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INFLUENCE OF ENZYMIC ACTIVITIES ON SUBSTRATE OXIDATIONS IN NORMAL AND DIABETIC RAT LIVER AND IN MAMMARY GLAND HOMOGENATE FRACTIONS

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SUMMARY

Activities of TPN-dependent enzymes that oxidize glucose 6-phosphate (glucose-6-phosphate and gluconic acid 6-phosphate dehydrogenases), isocitrate (isocitrate dehydrogenase) and malate (malic enzyme) were determined in particle-free supernatant fractions prepared from normal and diabetic rat liver homogenates and from lactating and nonlactating rat mammary-gland homogenates. These activities were compared with actual oxidations of their respective substrates under conditions where TPN availability was limited.

1. The decrease in activities of glucose 6-phosphate-oxidizing enzymes in diabetic rat livers was not associated with a reduction in the rate of oxidation of glucose 6-phosphate. Thus, reoxidation of TPNH limits the oxidation of glucose 6-phosphate.

2. The inhibitory effect of citrate on oxidation of glucose 6-phosphate and malate in livers of normal and diabetic rats is principally due to high activities of isocitrate dehydrogenase. The inhibitory effect of glucose 6-phosphate on oxidation of citrate and malate by lactating gland fractions is due mainly to high activities of glucose 6-phosphate-oxidizing enzymes.

3. Malic enzyme activity in diabetic liver was one-fourth that of normal liver.

4. Weaning decreased activities of glucose 6-phosphate-oxidizing and malic enzymes in mammary gland preparations.

5. Further evidence is presented for conversion of DPN to TPN in the presence of ATP in rat liver and lactating mammary gland fractions.

INTRODUCTION

Previous reports[1,2] have shown that the oxidation of citrate, glucose 6-phosphate and malate by a rat liver homogenate preparation freed of mitochondria is limited by the availability of TPN and thus depends upon the reoxidation rate of TPNH. It was also demonstrated that the oxidation of glucose 6-phosphate and malate was markedly inhibited by addition of citrate under conditions in which TPN availability was limited, whereas addition of glucose 6-phosphate and malate only slightly

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influenced citrate oxidation. These observations prompted us to investigate whether they resulted from different affinities of the enzymes for TPN or TPNH or from differences in the activities of TPN-dependent dehydrogenases.

MATERIAL AND METHODS

Female rats of the Long–Evans strain, weighing from 200 to 250 g, raised and maintained on an adequate stock diet, were used. Alloxan diabetes was induced by tail-vein injection of 40 mg of recrystallized alloxan monohydrate (Eastman Kodak)/kg body weight. In order to ensure the presence of diabetes in the injected rats, their weights, food and water intake and urine output were measured daily; only animals with blood sugar values in excess of 300 mg percent were selected for study. Lactating rats that had suckled at least 5 pups were killed between 18–21 days postpartum.

Preparations of tissue homogenates with 0.25 M sucrose solution and separation of particle-free supernatant fractions have been described elsewhere[3,4]. The techniques of incubation of tissue homogenate fractions[3] and isolation of $^{14}CO_2$ have also been reported previously[5].

Two methods for calculating TPNH generation, (a) from $^{14}CO_2$ production from [6-^{14}C]citrate and [4-^{14}C]malate and (b) from the difference between $^{14}CO_2$ production from [1-^{14}C]glucose 6-phosphate and [6-^{14}C]glucose 6-phosphate, have been presented[1,6].

Enzymic assays were performed on the particle-free supernatant fractions at 30° in a Beckman model DU spectrophotometer. All determinations of enzymic activities were made at pH 7.5 (0.15 M glycylglycine buffer) in the experiments with liver and at pH 7.2 (0.15 M glycylglycine buffer) in those with mammary gland preparations. Readings were taken of blank mixtures that contained all components except for the substrate studied.

