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SUBSTRATE CONCENTRATION AND METABOLIC PATHWAYS IN LIVER SLICES *IN VITRO*

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SUMMARY

To compare the quantitative metabolic pathways followed by substrates as a function of their concentration, liver slices from fed rats were incubated with 0.3 or 1 mM, and 20 or 40 mM concentrations of ^{14}C-labeled galactose, fructose, mannose, glycerol and pyruvate. In qualitative terms the patterns proved similar at the low and high concentrations, but there were differences which necessitate the consideration of substrate concentration in the quantitative evaluation of the metabolism of liver.

INTRODUCTION

In studies of metabolism with tissue slices *in vitro* high concentrations of substrate are frequently selected so that at the termination of incubation a significant quantity of substrate remains unused. Pathway patterns thus observed probably reflect conditions of saturation and indicate the maximum rates at which available pathways can be traversed under the experimental conditions.

Low concentrations offer the possibility of obtaining metabolic patterns reflecting the pathways selectively traversed under conditions of limited substrate availability. Thus, CAHILL *et al.*[1] determined the pathway followed by glucose at different concentrations in liver slices. While the use of trace quantities was not possible because of substrate dilution by glycogen breakdown, it was apparent that the relative pathways of CO_2 production and glycogen synthesis changed markedly with changing concentrations of glucose and that, at high substrate concentration, oxidation to CO_2 approached saturation, whereas glycogen synthesis did not.

Further, large substrate concentrations are for certain substances, for example pyruvate, unphysiological and the observed patterns may be the result of changes in normal pathways secondary to these concentrations[2]. On the other hand, small quantities of substrate may not provide sufficient nutrient for the maintenance of the integrity of tissue during the period of incubation.

EMERSON AND VAN BRUGGEN[3] have indicated the need to determine in systems *in vitro* the amount of substrate that will trace existing reactions at their normal rate or the rate *in situ*. They have examined the metabolism for [1-^{14}C]acetate in liver

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slices and have concluded that there is a critical concentration of a tracer in studies *in vitro* beyond which patterns observed are abnormal. This concentration may vary for different pathways.

The pathway patterns in liver slices using a low concentration of carbohydrate substrates (0.3 or 1.0 mM) compared with a high concentration (20 or 40 mM) have now been determined. Substrates have been selected which enter the metabolic scheme at various levels of the Embden–Meyerhof pathway. Particular attention has been directed toward pyruvate because of its focal position in relation to both the Embden–Meyerhof pathway and Krebs cycle.

METHODS AND MATERIALS

Animals

Male albino rats of the Wistar strain weighing about 200 g were used. They were either maintained *ad libitum* on purina chow until sacrifice or fasted 24 h prior to sacrifice which was by stunning followed by exsanguination.

Medium and substrates

Livers of the rats were sliced and a total of 1 g of slices was incubated in 12.0 ml of medium unless otherwise noted. The ionic composition (mM) of the medium was: K^+, 110; Mg^{2+}, 20; Ca^{2+}, 10; HCO_3^-, 40 and Cl^-, 130 mM, except that Cl^- was 90 mM when the substrate was pyruvate (40 mM). The low substrate concentration of D-[1-^{14}C]galactose, D-[^{14}C]fructose, D-[^{14}C]mannose, and [1,3-$^{14}C_2$]glycerol was 1.0 mM and of [2-^{14}C]pyruvate 0.3 mM*. Solutions were equilibrated with oxygen–carbon dioxide (95:5) to give a pH in the presence of the slices of 7.4–7.5. Incubations were for 90 min at 37° unless otherwise specified.

In time studies, either (a) identical flasks containing pyruvate in concentrations of 0.3 mM or 40 mM were incubated for 30, 60, and 90 min, or (b) identical flasks containing 11 ml of medium and no substrate or containing non-radioactive glucose (10 mM) were incubated. In the latter instance, 1.0 ml of medium, containing [2-^{14}C]pyruvate in an amount to give a final concentration of 0.3 mM, was added to one flask at the beginning of incubation and the incubation continued for 30 min, while into the other flask, 1.0 ml of pyruvate-containing medium was not added until incubation had proceeded for 60 min. The incubation was then terminated after another 30 min.

