad & Katz, 1966) that at high rates of lipogenesis in adipose tissue oxidation via the tricarboxylic acid cycle is small. This is also shown in Table 3. Without dinitrophenol, carbon dioxide formed in the tricarboxylic acid cycle constitutes from less than 1% to 4% of the utilized glucose carbon. With dinitrophenol, oxidation via the tricarboxylic acid cycle was increased three- to six-fold.

Moderate concentrations of dinitrophenol, which did not affect glucose utilization, decreased fatty acid synthesis to one-quarter of normal values. Higher concentrations of dinitrophenol depressed it to very low values, and the major product of glucose metabolism became lactate. Lactate synthesis without dinitrophenol varied in the range 0·1–0·4 mole of lactate/mole of glucose and increased to 1·5 moles/mole in its presence. Synthesis of lipid glycerol was relatively small, and was not greatly affected by dinitrophenol. It is not recorded in Table 3.

Effect of dinitrophenol on the pentose cycle and transhydrogenation. It has been shown (Katz & Rognstad, 1966) that the NADPH formed via the pentose cycle is used in adipose tissue nearly exclusively for the reduction of acetyl-CoA to fatty acids, but that with high rates of fatty acid synthesis this NADPH is not sufficient to produce all the reducing equivalents. Under such circumstances from one-half to two-thirds of the NADPH needed for fatty acid formation is provided by the pentose cycle and the rest by transhydrogenation from cytoplasmic NADH.

The last column of Table 3 shows the maximal contribution of the NADPH formed in the pentose

Table 1. *Glucose utilization and specific yields in the major products from ^{14}C-labelled glucose and succinate*

Epididymal fat-pad segments from starved–re-fed rats were incubated in 2 ml. of Krebs–Henseleit buffer with 1 unit of insulin and 25 μmoles of glucose (and 1 μmole of succinate in Expt. 2) for 3 hr. at 37°. Specific yield is defined in the text. n.d., Not determined.

Expt. no.	Labelled substrate: Glucose	Labelled substrate: Succinate	Concn. of dinitrophenol (mM)	Glucose utilization (μmoles/g./3 hr.)	Specific yields: CO_2	Specific yields: Fatty acids	Specific yields: Lipid glycerol	Specific yields: Lactate
1A	1-^{14}C		0	48	0·461	0·444	0·051	0·044
	6-^{14}C				0·020	0·840	0·051	0·089
	U-^{14}C				0·337	0·526	0·060	0·076
1B	1-^{14}C		0·25	51	0·349	0·176	0·035	0·440
	6-^{14}C				0·076	0·265	0·033	0·627
	U-^{14}C				0·227	0·153	0·035	0·583
2A	1-^{14}C		0	20	0·460	0·336	0·055	0·149
	6-^{14}C				0·028	0·662	0·076	0·234
	U-^{14}C				0·320	0·353	0·088	0·238
		1,4-^{14}C			0·971	0·029	—	n.d.
		2,3-^{14}C			0·225	0·775	—	n.d.
2B	1-^{14}C		0·15	22	0·326	0·122	0·045	0·507
	6-^{14}C				0·087	0·209	0·042	0·662
	U-^{14}C				0·202	0·108	0·042	0·647
		1,4-^{14}C			0·953	0·047	—	n.d.
		2,3-^{14}C			0·673	0·327	—	n.d.
2C	1-^{14}C		0·35	14	0·162	0·030	0·037	0·771
	6-^{14}C				0·082	0·037	0·023	0·857
	U-^{14}C				0·186	0·035	0·040	0·740
		1,4-^{14}C			0·970	0·030	—	n.d.
		2,3-^{14}C			0·909	0·091	—	n.d.
3A	1-^{14}C		0	25	0·541	0·296	0·107	0·056
	6-^{14}C				0·063	0·652	0·123	0·163
	U-^{14}C				0·396	0·418	0·103	0·084
3B	1-^{14}C		0·2	28	0·296	0·078	0·089	0·537
	6-^{14}C				0·154	0·134	0·053	0·659
	U-^{14}C				0·268	0·079	0·062	0·591
4A	1-^{14}C		0	19	0·494	0·339	0·065	0·102
	6-^{14}C				0·023	0·710	0·078	0·191
	U-^{14}C				0·374	0·415	0·067	0·144
4B	1-^{14}C		0·3	14	0·196	0·036	0·026	0·742
	6-^{14}C				0·114	0·061	0·024	0·802
	U-^{14}C				0·179	0·037	0·028	0·755

cycle to reductive fatty acid synthesis in the experiments of Table 1. Without dinitrophenol, the contributions are in the range 45–70%; with dinitrophenol, though the percentage metabolism of glucose by the pentose cycle is markedly decreased (Table 2), this decreased pentose cycle activity is sufficient to provide 90–100% of the required hydrogen equivalents. These results suggest the suppression of transhydrogenation by dinitrophenol.

This conclusion is supported by the T/^{14}C ratios in fatty acids from glucose labelled uniformly with ^{14}C and with tritium in positions 2, 3 and 4 (Table 4). The Appendix depicts the metabolism of hydrogen on these three positions of glucose. Dinitrophenol causes a large increase in the T/^{14}C ratio in fatty acids from [U-^{14}C,3-T]- or [U-^{14}C,2-T]-glucose, both of which produce NADPT in the pentose cycle. If the radioactivity of T and ^{14}C of glucose is 1·0, the relative specific radioactivity of the T in position 3 of glucose 6-phosphate will be $1/(1+2PC)$ and that of the acetyl unit will be 1/3. Hence when 12% of the glucose was metabolized by the pentose cycle in the presence of dinitrophenol (Table 2) the T/^{14}C ratio in palmitate should be $3 \times 7/1{\cdot}24 \times 8 = 2{\cdot}1$, and when 5% was so metabolized it should be $3 \times 7/1{\cdot}1 \times 8 = 2{\cdot}4$. Thus, if the pentose cycle is the sole provider of NADPH for fatty acid synthesis and all of it is used solely for fatty acid synthesis, the T/^{14}C ratio from [U-^{14}C,3-T]glucose should attain a ratio in the

Table 2. *Calculations of the percentage metabolism of glucose by the pentose cycle*

The experiments are those of Table 1. The methods of calculation and γ, δ and τ are defined in the Appendix. Method I uses eqn. (13) of the Appendix; method II uses eqn. (16) of the Appendix; method III uses eqn. (19) of Katz *et al.* (1966); method IV uses eqn. (17) of the Appendix. The average value quoted is that of methods II, III and IV, which do not require triose phosphate equilibration.