Reactions involving reductions or oxidations of pyridine nucleotides were measured at 340 mμ. Reactions involving formation of *cis*-aconitate were measured at 240 mμ. Enzymic activities were calculated from the linear slopes of extinction differences observed between 3 and 10 min. An extinction coefficient of $6.22 \cdot 10^6$ cm^2 per mole of reduced pyridine nucleotide[7] was used to calculate dehydrogenase activity, and of $3.54 \cdot 10^6$ cm^2/mole of *cis*-aconitate to calculate aconitase activity[8]. The activities of isocitric dehydrogenase[9], glucose 6-phosphate-oxidizing enzymes[10], and the malic enzyme[11] were determined under conditions in which substrate and TPN were present in excess.

RESULTS AND DISCUSSION

A comparison of substrate oxidations with activities of TPNH-generating enzymes

Experiments with particle-free supernatant fractions prepared from livers of normal and alloxan-diabetic rats: The activities of the TPN-dependent dehydrogenases were compared under conditions in which both substrate and TPN were present in excess. Under such conditions, isocitric dehydrogenase had by far the highest activity of the dehydrogenase enzymes in both normal and diabetic rat liver supernatant fraction (Table I). The activity of isocitric dehydrogenase in diabetic preparations was only slightly lower than that in normal preparations. The activities of the glucose 6-phos-

TABLE I

ACTIVITIES OF ENZYMES INVOLVED IN TPN-DEPENDENT SUBSTRATE OXIDATIONS BY PARTICLE-FREE SUPERNATANT FRACTIONS PREPARED FROM LIVERS OF NORMAL AND DIABETIC RATS, AND FROM LACTATING RAT MAMMARY GLANDS

TPN and the substrate were added in excess. The results (averages and standard errors) are expressed either as mμmoles TPNH formed/mg protein/min or as mμmoles *cis*-aconitate formed/mg protein/min. Figures in parentheses represent the number of animals studied in each enzyme assay.

Reaction	*Particle-free supernatant fractions prepared from:*		
	Normal liver	*Diabetic liver*	*Lactating mammary gland*
Glucose 6-phosphate + 2 TPN $\longrightarrow$ Pentose phosphate + CO_2 + 2 TPNH	40.7 ± 6.3 (7)	15.3 ± 2.4 (8)	485.2 ± 54.7 (15)
Malate + TPN $\longrightarrow$ Pyruvate + CO_2 + TPNH	6.2 ± 0.8 (17)	1.5 ± 0.2 (15)	97.5 ± 12.6 (13)
Citrate + TPN $\longrightarrow$ α-Ketoglutarate + CO_2 + TPNH	17.1 ± 0.8 (19)	17.5 ± 0.8 (11)	8.7 ± 1.0 (17)
Isocitrate + TPN $\longrightarrow$ α-Ketoglutarate + CO_2 + TPNH	133.4 ± 12.6 (12)	100.1 ± 5.3 (9)	19.9 ± 0.5 (27)
cis-Aconitate + TPN $\longrightarrow$ α-Ketoglutarate + CO_2 + TPNH	48.5 ± 1.5 (2)	39.9 ± 1.4 (5)	13.9 ± 0.7 (3)
Citrate $\longrightarrow$ *cis*-Aconitate	30.2 ± 2.3 (10)	24.4 ± 1.7 (3)	21.8 ± 2.9 (11)
Isocitrate $\longrightarrow$ *cis*-Aconitate	56.4 ± 5.3 (7)	48.6 ± 4.8 (3)	41.5 ± 4.5 (11)

phate-oxidizing enzymes were considerably lower than that of isocitric dehydrogenase. The observation of GLOCK AND MCLEAN[12] that glucose-6-phosphate dehydrogenase activity in diabetic liver preparations is less than half that in normal preparations is confirmed.

The activity of the malic enzyme in normal rat liver supernatant fractions was low, about 1/20 that of isocitric dehydrogenase (Table I). A definite decrease in the activity of the malic enzyme in diabetic rat liver preparations was observed. It had about $^1/_4$ the activity found in normal rat preparations*.

If it be assumed that changes in metabolic throughput are reflected in changes in enzymic activities, then the observation of a decrease in the malic enzyme activity observed in the liver of the alloxan-diabetic rat suggests that the increased gluconeogenesis in these livers does not proceed through the direct interconversion of maiate and pyruvate[13,14].