Methods

Procedures for the chemical and isotopic analyses of glucose and glycogen[4], CO_2 (see ref. 5), pyruvate[6], and fatty acids[7] were performed as previously described, except that when pyruvate was the substrate, glucose was isolated as the glucosazone following the passage of the medium down a Dowex-1 X1 anion exchange column. Lactate and pyruvate were isolated from the column and the lactate degraded[8]. When 0.3 mM pyruvate was substrate, carrier pyruvate and lactate were added. It was

* [1-^{14}C]-Galactose was obtained from Dr. H. S. ISBELL of the National Bureau of Standards, Washington, D.C.; [$^{14}C_6$]mannose from the Schwarz Laboratories, Inc., Mount Vernon, N.Y. (U.S.A.); [$^{14}C_6$]fructose and [2-^{14}C]pyruvate from Nuclear-Chicago Corp., Chicago, Ill. (U.S.A.). [1,3-$^{14}C_2$]Glycerol was provided by Dr. M. L. KARNOVSKY.

demonstrated that after incubation of pyruvate in the absence of liver slices, the pyruvate could be recovered unchanged. Galactose, fructose and mannose uptake were determined by adding carrier at the end of the incubation, incubating with glucose oxidase (EC 1.1.3.4), and then isolating and counting the sugars as their phenylosazones.

Calculations

Initial and final concentrations of glycogen, expressed as μmoles/g of liver, and medium glucose and lactate, expressed as μmoles/g of liver per period of incubation, were calculated from the data obtained by direct chemical analyses. When a low concentration of substrate was used, substrate uptake was calculated as the per cent of the counts added to the flask which were not found in the recovered substrate at the end of the incubation. When a high concentration of substrate was used, uptake was calculated as the difference between the quantity of substrate added to the flask, either 240 or 480 μmoles, and the quantity chemically determined to be present at the end of the incubations. These uptakes have been expressed for comparison as the per cent of the substrate added that disappeared from the medium. Radiochemical yields are calculated as the per cent of the uptake, or where uptake was not determined, the per cent of the counts added to the flasks, that were found in the various products (CO_2, glycogen, glucose, fatty acids, and lactate) at the termination of incubation.

RESULTS

In Table I, data obtained on incubating liver slices in media containing galactose, fructose, mannose, and glycerol at low and high concentrations for 90 min are presented. The results are recorded as the mean values ± the standard error of the mean. The number of experiments, each with the liver from a different rat, is given in parentheses. As will be noted in this and succeeding tables the mean glycogen content of the slices was between 129 and 217 μmoles and the medium glucose 54–89 μmoles at the completion of incubation, unless the rats were fasted, or supplementary glucose was added to the medium. Medium lactate was about 10 μmoles when substrate concentrations were 1 mM. At high substrate concentrations, larger quantities of lactate were formed.

When [1-^{14}C]galactose in a concentration of 1 mM was used, 56% disappeared and over twice as much radioactivity was incorporated into glycogen as was oxidized to CO_2. Only 0.3% of the uptake appeared in fatty acids. The pattern for galactose at 20 mM was not notably different except for a greater incorporation into fatty acids.

When [$^{14}C_6$]fructose was substrate at a concentration of 1 mM, the uptake was 61% and about twice as much fructose was oxidized to CO_2 as was incorporated into glycogen. At a concentration of 20 mM, a greater percentage of the fructose taken up was converted to glycogen than to CO_2. Incorporation into fatty acids was not different between the two concentrations.

When [$^{14}C_6$]mannose was the substrate, in a concentration of 1 mM, the uptake was only 30%, *i.e.*, about one-half that of galactose or fructose, and only small quantities of activity appeared in CO_2, glycogen, and fatty acids. However, the per cent converted to glycogen was about the same as that converted to CO_2.