					Percentage glucose metabolism by pentose cycle				
Expt. no.	Dinitrophenol	γ*	δ	τ	Method I (from γ)	Method II (from τ)	Method III (from γ, δ)	Method IV (from $^{14}CO_2$)	Average
1A	−	0·524 (F.A.)	1·00	0·545	23·2	21·8	22·3	21·5	22
1B	+	0·702 (Lac)	1·06	0·699	12·4	12·5	11·9	12·3	12
2A	−	0·507 (F.A.)	0·72	0·543	24·5	21·9	23·2	21·2	22
2B	+	0·766 (Lac)	1·07	0·723	9·3	11·3	8·7	10·4	10
2C	++	0·900 (Lac)	1·52	0·914	2·6	2·4	3·0	3·1	3
3A	−	0·454 (F.A.)	0·87	0·480	28·6	26·5	26·6	25·9	26
3B	+	0·815 (Lac)	1·68	0·814	7·0	7·1	5·8	6·4	6
4A	−	0·478 (F.A.)	0·82	0·506	26·7	24·5	25·2	23·8	25
4B	++	0·925 (Lac)	1·08	0·897	2·6	3·7	2·5	3·2	3

* Product used in calculating γ is given in parenthesis (F.A., fatty acids; Lac, lactate).

Table 3. *Calculated flows through the major metabolic pathways in the experiments of Table* 1

The methods used to derive, from the results in Table 1, values for the moles of CO_2, fatty acid acetyl units and lactate formed/mole of glucose and for the contribution of NADPH formed in the pentose cycle to fatty acid synthesis are described in the Appendix.

		Product formed (moles/mole of glucose)					Contribution of pentose cycle NADPH to fatty acid synthesis (%)
Expt. no.	Dinitrophenol	CO_2 via tricarboxylic acid cycle	CO_2 via pentose cycle	CO_2 via pyruvate decarboxylation	Fatty acids (acetyl units)	Lactate	
1A	−	<0·05	0·63	1·6	1·6	0·15	46
1B	+	0·36	0·36	0·64	0·46	1·1	90
2A	−	0·20	0·60	1·3	1·2	0·40	64
2B	+	0·36	0·36	0·51	0·33	1·3	100
2C	++	0·60	0·09	0·40	0·08	1·5	100
3A	−	0·22	0·78	1·3	1·2	0·17	70
3B	+	0·78	0·24	0·63	0·24	1·2	100
4A	−	0·18	0·72	1·3	1·2	0·30	65
4B	++	0·59	0·12	0·42	0·11	1·5	100

range 2·1–2·4. Ratios in the range 1·6–2·0 were observed (Table 4). The difference between these and the theoretical ratios could be due to some dilution of the acetyl-CoA pool with unlabelled material. The oxidation of 0·5 μmole of endogenous palmitate/g. of tissue/3 hr. could account for such a dilution.

The T/^{14}C ratio in fatty acids from [U-^{14}C,4-T]-glucose was depressed in the presence of dinitrophenol. Tritium from position 4 is transferred to NADH in the glyceraldehyde phosphate dehydrogenase reaction. Decrease of this ratio indicates a diminished role of the NADH → NADPH transhydrogenation mechanism in fatty acid biosynthesis. It is doubtful whether T/^{14}C ratios less than 0·05 are significant, since determination of small amounts of tritium in the presence of excess of ^{14}C is unreliable.

Dinitrophenol increased T/^{14}C ratios from [U-^{14}C,3-T]glucose into glycerol. This is in part due to the decrease in pentose cycle activity leading to an increase in the specific radioactivity of tritium in position 3. A small amount of tritium appears in lactate from [3-T]glucose. This could be due either to a small amount of NADPT being used to reduce pyruvate, or to some tritium in other than position 3 of the substrate.

Effect of dinitrophenol on succinate oxidation and randomization of ^{14}C in lactate. In Table 1 [1,4-^{14}C]- and [2,3-^{14}C]-succinate were used as the labelled substrates in the presence of unlabelled glucose in Expt. 2. In this experiment the ratio of the specific

Table 4. $T/^{14}C$ *ratios in glycerol, lactate and fatty acids*

The experiments are those described in Table 1. $T/^{14}C$ ratios are expressed relative to a substrate $T/^{14}C$ ratio of 1·0. n.d., Not determined.

Expt. no.	Label in glucose	Dinitrophenol	$T/^{14}C$ ratios in Glycerol	Lactate	Fatty acids
1A	6-^{14}C,6-T	−	0·99	0·79	0·19
	U-^{14}C,3-T	−	0·66	0·13	0·81
	U-^{14}C,4-T	−	0·64	0·22	0·17
	U-^{14}C,2-T	−	0·65	0·39	0·32
1B	6-^{14}C,6-T	+	1·03	0·85	0·22
	U-^{14}C,3-T	+	1·15	0·07	1·74
	U-^{14}C,4-T	+	0·89	0·57	0·08
	U-^{14}C,2-T	+	0·75	0·44	0·53
2A	6-^{14}C,6-T	−	1·02	0·77	0·20
	U-^{14}C,3-T	−	0·46	0·05	1·09
	U-^{14}C,4-T	−	0·63	n.d.	0·09
2B	6-^{14}C,6-T	+	0·93	0·94	0·22
	U-^{14}C,3-T	+	0·83	0·05	1·82
	U-^{14}C,4-T	+	0·87	n.d.	0·04
2C	6-^{14}C,6-T	++	0·91	0·97	0·24
	U-^{14}C,3-T	++	1·11	0·05	1·74
	U-^{14}C,4-T	++	0·95	n.d.	n.d.
3A	U-^{14}C,3-T	−	0·66	0·00	0·88
3B	U-^{14}C,3-T	+	1·13	0·00	2·01
4A	U-^{14}C,3-T	−	0·36	n.d.	0·91
	U-^{14}C,2-T	−	0·37	n.d.	0·28
4B	U-^{14}C,3-T	++	0·85	n.d.	1·58

yield of $^{14}CO_2$ from [1,4-^{14}C]succinate to the specific yield from [2,3-^{14}C]succinate decreased from 4·3 in the absence of dinitrophenol to 1·07 at the highest dinitrophenol concentration used. If succinate were oxidized completely in the tricarboxylic acid cycle, with no outflow from the cycle, the expected ratio would be 1. Ratios greater than 1 indicate inflow and outflow from the cycle (Katz & Chaikoff, 1955). Such outflow may represent utilization of a tri- or di-carboxylic acid by passage from the mitochondria to the cytoplasm for synthesis of amino acids or other intermediates. We have shown elsewhere (Rognstad, 1968) that a high ratio is consistent with an interacting 'pyruvate cycle'–tricarboxylic acid cycle model when the rate of fatty acid synthesis is high and the tricarboxylic acid cycle activity is low. The theory predicts that as fatty acid synthesis decreases and the tricarboxylic acid cycle activity increases this ratio should approach 1, which agrees with the data of Expt. 2C (see the Discussion section).