Aconitase activity, which was also measured in the particle-free liver supernatant fractions (Table I), was considerably lower than that of isocitric dehydrogenase. In diabetic liver preparations it was somewhat lower than in normal liver preparations. The ratio of reaction velocities of the formation of *cis*-aconitate from isocitrate to that from citrate was close to 2 (RACKER[8] has reported a ratio of 2.2). The over-all velocities for the two reactions *cis*-aconitate $\longrightarrow$ α-ketoglutarate + CO_2 and citrate $\longrightarrow$ α-ketoglutarate + CO_2, were significantly lower than those for the aconitase reactions. This observation calls for caution in the determination of enzymic activities by the use of combined reactions.

The observation that isocitric dehydrogenase had by far the highest enzymic activity of the TPN-specific enzymes in the particle-free preparations obtained from liver homogenates (Table I) could explain the inhibition of glucose 6-phosphate and malate oxidation by citrate and isocitrate shown in Table II. But the following considerations indicate that this might not be the case: (a) the velocity conversion of citrate to α-ketoglutarate + CO_2 was not higher than that of glucose 6-phosphate to pentose phosphate + CO_2 under conditions where TPN was present in excess (Table I), (b) certain enzymes may have higher affinities for TPN than others, (c) high concentrations of TPNH may inhibit various dehydrogenases to different degrees.

Experiments with particle-free supernatant fractions obtained from the mammary glands of lactating rats: Since, as shown in the preceding section, a clear answer to the question raised in the introduction could not be obtained from experiments with rat liver, we looked for a soluble preparation with an entirely different composition of enzymic activities. It was found in particle-free, supernatant fractions obtained from lactating rat mammary gland homogenates. In such preparations, the activities of the glucose 6-phosphate oxidizing enzymes are extremely high, whereas those of malic enzyme and isocitric dehydrogenase are considerably lower (Table I). Aconitase activity in these preparations was higher than that of isocitric dehydrogenase. Again, the velocities of the over-all reactions, *cis*-aconitate $\longrightarrow$ α-ketoglutarate + CO_2 and citrate $\longrightarrow$ α-ketoglutarate + CO_2, were significantly lower than that of the reaction catalysed by the enzyme with the lowest activity (isocitric dehydrogenase).

If the activities of the TPN-dependent dehydrogenase enzymes play an important role in the relative oxidation rates of their substrates, under conditions where more

* FITCH AND CHAIKOFF[13] reported a decline in hepatic malic enzyme activity in rats fasted 24 h which had been prefed a diet containing 60% of either glucose or fructose.

TABLE II

RELATIONS AMONG TPN-DEPENDENT SUBSTRATE OXIDATIONS IN RAT TISSUE HOMOGENATE FRACTIONS

For liver, 25–35 mg particle-free supernatant protein plus 5–7 mg microsomal protein, and for mammary gland, 10–22 mg particle-free supernatant protein were incubated at 30° for 2 h in air with 240 μmoles glycylglycine buffer (pH 7.5) for liver, (pH 7.2) for mammary gland, 60 μmoles reduced glutathione, 10 μmoles $KHCO_3$, 70 μmoles $MgCl_2$, 48 μmoles (liver) or 10 μmoles (mammary gland) ATP, 0.5 μmole TPN, 0.1 μmole CoA, 6 μmoles acetate, and/or 50 μmoles citrate and/or 40 μmoles glucose 6-phosphate and/or 50 μmoles (liver) or 100 μmoles (mammary gland) DL-malate in a final volume of 3.6 ml in the experiments with liver fractions and 3.5 ml in the experiments with mammary gland preparations. $2.5 \cdot 10^4$ counts/min of [6-^{14}C]-citrate or [1-^{14}C]glucose 6-phosphate, or [4-^{14}C]malate were incubated. Values represent averages and standard errors from 6 experiments with different rats.