TABLE I

CONCENTRATION EFFECTS ON THE PATTERNS OF GALACTOSE, FRUCTOSE, MANNOSE AND GLYCEROL METABOLISM

Substrate	Concentration (mM)	Final glycogen	Medium glucose	Lactate formed	Uptake (%)	Per cent of substrate taken up converted to:		
						CO_2	Glycogen	Fatty acids
[1-^{14}C]Galactose	1	129 ± 10 (8)	82 ± 4 (6)	9 ± 1 (3)	56 ± 3 (6)	8.0 ± 0.5 (9)	18.4 ± 2.8 (8)	0.3 ± 0.1 (6)
	20*	136 ± 11 (12)	85 ± 4 (8)	14 ± 8 (3)	12 ± 1 (8)	6.1 ± 0.7 (12)	12.9 ± 1.4 (12)	0.8 ± 0.2 (5)
[$^{14}C_6$]Fructose	1	138 ± 8 (10)	61 ± 4 (7)	9 ± 1 (4)	61 ± 3 (6)	18.4 ± 0.8 (10)	10.6 ± 2.1 (9)	0.9 ± 0.1 (7)
	20*	154 ± 7 (5)	89 ± 6 (5)	54 ± 2 (5)	41 ± 4 (5)	12.0 ± 1.1 (5)	15.7 ± 1.2 (5)	1.2 ± 0.2 (5)
[$^{14}C_6$]Mannose	1	137 ± 8 (9)	83 ± 5 (6)	10 ± 2 (3)	30 ± 4 (6)	2.0 ± 0.1 (9)	2.2 ± 0.3 (9)	0.11 ± 0.04 (6)
[1,3-$^{14}C_2$]Glycerol	1	167 ± 7 (4)	54 ± 2 (4)	9 ± 2 (4)	**	6.0 ± 0.3 (4)	8.8 ± 0.8 (4)	0.09 ± 0.01 (4)
	40	184 ± 31 (3)	74 ± 6 (3)	23 ± 2 (3)	31 ± 3 (3)	3.7 ± 0.3 (3)	8.1 ± 1.3 (3)	0.13 ± 0.009 (3)

* Portions of these data have been presented elsewhere[9].
** Uptake was not determined. Radiochemical yields are calculated as the per cent of the substrate counts added to the flask.

TABLE II

METABOLISM OF [2-^{14}C]PYRUVATE (0.3 mM) IN FED RATS AT 30-MIN INTERVALS DURING 90-MIN INCUBATION

Dietary state	Time	Initial glycogen*	Final glycogen	Pyruvate uptake	Per cent of substrate taken up converted to:		
					CO_2	Glycogen	Fatty acids
Fed	30	337	258	84.8	7.6	1.5	1.1
	60		235	92.6	27.9	2.1	2.9
	90		217	94.3	33.1	3.2	4.9
	SE	26	10	1.5	1.1	0.8	0.5

* Initial glycogen in this and subsequent tables is the content of glycogen in μmoles/g of liver slices at zero time.

When [1,3-$^{14}C_2$]glycerol was the substrate, the ^{14}C patterns were not remarkably different at the two concentrations. The small incorporation into fatty acids at both concentrations is to be noted.

In Table II and subsequent tables, the response to different periods of incubation are recorded. In each experiment, for a given concentration, slices from the liver of the same rat were compared at the different time periods. The results recorded in each table are the mean of three individual experiments. The standard error of the mean given for each time period is obtained from the analysis of variance[10]. Such a standard error reduces the effect of animal variation and thus presents a measure of the variations due to the changes in the experimental conditions.

Table II presents the results of the incubation of [2-^{14}C]pyruvate (0.3 mM) for 30, 60 or 90 min. Glycogen breakdown was greatest, and over 80% of the pyruvate was taken up in the first 30 min. Of the total quantity of C-2 of pyruvate oxidized to $^{14}CO_2$ in the 90-min period (33.1%), the largest amount was formed during the first 30 min (17.6%). In contrast the incorporation of ^{14}C into fatty acids appeared to increase in the later periods.