In Table 5 the degradation of lactate formed from [6-^{14}C]glucose is shown. In the absence of dinitrophenol there is a considerable randomization of ^{14}C into position 2 of lactate. In the presence of dinitrophenol there is very little randomization of

Table 5. *Degradation patterns of lactate formed on incubation of adipose tissue with* [6-^{14}C]*glucose*

The experiments are those described in Table 1 and the method of lactate degradation is described in the Methods section.

Expt. no.	Dinitrophenol	Relative specific radioactivity of carbon atoms of lactate C-3	C-2	C-1
3A	−	100	21	3
3B	+	100	4	3
4A	−	100	21·6	2·1
4B	+	100	1·5	1·2

^{14}C. (In control degradations of [3-^{14}C]lactate about 1% of the total radioactivity is recovered in C-1 and C-2, probably owing to overoxidation in the procedure.) These results are consistent with interconversion of pyruvate and fumarate via oxaloacetate. Scheme 4 of the Appendix illustrates the pathway probably followed, involving the enzymes of the 'pyruvate cycle' as well as mitochondrial malate dehydrogenase and fumarase. Little or no carboxylation of pyruvate appears to occur in the presence of dinitrophenol. The

Table 6. *Effects of dinitrophenol on the metabolism of pyruvate and lactate by adipose tissue*

Epididymal fat-pad segments from starved–re-fed rats (or rats starved overnight, Expt. 7) were incubated in 2ml. of Krebs–Henseleit buffer for 3hr. at 37°. The substrate concentration was 0·01M. The dinitrophenol concentration was 0·4mM (Expt. 5), 0·5mM (Expt. 6) or 0·3mM (Expts. 7 and 8). Thyroxine-treated rats were injected daily for 1 week with 0·2ml. of 0·5% thyroxine solution.

Expt. no.	Dietary status	Dinitrophenol	Substrate	Utilization (μmoles/g./3hr.)	% of added ^{14}C in CO_2	Fatty acids	Glycerol	Lactate	Pyruvate
5	Starved–re-fed	−	[U-^{14}C]Pyruvate	32	32	18	2·2	4·8	
		+		21	29	0·3	0	7·6	
		−	[U-^{14}C]Lactate	10	11	5·0	0·6	—	0·8
		+		1·7	2·6	0·1	0	—	0·2
6	Starved–re-fed	−	[U-^{14}C]Pyruvate	44	42	24		9·3	—
		+		58	67	3·1		17	—
		−	[U-^{14}C]Lactate	34	29	23		—	
		+		5·4	7·5	0·6		—	
7	Starved	−	[U-^{14}C]Pyruvate	37	18	8·7		10	—
		+		41	28	1·3		12	—
		−	[U-^{14}C]Lactate	5·2	4·3	0·2		—	0·6
		+		3·2	2·6	0		—	0·6
8A	Starved–re-fed	−	[U-^{14}C]Pyruvate	26·0	40·5	15·2		9·2	—
		+		27·8	58·1	1·4		9·9	—
		−	[U-^{14}C]Lactate	3·2	7·4	0·7		—	
		+		1·0	2·5	0		—	
8B	Starved–re-fed (thyroxine-treated)	−	[U-^{14}C]Pyruvate	44·4	34·6	6·5		14·2	—
		+		47·2	46·1	0·4		12·7	—
		−	[U-^{14}C]Lactate	8·4	10·2	0·4		—	—
		+		3·5	4·4	0		—	

significance of this finding is considered in the Discussion section.

Oxidation of lactate and pyruvate. Table 6 presents the results of experiments when either pyruvate or lactate was used as sole substrate for adipose-tissue slices. In tissue of fed rats, without dinitrophenol, both substrates are metabolized well by adipose tissue, and lipogenesis is quite high. In the presence of dinitrophenol, pyruvate is still extensively oxidized, although very little lipid synthesis occurs. Lactate utilization, however, is markedly decreased by dinitrophenol. The possible cause of the divergent effects of dinitrophenol on pyruvate and lactate utilization is considered in the Discussion section.

DISCUSSION

ATP balance. In the metabolism of glucose, ATP is generated in the cytoplasm in the glyceraldehyde phosphate dehydrogenase and pyruvate kinase reactions. By subtracting the ATP required for phosphorylation of glucose and fructose 6-phosphate, the amount of ATP available for biosynthesis and other metabolic functions is obtained. The amount of such available cytoplasmic ATP was calculated from the data of Tables 1 and 3 and is reported in Table 7. In the synthesis of fatty acids, two molecules of ATP per C_2 unit are required in the cytoplasm and one in the mitochondria. The cytoplasmic ATP requirement can then be calculated on the basis of a need for 15 ATP molecules for the synthesis of one molecule of palmitate (eight for citrate cleavage, seven for malonyl-CoA synthesis). Two ATP molecules (one pyrophosphate group) are required for re-esterification of a fatty acid molecule formed by lipolysis of endogenous fat. Re-esterification was calculated from the amount of glycerol synthesis in excess of that needed to esterify newly synthesized fatty acids. The results are given in Table 7 and the details of the calculation are given in the legend to the Table.

In the absence of dinitrophenol, the supply of cytoplasmic ATP was only about half the requirement, and a flow of ATP from mitochondria must have supplied the excess needed. The main source for mitochondrial ATP is the NADH formed in the oxidation of pyruvate. We have shown previously (Rognstad & Katz, 1966) that there is a fairly close balance in adipose tissue under conditions of high lipogenesis between ATP formation and utilization of ATP.

Table 7. *Cytoplasmic ATP and reduced nicotinamide nucleotide balances*

The experiments are those of Table 1.

		Cytoplasmic ATP (moles/mole of utilized glucose)		Cytoplasmic reduced nicotinamide nucleotide (moles/mole of utilized glucose)		
					Formed	
Expt. no.	Dinitrophenol	Available*	Required for lipogenesis†	Used for synthesis‡	Total§	After malate shuttle‖
1A	−	1·4	3·0	3·0	3·0	3·0
1B	+	1·6	0·9	2·1	2·6	2·2
2A	−	1·3	2·3	2·5	2·9	2·5
2B	+	1·6	0·5	2·0	2·5	2·2
2C	++	1·7	0·3	1·8	2·1	2·0
3A¶	−	1·1	2·5	2·8	3·1	2·7
3B¶	+	1·6	0·6	1·9	2·2	2·0
4A	−	1·3	2·4	2·8	3·0	3·0
4B	++	1·8	0·3	1·8	2·1	2·0

* ATP produced by phosphoglycerate kinase and pyruvate kinase less that used by hexokinase and phosphofructokinase.

