The Utilization of ^{14}C-Labeled Lactate, Pyruvate and Alanine by Prelactating Tissues and Hyperplastic Alveolar Nodule Outgrowths[1] (37154)

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The investigation of primary metabolic lesions has been greatly facilitated by the availability of two types of tissues: minimal-deviation tumors and naturally occurring hyperplastic alveolar nodule outgrowths. These hyperplastic alveolar nodule outgrowths give rise to tumors with greater frequency and in less time than do normal tissues (1, 2). Heretofore, these hyperplastic alveolar nodule outgrowths were found to be indistiguishable from lobules of prelactating tissue taken from pregnant mice by cytologic (1, 2) and metabolic (3–8) criteria.

In a previous communication, we suggested (9) that decreased entry of 3-carbon glycolytic intermediates into the Krebs cycle in the mammary adenocarcinoma could be due to low activities of pyruvate dehydrogenase [pyruvate:lipoate oxidoreductase, acceptor acetylating (EC 1.2.4.1)] and the citrate condensing enzyme (citrate oxaloacetate-lyase (CoA-acetylating) (EC 4.1.3.7)]. This proposal, and possibly other mechanisms as well (10–20), may result in augmented glycolysis and in deficiency of respiratory energy. In our search for primary metabolic lesions, therefore, we considered it imperative to study the pattern of utilization of ^{14}C-labeled lactate, pyruvate and alanine and the citrate condensing enzyme in the hyperplastic alveolar nodule out-outgrowths, a preneoplastic state in the development of the mammary adenocarcinoma.

A comparison of these parameters in tissue samples of the prelactating tissue and the hyperplastic alveolar nodule outgrowths is presented.

Materials and Methods. Animals, tissue preparation and analytical methods. Mice were obtained from the Cancer Research and Genetics Laboratory, University of California, Berkeley, CA, and were treated as previously described (6). Tissue specimens were prepared (6) and incubated in a Krebs–Henseleit bicarbonate buffer (21) (pH 7.4), in a total volume of 0.2 ml and all other incubation procedures were identical to those previously described (6). The labeled substrates and their specific radioactivities were given in tables. The following analytical procedures were performed as described elsewhere (22): determination of respiratory $^{14}CO_2$ activity, isolation and determination of total ^{14}C-activity in fatty acids, chromatographic analyses of amino acids and lactate in the incubation medium.

Preparation of tissue homogenates and assay of the citrate condensing enzyme. Washed specimens of prelactating tissue and hyperplastic alveolar nodule outgrowths were homogenized in 5 vol of 95% ethanol and 0.5 *M* KCl (1:5, by vol), spun and assayed for activity of the citrate condensing enzyme (23). In our hands, this extraction procedure gave values similar to or higher than those of the isolated mitochondria and was, therefore, used in this study. Citrate condensing enzyme activity was measured at 30°. One unit is equal to the utilization of 1 nmole of substrate/min/mg protein. The assay mixture consisted of 100 μmoles of Tris–HCl buffer (pH 8.0), 1 μmole of DTNB, 0.1 μmole of acetyl-CoA, 2 μmoles of oxaloacetate and enzyme preparation, in a total volume

[1] This work was aided by grants from the American Cancer Society and by Grant CA-08748 from the National Cancer Institute.

TABLE I. Metabolism of ^{14}C-Labeled Lactate, Pyruvate and Alanine by Slices of Prelactating Mouse Mammary Gland and Hyperplastic Alveolar Nodule Outgrowths.[a]

Labeled substrates	Glucose added (μmoles)	Prelactating mammary gland; percentage of added ^{14}C recovered in:		Hyperplastic alveolar nodule outgrowths; percentage of added ^{14}C recovered in:	
		CO_2	Fatty acids	CO_2	Fatty acids
Lactate-1-^{14}C	0	20.4	0	12.3	0
	2.5	16.1	0	10.3	0
Lactate-2-^{14}C	0	9.4	0.7	5.8	0.5
	2.5	6.7	2.7	5.1	1.8
Lactate-3-^{14}C	0	6.5	0.8	3.7	0.5
	2.5	5.4	3.3	3.1	1.9
Pyruvate-1-^{14}C	0	40.1	0	20.6	0
	2.5	35.4	0	18.1	0
Pyruvate-2-^{14}C	0	18.4	1.2	8.9	0.7
	2.5	12.1	4.0	5.4	2.5
Pyruvate-3-^{14}C	0	13.7	1.2	6.1	0.7
	2.5	9.6	5.4	4.6	3.2
Alanine-2-^{14}C	0	5.0	0.6	2.6	0.4
	2.5	3.6	2.4	2.0	1.8
Alanine-3-^{14}C	0	3.4	0.7	1.8	0.5
	2.5	3.0	3.0	1.7	2.2

[a] Slices (25 mg) were incubated for 90 min at 37° in a medium consisting of 0.2 ml of Krebs–Henseleit bicarbonate buffer (pH 7.4) and 25 μmoles of the labeled substrates recorded above. The specific radioactivity for all substrates was about 1×10^5 cpm/μmole. Glucose was added as indicated. Gas phase, O_2 and CO_2 (95:5, by vol). Each value is the average of 3 closely agreeing results from experiments with different mice. See text for further experimental details.

of 1 ml. All determinations were corrected for acetyl-CoA deacylase activity by incubating the enzyme preparation without oxaloacetate. Protein was determined by the biuret method (24) with defatted human serum albumin as standard (25).