Substrates added				*mμmoles TPNH generated from labeled substrate/mg supernatant protein from:*					
Labeled		*Unlabeled*		*Lactating mammary gland*		*Normal liver*		*Diabetic liver*	
				Supernatant		*Supernatant + microsomes*		*Supernatant + microsomes*	
Kind	*Amount (μmoles)*	*Kind*	*Amount (μmoles)*	*Average*	*Total*	*Average*	*Total*	*Average*	*Total*
[6-^{14}C]Citrate	50			628 ± 23		921 ± 52		1165 ± 91	
[1-^{14}C]Glucose 6-phosphate	40			940 ± 67		870 ± 35		952 ± 48	
[4-^{14}C]Malate	50*			955 ± 61		200 ± 13		225 ± 45	
[6-^{14}C]Citrate	50	Glucose 6-phosphate	40	120 ± 14	936	715 ± 68	905	986 ± 99	1179
[1-^{14}C]Glucose 6-phosphate	40	Citrate	50	816 ± 110		190 ± 17		193 ± 23	
[6-^{14}C]Citrate	50	Malate	50*	404 ± 14	1023	873 ± 53	943	1057 ± 50	1151
[4-^{14}C]Malate	50*	Citrate	50	619 ± 59		70 ± 7		94 ± 11	
[1-^{14}C]Glucose 6-phosphate	50	Malate	50*	642 ± 76	923	742 ± 26	831	781 ± 35	936
[4-^{14}C]Malate	50*	Glucose 6-phosphate	40	281 ± 52		99 ± 15		156 ± 28	

* 100 μmoles DL-malate in experiments with mammary gland. 50 μmoles DL-malate in experiments with normal and diabetic liver.

than one of these substrates is present in excess and TPN availability is limited, glucose 6-phosphate must have a significant inhibitory effect on the oxidation rates of citrate and malate. This was found to be the case in earlier experiments with mammary gland preparations[15].

In the oxidation studies recorded in Table II, where a limited amount of TPN was added, the amounts of TPNH generated from (a) glucose 6-phosphate, (b) malate, (c) citrate + glucose 6-phosphate, (d) citrate + malate and (e) glucose 6-phosphate + malate were about the same. This indicates that availability of TPN is the limiting factor in oxidations requiring this pyridine nucleotide by lactating rat mammary gland supernatant fractions. Since the amount of TPN added to these preparations was small as compared with the amount of TPNH produced, and furthermore since all of the TPN added was converted to TPNH within the first minute, regardless of the TPNH-generating substrate used (citrate, glucose 6-phosphate or malate), the reoxidation rate of TPNH must be the limiting factor in the oxidation of these three substrates.

Citrate, when used as sole substrate, yielded considerably less TPNH than did any combination of TPNH-generating substrates (Table II). This is obviously due to the limited enzyme activities (aconitase and isocitric dehydrogenase) in the reaction chain involved in the conversion of citrate to α-ketoglutarate + CO_2. (Compare this with the small TPNH generation observed in liver preparations when malate was the sole substrate.)

In the lactating mammary gland supernatant fractions, citrate oxidation was inhibited to about 80 % in the presence of excess glucose 6-phosphate and to about 40 % by excess malate (Table II). Malate oxidation was inhibited about 70 % in the presence of excess glucose 6-phosphate and about 35 % by excess citrate. Glucose 6-phosphate oxidation was only slightly inhibited by the addition of excess citrate (13 %) and somewhat more so by excess malate (30 %).

When the results obtained in the substrate oxidation studies (Table II) with liver and mammary gland preparations are compared, it becomes clear that different affinities of the enzymes for TPN or different inhibitions of the enzyme reactions by TPNH cannot explain the influence of one substrate on the oxidation rate of another. For example, in liver preparations, glucose 6-phosphate oxidation is strongly inhibited by citrate, whereas in mammary gland preparations, citrate oxidation is strongly inhibited by glucose 6-phosphate. Unless it be assumed that the enzymes involved in these reactions in the two tissues have quite different reaction characteristics, we can rule out the possibility that their affinity for TPN or TPNH has a pronounced effect on the oxidation velocity of one substrate in the presence of another. Thus, when the availability of TPN is limited (presumably this is the case in the intact cell[16, 17]), the oxidation rates of TPNH-generating substrates are dependent mainly upon the relative enzymic activities of enzymes catalysing the oxidation of substrates.