† For citrate cleavage, malonyl-CoA synthesis and fatty acid re-esterification.

‡ Of fatty acids, lactate and glycerol (does not include cytoplasmic synthesis of intermediate malate).

§ Pentose cycle and glyceraldehyde phosphate dehydrogenase.

‖ As in §, but less the NADH removed by 'malate shuttle' (see the Discussion section). Example of 'malate shuttle' calculation (Expt. 1B): From the [U-^{14}C]glucose yield in fatty acids, 0·46 (= 3 × 0·153) mole of oxaloacetate is produced by the citrate-cleavage enzyme. This is reduced to malate by cytoplasmic NADH. The NADPH required for fatty acid synthesis is 0·81. The NADPH produced by the pentose cycle is 0·72. The difference between these two values is the NADPH produced by the transhydrogenase mechanism, involving the 'malic' enzyme. Subtraction of the flow via the 'malic' enzyme from the malate formed gives the malate that flows into the mitochondria (0·46 − 0·09 = 0·37). This value is subtracted from the total reduced nicotinamide nucleotide formed to give the values shown in the last column.

¶Flasks in Expt. 3 also contained a small amount of pyruvate. Flasks with ^{14}C-labelled pyruvate plus unlabelled glucose and ^{14}C-labelled glucose plus unlabelled pyruvate were used. The NADH for lactate synthesis and the NADPH for fatty acid synthesis from [U-^{14}C]pyruvate are added to the values obtained from [^{14}C]glucose.

In the presence of dinitrophenol the situation is radically different. ATP production in the cytoplasm is somewhat greater, but the ATP requirement much less. A flow of excess of ATP from cytoplasm to mitochondria must occur, where it is presumably hydrolysed by the adenosine triphosphatase activity induced by dinitrophenol.

Balance of reduced nicotinamide nucleotides in the cytoplasm. In Table 7 the production of reducing equivalents and their utilization are compared. In the absence of dinitrophenol production and utilization nearly balance out. This has been previously shown (Rognstad & Katz, 1966). In the presence of dinitrophenol, there is some excess, about 10–20%, of reduced nicotinamide nucleotides. As discussed above (see Table 3), the NADPH formed in the pentose cycle suffices for fatty acid synthesis and the excess is in the form of NADH. A way of disposing of this excess of reducing equivalents and regenerating NAD^+ is required.

In the formation of cytoplasmic acetyl-CoA by the citrate-cleavage enzyme one molecule of oxaloacetate is produced in the cytoplasm. Under normal conditions when fatty acid synthesis is extensive, the oxaloacetate is reduced by the excess of cytoplasmic NADH to malate, and the malate is decarboxylated to pyruvate and NADPH. Pyruvate re-enters the mitochondria, where it serves to re-form citrate. This constitutes a system, designated the 'pyruvate cycle', which serves for acetyl-CoA transfer and transhydrogenation from NADH to NADPH. In the presence of dinitrophenol such a transhydrogenation does not occur. We suggest that, in the presence of dinitrophenol, an excess of NADH reduces oxaloacetate to malate, which is not decarboxylated, but passes into the mitochondria, where it is reoxidized.

In the last column of Table 7 the extra NADH, presumably transferred into mitochondria, is subtracted from the available cytoplasmic reducing equivalents. This gives, within experimental error, a close balance of reducing equivalents. A number of observations supporting the operation of the 'malate shuttle' for reducing equivalents are given below.

Pyruvate carboxylation. When the tricarboxylic acid cycle operates essentially without outflow, addition of trace amounts of a labelled dicarboxylic

acid will yield only carbon dioxide, and the ratio of the $^{14}CO_2$ from carboxyl groups to the $^{14}CO_2$ from inner carbon atoms will be close to 1·0. If outflow of citrate into the cytoplasm occurs, the ratio will still be 1·0 provided that the oxaloacetate formed after citrate cleavage is returned and reutilized. However, should the oxaloacetate be decarboxylated to pyruvate, which is either converted into lactate or further decarboxylated to acetyl-CoA, the ratio will be greater than 1·0, since in this case there is inflow and outflow of isotope from the cycle. A detailed mathematical analysis of the effect of inflow–outflow on the $^{14}CO_2$ ratio from succinate has been provided elsewhere (Rognstad, 1968). The decrease of the '$^{14}CO_2$ ratio' (Table 1, Expt. 2) from 4 to 1 in the presence of dinitrophenol provides support for the normal occurrence of extensive carboxylation–decarboxylation reactions between pyruvate and oxaloacetate, and the virtual absence of these reactions in the presence of dinitrophenol.

The degradation of the lactate formed from [6-^{14}C]glucose also supports this conclusion. Lactate from [6-^{14}C]glucose is partially randomized under normal conditions from C-3 into C-2, and not randomized in the presence of dinitrophenol (Table 6). This indicates the absence of exchange of pyruvate carbon with dicarboxylic acids in the presence of dinitrophenol, probably owing to the absence of pyruvate carboxylation, and supports the operation of the modified 'pyruvate cycle', with a return of malate into the mitochondria.

The suppression of pyruvate carboxylation in the presence of dinitrophenol is an attractive hypothesis, because it serves to conserve the limited supply of ATP.

Dinitrophenol and oxidation of lactate and pyruvate. When pyruvate is the sole substrate, all ATP and reducing equivalents for lipogenesis must originate in the mitochondria, except for those formed from endogenous glycogen. Hence lipogenesis from pyruvate is suppressed to a greater extent by dinitrophenol than that from glucose.

The observation that in the presence of dinitrophenol oxidation of pyruvate is stimulated or not affected, but that of lactate is suppressed, is of considerable interest. The likely explanation is the absence of a carrier system to transport cytoplasmic NADH formed in the lactate dehydrogenase reaction into mitochondria. It appears that sufficient oxaloacetate is not synthesized in mitochondria, or that it cannot pass into the cytoplasm, to serve as a carrier for reducing equivalents.

This interpretation implies that an α-glycerophosphate shuttle functions little if at all in adipose tissue. Adipose tissue is known to contain mitochondrial α-glycerophosphate dehydrogenase (Lee & Lardy, 1965), which is stimulated by the administration of thyroxine. Cytoplasmic NADH-linked α-glycerophosphate dehydrogenase is, of course, present in adipose tissue, and functions in the synthesis of lipid glycerol. A function of the glycerophosphate shuttle in moving reducing equivalents from the cytoplasm to the mitochondria is clearly established in the flight muscle of insects (Zebe, Delbrück & Bücher, 1957). It may function also in other tissues. However, we have found as yet no evidence for its operation in adipose tissue, even in that of thyroxine-treated rats, when the activity of α-glycerophosphate dehydrogenase is greatly elevated (Lee & Lardy, 1965). We have some indication of a 'malate shuttle' in adipose tissue, but the evidence cannot be considered conclusive. However, this shuttle, if present, apparently can only operate when lipogenesis occurs, during which an oxaloacetate moiety is carried out of the mitochondria as citrate.