Table III presents the results of the incubation of 0.3 mM [2-^{14}C]pyruvate in identical flasks, the substrate being added at zero time or at 60 min and the incubation being continued for 30 min after addition of the pyruvate. In the first series of the table, the liver slices were from rats fed until the time of sacrifice. By the end of 30 min most of the glycogen breakdown had occurred. Pyruvate uptake was the same for both 30-min periods as was the oxidation of the pyruvate to CO_2. Incorporation of pyruvate into glycogen appeared greater in the 0–30-min period than in the 60–90-min period while the incorporation into fatty acids appeared to be greater in the latter period. In the second series of the table, the liver slices were from rats fasted for 24 h. Initial glycogen levels were very low and there was a further decrease during the incubation. Medium glucose at the end of the incubations was more than could be accounted for by glycogen breakdown. Pyruvate uptake was about 90% in both of

TABLE III

COMPARISON OF [2-^{14}C]PYRUVATE (0.3 mM) METABOLISM DURING INITIAL AND FINAL 30-MIN TIME PERIODS

Dietary state	*Time period (min)*	*Initial glycogen*	*Final glycogen*	*Medium glucose*	*Pyruvate uptake*	*Per cent of substrate taken up converted to:*		
						CO_2	*Glycogen*	*Fatty acids*
Fed	0–30	221	159	49	83	15.4	1.5	1.6
	60–90		141	69	81	15.5	0.2	3.1
	SE	43	16	4	1	0.4	0.6	1.0
Fasted	0–30	12	4	21	90	13.7	2.1	0.03
	60–90		3	30	91	14.9	1.2	0.06
	SE	10	2	4	1	1.0	0.4	0.02
Fasted*	0–30	16	18	129	82	13.9	2.8	0.06
	60–90		21	135	82	14.8	0.5	0.14
	SE	2	9	3	1	0.8	1.3	0.03

* Initial medium glucose concentration was 10 mM.

the 30-min periods. CO_2 production was similar to that in the fed series. As in the series with fed animals, incorporation of [2-^{14}C]pyruvate into glycogen appeared greater in the 0–30 min period. Incorporation into fatty acids in the fasted rat-liver slices was much less than in the fed rat-liver slices. In the last series, conditions were identical with those in the previous series except that the medium also contained non-radioactive glucose (10 mM). Under these conditions, there was little change in liver glycogen during the course of the incubation. The uptake of pyruvate was about 80%. The distribution of isotope in CO_2, glycogen, and fatty acids was similar to that observed in the absence of added glucose.

TABLE IV

METABOLISM OF [2-^{14}C]PYRUVATE (40 mM) AT 30-MIN INTERVALS DURING 90-MIN INCUBATION

Dietary state	*Time (min)*	*Initial glycogen*	*Final glycogen*	*Medium glucose*	*Lactate formed*	*Pyruvate uptake (%)*	*Per cent of substrate taken up converted to:*				*Lactate**	
							CO_2	*Glycogen*	*Glucose*	*Fatty acids*	*C-1*	*C-2,3*
Fed	30	245	174	61	61	25	18.1	3.2	7.2	0.7	4	37
	60		157	73	85	41	18.0	5.1	8.0	1.4	2	26
	90		160	87	97	59	17.1	6.4	7.0	2.1	2	27
	SE	35	5	2	12	4	6.4	1.5	2.0	0.4		
Fasted	30	4.9	4.4	24	46	16	24.4	5.8	11.2	0.2	9	52
	60		8.7	29	72	33	20.6	6.8	12.2	0.4	5	52
	90		12.6	38	79	52	21.3	6.9	13.3	0.4	3	35
	SE	1.2	2.5	2	12	2	2.2	1.5	1.2	0.1		

* Means of 2 determinations.