Experiments with particle-free supernatant fractions prepared from mammary glands of rats in which lactation was interrupted by weaning of the pups: Table III shows that the activities of glucose 6-phosphate-oxidizing enzymes and of the malic enzyme in the rat mammary gland preparation declined rapidly on the first day after cessation of suckling. In contrast to this, the activity of isocitric dehydrogenase rose significantly. Under conditions where TPN availability was limited, the oxidation of all

TABLE III

CHANGES IN ENZYMIC ACTIVITIES AND SUBSTRATE OXIDATIONS, IN RAT MAMMARY GLAND SUPERNATANT FRACTIONS, RESULTING FROM INTERRUPTION OF LACTATION

For experimental details and explanation of data see Table I (enzyme studies where TPN was present in unlimiting amounts) and Table II (substrate oxidation studies where TPN was added in limiting amounts). The values (averages of separate results obtained with three rats in each group, and their standard errors) refer to mμmoles of TPNH generated/mg supernatant protein/min.

State	*Days postpartum*	*TPNH-generating substrate*						
		Glucose 6-phosphate		*Malate*		*Citrate*		*Isocitrate*
		Enzyme study	*Oxidation study*	*Enzyme study*	*Oxidation study*	*Enzyme study*	*Oxidation study*	*Enzyme study*
Lacting	20	572 ± 27	7.1 ± 0.4	59 ± 3	7.9 ± 1	7.8 ± 2.0	4.5 ± 0.3	17.9 ± 1.2
1 Day weaned	22	217 ± 26	4.9 ± 0.4	35 ± 5	5.0 ± 0.4	7.5 ± 1.3	3.4 ± 0.8	25.6 ± 2.5
2 Days weaned	23	111 ± 11	3.0 ± 0.5	22 ± 1	2.7 ± 0.6	9.3 ± 1.6	2.8 ± 0.6	38.7 ± 2.0
1 Day weaned and 1 day suckled	23	248 ± 9	5.4 ± 0.5	26 ± 1.5	5.5 ± 0.3	8.6 ± 0.2	5.4 ± 0.5	19.1 ± 3.0

three substrates (glucose 6-phosphate, malate and citrate) decreased markedly after weaning, a result which indicates that TPNH utilization also declines after weaning.

Both effects of weaning, the changes in enzymic activities and the decrease in total TPNH oxidation, can be arrested when, after one day of weaning, the pups are returned to the mother for one day.

Enzymic reactions involving the use of DPN

Conversion of DPN to TPN: In spectrophotometric studies, no conversion of DPN to DPNH was observed when citrate or glucose 6-phosphate or malate was incubated with supernatant fractions prepared from liver or lactating mammary gland. When, however, the oxidation of these substrates was measured in the presence of DPN and ATP but in the absence of TPN, considerable oxidation occurred (Table IV).

TABLE IV

TPNH PRODUCTION FROM CITRATE AND GLUCOSE 6-PHOSPHATE AS SOLE SUBSTRATES WITH VARYING AMOUNTS OF PYRIDINE NUCLEOTIDES

For experimental details see Table II. Each value is the average of the results obtained with 4 rats, and its standard error, and is expressed as μmoles TPNH produced in 2 h. The supernatant protein concentrations varied from 28 to 32 mg per incubation mixture in the experiments with normal liver fractions and from 15 to 22 mg in mammary gland experiments.