Energy balance and metabolism in adipose tissue. The effects of dinitrophenol on glucose metabolism can be well explained on the basis of limited availability of mitochondrial ATP. We suggest the limiting step to be the mitochondrial carboxylation of pyruvate to oxaloacetate. Our work emphasizes the value of balances of energy and reduced nicotinamide nucleotides in the interpretation of metabolic interrelationships. Dinitrophenol is a useful tool to study the effects of altered energy on metabolic pathways.

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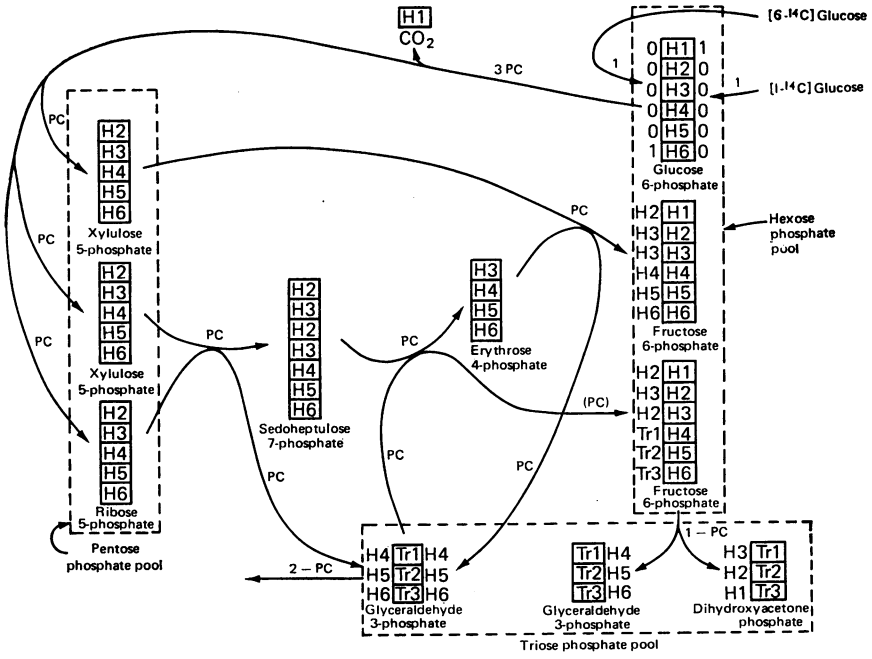
APPENDIX

Determination of pentose cycle activity

The methods used have been given previously (Katz & Wood, 1960; Katz, Landau & Bartsch, 1966; Landau & Katz, 1965) and are described here in an abbreviated form. Only methods based on the use of [1-^{14}C]- and [6-^{14}C]-glucose are discussed. Scheme 1 illustrates the flow of carbon in the pentose cycle. In this model it is assumed that the hexose phosphates and triose phosphates are in isotopic equilibrium, and that transaldolase and transketolase operate only in the direction of the forward pentose cycle. Fructose diphosphatase is absent from adipose tissue, and the net result of pentose cycle metabolism is the production of one molecule of glyceraldehyde phosphate and three molecules of carbon dioxide from one molecule of glucose 6-phosphate. The model used here is more complete than the one used in our earlier papers, and labelling of C-6 of hexose 6-phosphate, neglected at that time, is considered. Using Scheme 1, one can calculate the specific radioactivity of C-3 of triose phosphate as a function of the rate of the pentose cycle when either [1-^{14}C]- or [6-^{14}C]-glucose is the substrate. At steady state, inflow and outflow of isotope into any carbon atom are equal. H1, H6 and Tr3 are defined as the molar specific radioactivity of C-1 and C-6 of hexose phosphate and C-3 of triose phosphate respectively (i.e. counts/min. in the carbon atom designated/mole of the compound). The rate of glucose utilization (moles/unit time) and the molar specific radioactivity of the substrate glucose are set equal to 1. Superscripts in parentheses denote the position of label in the substrate glucose.

For [1-^{14}C]glucose the equations are (where PC represents pentose cycle activity):



Scheme 1. Metabolism of [1-^{14}C]- and [6-^{14}C]-glucose in the pentose cycle. The compounds are pictured as rectangles with each separate box representing individual carbon atoms. The numbers inside the boxes designate the specific radioactivities of the carbon atoms. Since the model assumes no reversible transaldolase and transketolase reactions, the specific radioactivities of the pentose phosphates, sedoheptulose phosphate and erythrose phosphate are given directly in terms of the specific radioactivities of the hexose phosphates. The numbers outside the boxes denote the specific radioactivities of the carbon atoms that flow into the compound by the reaction involved. The hexose phosphates are assumed to be in complete isotopic equilibrium, as are the pentose phosphates and triose phosphates.

C-1 of hexose phosphate

$$1 = H1^{(1)}(3PC+1-PC) = H1^{(1)}(1+2PC) \quad (1)$$

C-6 of hexose phosphate

$$PC \times Tr3^{(1)} = H6^{(1)}(1+PC) \quad (2)$$

C-3 of triose phosphate

$$(1+PC)H6^{(1)}+(1-PC)H1^{(1)} = Tr3^{(1)}(2-PC) \quad (3)$$

Solving these three equations with three unknowns yields:

$$H1^{(1)} = \frac{1}{1+2PC} \quad (4)$$

$$H6^{(1)} = \frac{PC}{2(1+PC)(1+2PC)} \quad (5)$$

$$Tr3^{(1)} = \frac{1}{2(1+2PC)} \quad (6)$$

For [6-^{14}C]glucose the equations are:

C-6 of hexose phosphate

$$1+PC \times Tr3^{(6)} = H6^{(6)}(1+PC) \quad (7)$$

C-3 of triose phosphate

$$(1+PC)H6^{(6)} = Tr3^{(6)}(2-PC) \quad (8)$$

Solving these equations yields:

$$H6^{(6)} = \frac{2-PC}{2(1-PC)(1+PC)} \quad (9)$$

$$Tr3^{(6)} = \frac{1}{2(1-PC)} \quad (10)$$

The ratio of the specific radioactivities of triose phosphate from [1-^{14}C]- and [6-^{14}C]-glucose is:

$$\frac{Tr3^{(1)}}{Tr3^{(6)}} = \frac{1-PC}{1+2PC} \quad (11)$$

Experimental values of the ratios of specific yields from [1-^{14}C]- and [6-^{14}C]-glucose in products derived from the triose phosphate pool, such as fatty acids or lactate, can be used to calculate the pentose cycle activity. By using square brackets to denote specific yields, and setting $[F.A.]^{(1)}/[F.A.]^{(6)} = \gamma$ (where F.A. represents fatty acids), from eqn. (11):

$$\gamma = \frac{1-PC}{1+2PC} \quad (12)$$

Rearranging:

$$PC = \frac{1-\gamma}{1+2\gamma} \quad (13)$$

Another method is to use specific yields in $^{14}CO_2$ from [1-^{14}C]- and [6-^{14}C]-glucose. The $^{14}CO_2$ produced in the pentose cycle from [1-^{14}C]glucose is equal to $3PC \times H1^{(1)}$, or, from eqn. (1), $3PC/(1+2PC)$. The $^{14}CO_2$ produced in reactions other than the pentose cycle from [1-^{14}C]glucose is equal to γ times the $^{14}CO_2$ produced from [6-^{14}C]glucose, since γ is the ratio of specific radioactivities in glyceraldehyde phosphate from [1-^{14}C]- and [6-^{14}C]-glucose, and all non-(pentose cycle) carbon dioxide is derived from this pool. If the original assumption is correct, that the triose phosphates are in isotopic equilibrium, $\gamma = (1-PC)/(1+2PC)$. Hence:

$$\underbrace{[CO_2]^{(1)}}_{\text{Total}} = \underbrace{\frac{3PC}{1+2PC}}_{\substack{\text{Pentose}\\ \text{cycle}}} + \underbrace{\left(\frac{1-PC}{1+2PC}\right)[CO_2]^{(6)}}_{\substack{\text{Non-pentose}\\ \text{cycle}}}$$

Rearranging this yields:

$$PC = \frac{[CO_2]^{(1)}-[CO_2]^{(6)}}{3-2[CO_2]^{(1)}-[CO_2]^{(6)}} \quad (14)$$

This equation is identical with that given by Katz *et al.* (1966), where the term S was used.

Both of the above methods require that triose phosphates be in isotopic equilibrium. However, it has been shown (Katz *et al.* 1966), and it is apparent from the data of Table 1 of the main paper that triose phosphate isomerase is not sufficiently active to bring glyceraldehyde 3-phosphate and dihydroxyacetone phosphate into complete isotopic equilibrium. The ratio of specific yields in glycerol from [1-^{14}C]- and [6-^{14}C]-glucose is always larger than the corresponding ratio in lactate or fatty acids. Since glycerol is not a major product except in tissue of starved rats or when a lipolytic hormone is added, equations that neglect incomplete equilibration of triose phosphates do not cause large errors. One procedure to correct for non-equilibration is to calculate an average triose phosphate ratio (τ) which is the ratio of the yields in the major metabolic products, derived from the triose phosphate pool, from [1-^{14}C]- and [6-^{14}C]-glucose. Thus:

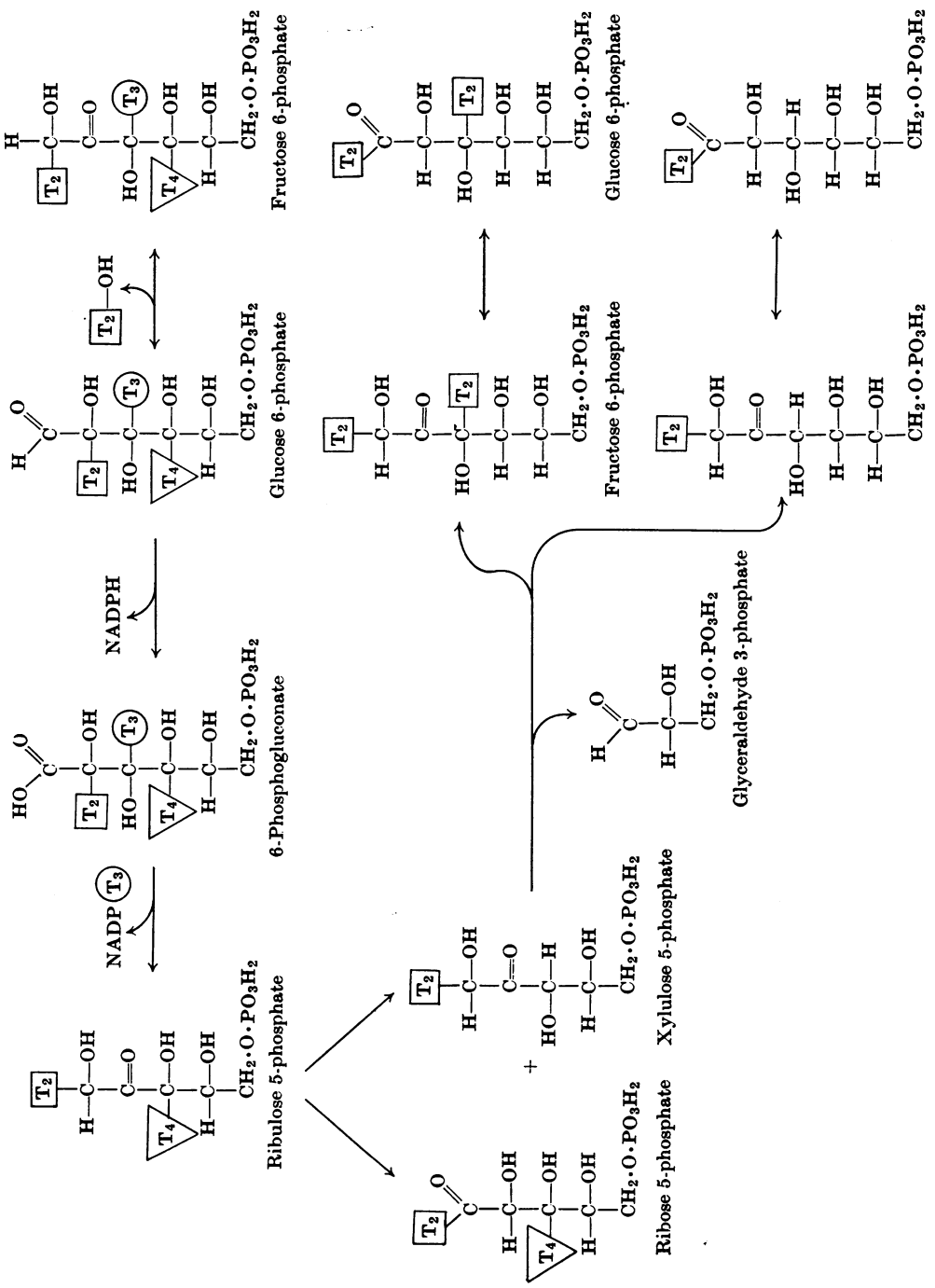
$$\tau = \frac{[F.A.]^{(1)}+[lactate]^{(1)}+[glycerol]^{(1)}}{[F.A.]^{(6)}+[lactate]^{(6)}+[glycerol]^{(6)}} \quad (15)$$