In Table IV, 30-, 60-, and 90-min values for the incubation of [2-^{14}C]pyruvate (40 mM) are given. In liver slices from fed rats, glycogen breakdown and lactate formation were greatest during the first 30 min and could be accounted for by the quantity of glycogen breakdown. Pyruvate uptake, while variable, appeared to be nearly linear with time. Throughout the time periods the same percentage of the [2-^{14}C]pyruvate uptake was oxidized to CO_2. The percentage of the pyruvate incorporated into glycogen and fatty acids increased with time while the percentage into glucose did not change. Most of the lactate formed was derived from pyruvate, with a small but significant quantity of activity appearing in the lactate carboxyl group. In comparison, when the pyruvate concentration was 0.3 mM (Table II), oxidation to CO_2 and incorporation into fatty acids was greater but incorporation into glycogen was less.

In the lower series of Table IV, the results of incubating [2-^{14}C]pyruvate (40 mM) with liver slices from fasted rats are presented. As the incubation proceeded, there was a net synthesis of glycogen as well as the appearance of glucose in the medium. Almost 50% of the glucose was derived from pyruvate. Most of the lactate formation occurred during the first 30 min and the lactate was primarily derived from pyruvate. Pyruvate uptake and oxidation to CO_2 was nearly linear with time. Incorporation into fatty acids was less in the fasted than in the fed series. This was also the case in the studies where the pyruvate was 0.3 mM (Table III).

DISCUSSION

The pattern of metabolism for substrates in low concentration is, in general, consistent with the accepted sites of entrance of the substrates into the metabolic pathways. Thus, the large percentage of galactose incorporated into glycogen relative to CO_2 production, in comparison with the other substrates used, is in agreement with the initial entrance of galactose into the metabolic scheme as galactose 1-phosphate. The small quantity of mannose appearing in the products and the smaller uptake of mannose than of galactose or fructose are perhaps related to competition with glucose for entrance via the hexokinase reaction[11]. While fructose and glycerol are believed to enter the metabolic reactions at the triose level, there was much less glycerol than fructose incorporated into fatty acids at low as well as high substrate concentration. The formation of a significant quantity of reduced NAD during the entry at a 40 mM concentration of glycerol via dihydroxyacetone phosphate into the Embden–Meyerhof pathway could result in a reduced milieu, more favorable for hexose synthesis than for glycolysis via glycerate. However, this explanation would seem inadequate to explain the low incorporation into fatty acids observed at a glycerol concentration of 1 mM.

The instances of decreased incorporation of ^{14}C into fatty acids, at low compared to high substrate concentrations, could be a reflection of the need for intermediates for synthesis, paralleling the decreased fatty acid synthesis observed in livers of fasted compared to livers of fed animals[12]. Glucose does not satisfy this need in slices from fasted rats (Table III). Time studies with pyruvate also provide evidence of the need for an incubation period before metabolic conditions are favorable for fatty acid synthesis. For all substrates studied, a greater percentage of the substrate was oxidized to CO_2 at low concentrations than at high concentrations.

When slices of liver from fed rats were incubated in the presence of any of the substrates studied, there was a rapid breakdown of glycogen, presumably associated with phosphorylase activation[13], and glucose appeared in the medium in quantities consistent with the amount of glycogen breakdown. Only a small quantity of lactate was formed at low substrate concentrations. When 40 mM pyruvate was present, a considerable portion of this substrate was reduced directly to lactate. Some of the lactate, as evidenced by the activity in the carboxyl group of the lactate, was derived from pyruvate metabolized prior to reduction. When slices from fasted rats were incubated in the presence of 0.3 mM pyruvate, glycogen breakdown occurred. However, in the presence of 40 mM pyruvate, as previously reported, net glycogen synthesis occurred[4].

It is concluded that, while differences exist in the metabolic patterns depending on whether low or high concentrations of substrates are employed, they are qualitatively similar and results at both concentrations would appear to have relevance. However, it is apparent from these observations that the substrate concentrations exert a quantitative influence on the pathways taken by various substrates in the course of their metabolism by liver.

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