Results and Discussion. The utilization of ^{14}C-labeled lactate, pyruvate, alanine and succinate by specimens of prelactating tissue and hyperplastic alveolar nodule outgrowths. As shown in Table I, CO_2 production from all three carbons of lactate, pyruvate and alanine in the absence of glucose was considerably lower in the hyperplastic alveolar nodule outgrowths than in the prelactating mammary gland. The addition of glucose decreased CO_2 production from all three carbons of these substrates; this decrease was proportionately similar in both tissues (Table I).[2] Fatty acid formation from the second and third carbons of lactate, pyruvate and alanine was lower in the hyperplastic alveolar nodule outgrowths than in the prelactating mammary tissue. This is in contrast to results on the formation of fatty acids from acetate and glutamate in these tissues (6). Glucose stimulated fatty acid production from these substrates in both tissues to a similar extent. It should be noted that in the prelactating tissue, the value for the ratios of $^{14}CO_2$ from lactate-1-^{14}C/$^{14}CO_2$ from lactate-2-^{14}C, and of $^{14}CO_2$ from lactate-2-^{14}C/$^{14}CO_2$ from lactate-3-^{14}C were 2.2 and 1.5, respectively, in the absence of glucose (Table I). The corresponding values of these ratios in the hyperplastic alveolar nodule outgrowths were 2.1 and 1.5, respectively (Table I). Similar values for these ratios can be derived for ^{14}C-labeled pyruvate and alanine in both tissues (Table I). The similarity of the values of these ratios for all substrates indicates that neither lactate dehydrogenase nor alanine transaminase were

[2] The values given in Ref. (6) on the metabolism of lactate-2-^{14}C were misplaced; those given in Table I belong in Table II and vice versa.

rate limiting in the conversion of lactate and alanine to pyruvate by the hyperplastic alveolar nodule outgrowths. These ratios also indicate that reactions leading to CO_2 formation from the second and third carbons of lactate, pyruvate and alanine (via the Krebs cycle) are not responsible for the reduced capacity of the hyperplastic alveolar nodule outgrowths to utilize these substrates. The latter conclusion is substantiated by the similarity of the oxidation of succinate-1-^{14}C and -2-^{14}C in both tissues (Table II), and from experiments on the oxidation of acetate-1-^{14}C and -2-^{14}C and on the activities of various Krebs cycle enzymes reported elsewhere (4, 6). It would appear, therefore, that the derangement of the reactions involved in the utilization of these three substrates must be localized at a stage prior to their entry into the Krebs cycle, and past the lactate dehydrogenase and the alanine transaminase reactions (9).

Chromatographic analyses of some amino acids formed from ^{14}C-lactate by specimens of prelactating mammary tissue and hyperplastic alveolar nodule outgrowths. As shown in Table III, about 37% of the lactate added (av for all 3 carbons), was recovered from the incubation medium of the prolacting mammary tissue. The corresponding value for the hyperplastic alveolar nodule outgrowths was about 61%. These results are in agreement with data shown in Table I which demonstrated decreased conversion of lactate, pyruvate and alanine carbons into CO_2 and fatty acids by the hyperplastic alveolar nodule outgrowths. The incorporation of ^{14}C-lactate into amino acids, however, was considerably higher in the hyperplastic alveolar nodule outgrowths, than in the prelactating tissue, that of alanine in particular. The significance of such a finding in relation to entry of 3-carbon glycolytic intermediates in the mammary adenocarcinoma has been discussed elsewhere (9). The incorporation of ^{14}C-lactate into glutamine, however, was similar in both the prelactating tissue and the hyperplastic alveolar nodule outgrowths. In this respect, low glutamine content has been found in most neoplasms thus far investigated and has been attributed to both its reduced synthesis and its rapid utilization (26).