and

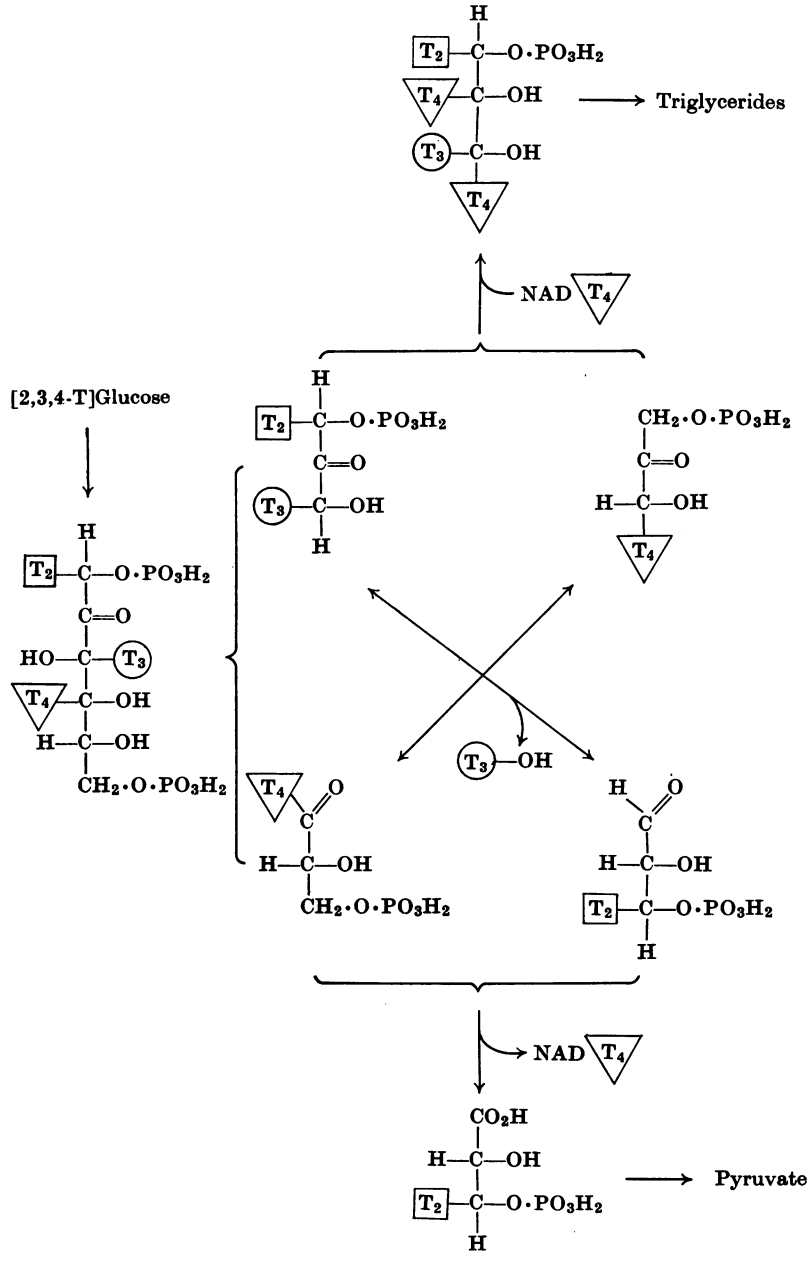
$$PC = \frac{1-\tau}{1+2\tau} \quad (16)$$

This expression for τ is an adequate approximation if carbon dioxide yield via the tricarboxylic acid cycle is small.

Another method that corrects for unequal triose phosphate equilibration is to use $^{14}CO_2$ yields, as above, together with an experimental value of γ,



Scheme 2. Metabolism of [2,3,4-T]glucose by the pentose cycle and hexose phosphate isomerase. For details see the text of the Appendix.



Scheme 3. Metabolism of [2,3,4-T]glucose in the Embden–Meyerhof pathway, including hydrogen exchange in the triose phosphate isomerase reaction. For details see the text of the Appendix.

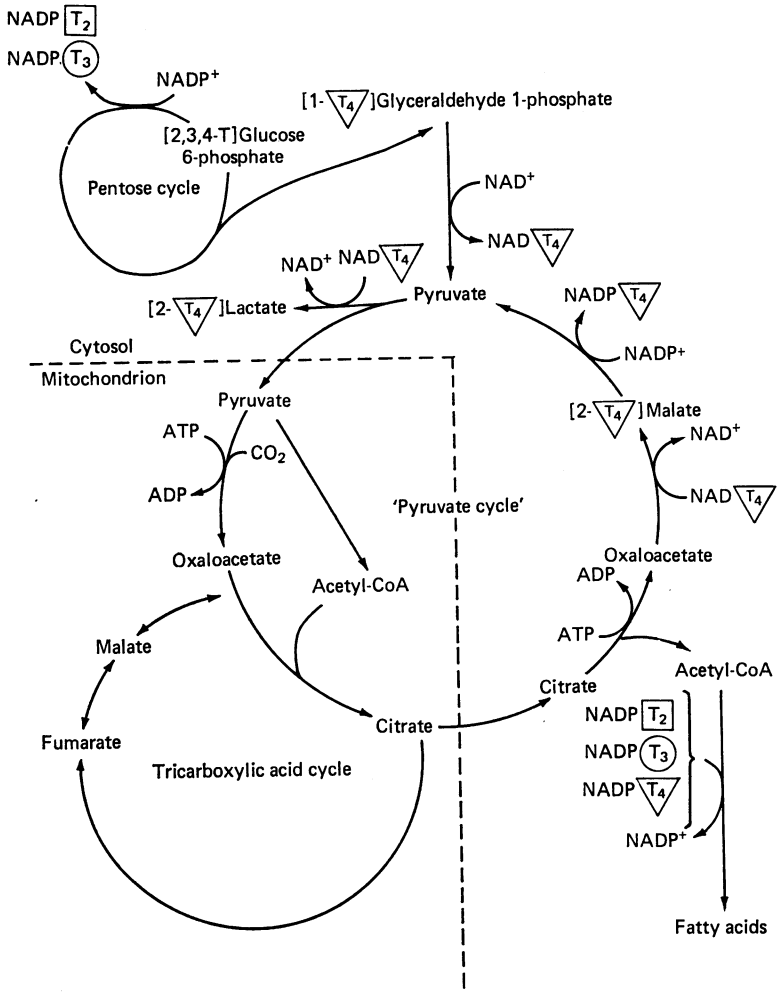
such as $[F.A.]^{(1)}/[F.A.]^{(6)}$, rather than the value of eqn. (12). Then:

$$[CO_2]^{(1)} = \frac{3PC}{1+2PC} + \gamma[CO_2]^{(6)}$$

Rearranging this expression yields:

$$PC = \frac{[CO_2]^{(1)} - \gamma[CO_2]^{(6)}}{3 + 2\gamma[CO_2]^{(6)} - 2[CO_2]^{(1)}} \quad (17)$$