Supernatant fractions prepared from:	*Pyridine nucleotide added*		*TPNH production calculated from:*	
		(μmoles)	*Citrate (μmoles)*	*Glucose 6-phosphate (μmoles)*
Normal liver	None		20.8 ± 1.2	20.3 ± 0.6
	TPN	0.5	29.0 ± 1.2	28.6 ± 0.4
	TPN	1.0	29.8 ± 0.6	29.5 ± 0.8
	TPN	2.0	30.4 ± 1.5	30.2 ± 1.0
	DPN	0.5	21.5 ± 2.3	21.3 ± 0.5
	DPN	1.0	24.7 ± 4.8	24.3 ± 0.7
	DPN	2.0	24.9 ± 2.0	24.6 ± 2.5
Normal liver (Dowex-1-treated)	None		3.2 ± 0.1	3.8 ± 0.2
	TPN	0.5	25.0 ± 1.4	25.2 ± 1.9
	DPN	0.5	14.3 ± 0.8	16.3 ± 1.5
	TPN + DPN	0.5 + 0.5	25.0 ± 2.2	24.1 ± 1.0
Lactating mammary gland	None		3.1 ± 0.3	6.9 ± 0.7
	TPN	0.5	18.2 ± 1.9	25.5 ± 2.0
	TPN	1.0	17.7 ± 0.9	26.0 ± 1.1
	TPN	2.0	17.8 ± 1.2	28.5 ± 1.0
	DPN	0.5	7.1 ± 0.4	13.9 ± 0.7
	DPN	1.0	10.8 ± 2.5	17.7 ± 0.5
	DPN	2.0	14.7 ± 1.5	17.9 ± 2.5

In these experiments the supernatant liver fractions had been treated with Dowex-1-chloride[3] to remove endogenous TPN. In the mammary gland supernatant fraction* and in the Dowex-treated liver supernatant fraction, oxidation of TPNH-generating substrates was negligible over a period of 2 h, when neither pyridine nucleotide was added (Table IV). The addition of 0.5 μmole of DPN and of ATP (48 μmoles for liver and 10 μmoles for mammary gland) resulted in a TPN-dependent substrate oxidation

* It was not necessary to treat the mammary gland supernatant fraction with Dowex-1-chloride because the endogenous levels of TPN are low in that tissue (see Table IV).

which was about half that observed with 0.5 μmole of TPN alone. These findings suggest that mammary gland and liver supernatant fractions contain the enzymic equipment needed for the conversion of DPN to TPN in the presence of ATP and other cofactors. The ability of rat liver[18] and lactating rat mammary gland preparations[4] to convert DPN to TPN in the presence of ATP has been previously demonstrated.

Activities of DPN-dependent enzymes: Malic and lactic dehydrogenases were highly active in both liver and mammary gland preparations. Triosephosphate dehydrogenase, determined as described[19], was demonstrated only in the presence of arsenate. In the absence of arsenate, DPNH generation from fructose 1,6-diphosphate could not be detected. Instead, a rapid oxidation of DPNH was observed with hexose diphosphate (18.8 ± 0.5 mμmoles DPNH/mg protein/min were oxidized by liver preparations, and 20.1 ± 1.3 mμmoles DPNH/mg protein/min were oxidized by mammary gland preparations). This oxidation of DPNH with hexose diphosphate as substrate can be explained by the presence of α-glycerophosphate dehydrogenase in the liver and mammary gland preparations.

By the use of [1,5-$^{14}C_2$]citrate, some activity of α-ketoglutaric dehydrogenase could be demonstrated in all preparations*. This activity was determined by calculating the release of $^{14}CO_2$ by oxidation of the labeled citrate by the α-ketoglutaric dehydrogenase system**. This system was capable of oxidizing only 0.50 ± 0.15 mμmole of α-ketoglutarate/mg protein/min in the experiments with liver, and 0.45 ± 0.08 in the experiments with lactating mammary tissue. Spectrophotometric measurements, usually performed over short intervals of time, failed to detect the activity of this enzyme, but by a study of the oxidation of ^{14}C-labeled compounds over an extended period, a low activity of this enzyme could be detected in the supernatant fraction. It should be mentioned, however, that the bulk of α-ketoglutarate dehydrogenase activity is associated with the mitochondria.

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* The possibility of the citrate cleavage enzyme's contributing to the $^{14}CO_2$ production should also be considered[20].

** $^{14}CO_2$ is produced through the following reactions: [1,5-$^{14}C_2$]citrate → [1,5-$^{14}C_2$]aconitate → [1,5-$^{14}C_2$]isocitrate → α-[1,5-$^{14}C_2$]ketoglutarate → [4-^{14}C]succinate + $^{14}CO_2$.

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