Activity level of the citrate condensing enzyme in homogenate fractions of prelactating mammary tissue and hyperplastic alveolar nodule outgrowths. The activity of the citrate condensing enzyme was about 3-fold higher in the prelactating mammary tissue than in the hyperplastic alveolar nodule outgrowths, *i.e.*, 99 and 32 nmoles/min/mg protein. Acetyl-CoA deacylase activity, however, was about 2.5-fold higher in the latter tissue (unpublished data). These results, taken in the context of a decrease in lactate uptake and in lactate, pyruvate and alanine utilization and increased amino acid formation by the hyperplastic alveolar nodule outgrowths, provide for the first time a significant metabolic difference with regard to the prelactating tissue on the one hand, and a metabolic condition analogous to that

TABLE II. Metabolism of ^{14}C-Labeled Succinate by Slices of Prelactating Mouse Mammary Gland and Hyperplastic Alveolar Nodule Outgrowths.[a]

Labeled substrates	Glucose added (μmoles)	Prelactating mammary gland; percentage of added ^{14}C recovered in[b] CO_2	Hyperplastic alveolar nodule outgrowths; percentage of added ^{14}C recovered in[b] CO_2
Succinate-1-^{14}C	0	4.3	4.1
	2.5	3.9	3.4
Succinate-2-^{14}C	0	3.0	2.7
	2.5	2.6	2.6

[a] ^{14}C-Succinate (1 μmole) was incubated as described in Table I. The specific radioactivity was about 2×10^5 cpm/μmole. Each value is the average of 3 experiments with different mice.

[b] No ^{14}C was recovered as fatty acids.

TABLE III. Chromatographic Analysis of Some Water-Soluble Compounds Formed from Incubation of ^{14}C-Labeled Lactate with Prelactating Mouse Mammary Gland and Hyperplastic Alveolar Nodule Outgrowths.[a]

Lactate carbon labeled	Compounds recovered on chromatogram	Percentage of added ^{14}C recovered in experiments with: Prelactating mammary gland (A)	Hyperplastic alveolar nodule outgrowths (B)	Ratio B/A
1	Lactate	41	65	1.6
2	Lactate	39	62	1.6
3	Lactate	31	58	1.9
1	Alanine	1.0	5.1	5.0
2	Alanine	1.4	5.9	4.2
3	Alanine	0.9	4.7	5.2
1	Glutamate	0	0	—
2	Glutamate	1.9	4.0	2.1
3	Glutamate	2.9	5.7	2.0
1	Glutamine	0	0	—
2	Glutamine	2.0	2.3	1.2
3	Glutamine	2.3	2.4	1.1
1	Aspartate	0	0	—
2	Aspartate	1.1	1.9	1.7
3	Aspartate	1.0	2.1	2.0

[a] The variously labeled ^{14}C-lactates were incubated as described in Table I and text.

existing in the fully developed mammary adenocarcinoma (9) on the other. It would follow then that one of the early events in the development of this tumor is a decreased entry of 3-carbon glycolytic intermediates into the Krebs cycle. In this respect, formation of fatty acid from acetate and glutamate was higher (6) but that from lactate, pyruvate and alanine was lower (Table I) in the hyperplastic alveolar nodule outgrowths than that in the prelactating tissue. The preferential synthesis of alanine from lactate may also be a consequence of this lesion, although formation of all amino acids in the hyperplastic alveolar nodule outgrowths and in the mammary adenocarcinoma (9) was considerable. Whether this finding is due to the observed decrease in the activities of pyruvate dehydrogenase and the citrate condensing enzyme and higher acetyl-CoA deacylase activity in the hyperplastic alveolar nodule[3] and in the mammary adenocarcinoma (9) remains to be established. Thus, as was pointed out by Warburg (11) and others [for review, see (10)], augmented aerobic and anaerobic glycolysis may indeed reflect one of the prime metabolic lesions during neoplastic transformation that occurs as a result of defective mechanisms for the utilization by the mitochondria of glycolytic substrates and other cytosolic compounds as well (9–17). The possibility of regulation by negative effectors (9, 18–20) remains to be shown.

Summary. The utilization of ^{14}C-labeled lactate, pyruvate and alanine by specimens of prelactating tissue taken from pregnant mice and hyperplastic alveolar nodule outgrowths from C3H mice was investigated. In the absence of glucose, CO_2 production and fatty acid synthesis from these substrates was higher in the prelactating mammary gland than in the hyperplastic alveolar nodule outgrowths. Addition of glucose effected a decrease in CO_2 production from all 3 carbons, but caused an increase in fatty acid formation from the second and third carbons of these substrates. This effect by glucose was proportionately similar in both tissues. Lactate uptake by the prelactating mammary gland was about 60% higher than

[3] In the present work, study of the levels of pyruvate dehydrogenase was not feasible for lack of experimental material.

that by the hyperplastic alveolar nodule outgrowths, but amino acid formation from this substrate was markedly higher in the latter tissue. The activity of the citrate condensing enzyme (EC 4.1.3.7) was considerably lower in the hyperplastic alveolar nodule outgrowths than in the prelactating mammary tissue. An enzymatic alteration at this site would slow entry of 3-carbon glycolytic intermediates into the Krebs cycle and may represent a prime metabolic lesion in the development of the hyperplastic alveolar nodule outgrowths.

The project was initiated in the laboratory of the late Professor I. L. Chaikoff, Department of Physiological Chemistry, University of California, Berkeley, CA. We acknowledge with pleasure the able technical assistance of Mr. Weston Kane and Mrs. Hope McGrath.

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Received Nov. 17, 1972. P.S.E.B.M., 1973, Vol. 142.