An additional procedure is given by eqn. (19) of



Scheme 4. Metabolism of [2,3,4-T]glucose in the 'pyruvate cycle' and in fatty acid and lactate synthesis. For details see the text of the Appendix.

Katz *et al.* (1966). This method requires determination of γ, and also δ, defined as [glycerol](1)/[glycerol](6), and is valid when triose phosphate equilibration is incomplete.

Estimation of tricarboxylic acid cycle activity

When pyruvate from [U-^{14}C]glucose is further metabolized to fatty acids, the amount of $^{14}CO_2$ produced in the pyruvate dehydrogenase reaction is equal to one-half the amount of [^{14}C]acetyl-CoA produced, i.e. to one-half the specific yield in fatty acids. By subtracting this carbon dioxide, and also the carbon dioxide produced in the pentose cycle, from the total $^{14}CO_2$ specific yield from [U-^{14}C]glucose, the amount of pyruvate oxidized completely to $^{14}CO_2$ is found. Two-thirds of this is formed in the tricarboxylic acid cycle and one-third in the pyruvate dehydrogenase reaction. When the tricarboxylic acid cycle activity is low, this estimate is subject to considerable error, since it involves a small difference between rather large values. A preferable method would be to use glucose labelled with ^{14}C in C-4, C-5 and C-6 only. With this substrate no $^{14}CO_2$ is produced in the pentose cycle, and the tricarboxylic acid cycle $^{14}CO_2$ yield is equal to $\frac{2}{3}([CO_2]-\frac{1}{2}[F.A.])$.

Another procedure using the $^{14}CO_2$ yield from [6-^{14}C]glucose has been described (Katz *et al.* 1966). This method assumes equal oxidation of C-3 and C-2 of the pyruvate produced from [6-^{14}C]glucose.

Though this assumption is not proved, when the tricarboxylic acid cycle activity is very low this method is probably the only practical method, since other methods involve small differences between very large quantities.

All the above methods neglect the contribution of endogenous fatty acid oxidation. Although in the body fat is the major source of respiratory carbon dioxide, in adipose tissue from fed rats (Flatt & Ball, 1964) this is a relatively minor pathway. However, this inflow is not always negligible and the calculated values of the tricarboxylic acid cycle can only be accepted with caution.

Proportion of NADPH required for fatty acid synthesis from the pentose cycle

From the pentose cycle determinations one can readily calculate the amount of NADPH produced by these reactions, since for each molecule of carbon dioxide produced two molecules of NADPH are formed, one each in the glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) reactions. The amount of NADPH formed by the pentose cycle (see Scheme 1) is thus 6PC. The amount of fatty acid synthesis is calculated from the [U-^{14}C]glucose specific yield. This is multiplied by 3 to convert molecules of glucose into molecules of acetyl groups. Most of the fatty acids synthesized in adipose tissue is palmitate. To convert acetyl-CoA into fatty acids requires two molecules of NADPH/C_2 group, except for the methyl terminal group. Thus $[\text{F.A.}]^{(U)} \times 3 \times 2 \times \frac{7}{8} = \text{NADPH}$ required for fatty acid synthesis.

Metabolism of [2-T]-, [3-T]- *and* [4-T]-*glucose*

Scheme 2 illustrates the fate of tritium atoms on C-2, C-3 and C-4 of glucose in the pentose cycle, and Scheme 3 shows their fate in the Embden–Meyerhof pathway. Scheme 4 indicates their distribution in the 'pyruvate cycle' and in lipid biosynthesis. Tritium on C-2 of glucose 6-phosphate remains carbon-bound during the first turn of the pentose cycle, but is rearranged (along with carbon) into positions 1 and 3 of the fructose 6-phosphate formed (Lienhard & Rose, 1964; Bloom, 1964; Katz & Rognstad, 1969). Tritium on C-1 of fructose 6-phosphate is in the 'glu' position, where 'glu' denotes the hydrogen that remains carbon-bound in the hexose phosphate isomerase reaction (Dorrer, Fedtke & Trebst, 1966). The proportion of [1-T^{glu},3-T]fructose 6-phosphate that undergoes a second turn of the pentose cycle will produce NADPT during this cycle. Tritium from C-4 of glucose is lost to water during the pentose phosphate epimerase (EC 5.1.3.1) reaction. Scheme 2 also shows that tritium from position 2 of glucose is partially lost to water in the hexose phosphate isomerase reaction, and partly transferred to the 'iso' position on C-1 of fructose 6-phosphate (Rose & O'Connell, 1961; Katz & Rognstad, 1969).

From Scheme 3 it is evident that tritium on C-3 of glucose will appear on C-1 of dihydroxyacetone phosphate and thus in lipid glycerol. This tritium is exchanged with water in the triose phosphate isomerase reaction (Rieder & Rose, 1959; Katz & Rognstad, 1966). Tritium from C-4 of glucose appears on C-1 of glyceraldehyde 3-phosphate, and subsequently forms NADT in the glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) reaction. This tritium on C-1 of glyceraldehyde 3-phosphate remains carbon-bound in the triose phosphate isomerase reaction and appears on C-1 of lipid glycerol. Position 2 of glycerol phosphate will also become labelled, by reduction of dihydroxyacetone phosphate with NADT. However, in the reverse aldolase reaction the tritium on C-1 of dihydroxyacetone phosphate will exchange with water (Katz & Rognstad, 1966). The result of this exchange reaction plus the loss of tritium in the pentose phosphate epimerase reaction is a marked dilution of the specific radioactivity of the [1-T]glyceraldehyde 3-phosphate, and hence of the NADT.

In the 'pyruvate cycle' (Scheme 4) (Katz & Rognstad, 1966), tritium from NADT is transferred to NADPT; thus tritium from [4-T]glucose will also appear in fatty acids when this cycle operates.

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