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Substrate Utilization in Bovine Kidney Culture Cells Infected with Foot-and-Mouth Disease Virus. (27118)

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Infection of bovine calf kidney culture cells with foot-and-mouth disease virus (FMDV) results in an increased use of glycolytic pathways, the metabolic sequences previously found necessary for virus production(1,2,3). A further study of the acids produced during the metabolism of glucose revealed only pyruvic, lactic, and acetic acids,* the quantities of which were influenced by the presence of lactalbumin hydrolyzate in the maintenance medium.

The present investigation was undertaken to study in more detail the metabolic interactions of normal and infected cells, partially depleted of endogenous nutrients, in contact with a defined medium(4) containing varying quantities of glucose, pyruvate, acetate, and lactalbumin hydrolyzate.

Materials and methods. Tissue culture. Primary monolayer cultures from bovine kidney tissue were prepared in 4-oz. prescription bottles as previously described(5). The growth medium consisted of 0.5% lactalbumin hydrolyzate and 6% bovine serum in Hanks' balanced salt solution. Resting cells were obtained by storage of 6-day-old confluent cultures, each containing from 11 to 13 million cells, for 5-9 days at 30°C without change of culture fluid.

Virus. High-titer stocks of FMDV-A119 were produced from infected tissue culture fluids by precipitation with methanol, and the alcohol removed by subsequent dialysis (1).

Virus assay. The plaque method using the monolayer cultures in prescription bottles was used(5).

Starvation. Cells were depleted of endogenous nutrients by washing 3 times with 5-ml portions of 1% tris(hydroxymethyl) amino methane (Tris) buffer, pH 7.5. They were then incubated at 37°C for 2 hours in presence of 5 ml of Hanks' balanced salt solution without glucose made up in 1% Tris buffer in which the sodium chloride concentration was reduced to 2.1 g per liter to maintain isotonicity (TB-H).

Respiration. Cell suspensions were prepared by scraping cells from ½-hour starved

* Unpublished data.

TABLE I. Glucose Utilization and Lactic Acid Production as a Function of Glucose Concentration in Normal Bovine Kidney Cultures.

Glucose conc., M	Glucose used, μM/3 hr/culture*	$\frac{\mu M \text{ glucose used}}{\mu M \text{ glucose available}} \times 100$	Lactic acid produced, μM/3 hr/culture*	$\frac{\text{Lactic acid produced}}{\text{Glucose used}}$†
5.0×10^{-2}	11.5	4.6	18.1	1.6
1.0×10^{-2}	10.7	21.4	17.8	1.7
2.5×10^{-3}	6.15	49.2	9.90	1.6
5.0×10^{-4}	1.47	58.8	2.54	1.7
5.0×10^{-5}	.12	46.8	.18	1.5

* Avg of 6 independent samples. Avg stand. dev. for glucose analyses was 3.5%; for lactic acid analyses 6%.

† Cell cultures occasionally showed ratios as low as 1.1. In these cells, glucose utilization (at 1×10^{-2} M level) ran erratically lower than the usual value.

cultures, packing them at 300 g for 5 minutes, and resuspending in TB-H at concentrations of 10-20 $\times$ 10^6 cells per ml. Warburg flasks contained 1 ml of cell suspension, ½ ml of substrate prepared in TB-H, and 0.20 ml of 10% KOH in the central well. Flasks were equilibrated at 37°C for 45 minutes, sufficient time for all carbon dioxide to be removed from the medium by the alkali. Substrate addition from the sidearms was made after a total of 2 hours cell starvation, and oxygen uptake was then measured.

Typical experimental run. Starved control cultures were incubated with 5 ml of TB-H containing the test substrate for 3 hours, with occasional 5-hour periods to measure constancy of utilization with time. Cultures to be exposed to virus were starved for only 1½ hours before being infected with 3-5 plaque forming units per cell for 30 minutes. They were then washed 5 times with 5-ml portions of Tris buffer (as were the control cultures), and incubated with 5 ml of TB-H containing the test substrate for 4½ hours (5 hours virus exposure), the time at which maximum virus titer is reached(1). The cells were then lysed by addition of 2 ml of 1% sodium lauryl sulfate, assayed for virus if infected, and the cell lysate with medium was deproteinized with barium hydroxide-zinc sulfate. Chemical analyses were done for glucose (Worthington Glucostat reagent), pyruvate(6), acetate(7), and lactate(8). All data were corrected for zero time controls.

Results. Glucose utilization was proportional to concentration in normal cultures exposed to varying amounts of substrate, although efficiency of glucose uptake was highest at the low level of 5×10^{-4}M. Table I shows lactic acid production to be comparable to glucose utilization at 5×10^{-5} M to 5×10^{-2} M glucose concentration.

Lactate was only produced in significant quantities from glucose. Table II shows that neither the endogenous metabolism, acetate, nor lactalbumin hydrolyzate yielded any lactate, while pyruvate did give a very slight amount. However, lactalbumin hydrolyzate

TABLE II. Lactate Production from Different Substrates in Normal and FMDV Infected Bovine Kidney Culture Cells and Corresponding Virus Production.

Substrate addition	Glucose utilization		Lactic acid production		Virus titer, log units‡
	Normal*	Infected†	Normal*	Infected†	
None	—	—	None	None	5.4
.01 M acetate	—	—	"	"	5.5
.01 M pyruvate	—	—	.17	.18	5.4
.5% lactalbumin hydrolyzate	—	—	None	None	6.0
.01 M glucose	2.24	2.50	3.53	3.81	7.0
.01 M glucose + .5% lactalbumin hydrolyzate	2.72	2.91	4.46	4.48	6.9

* μM/hr/culture based on 3 hr period.

† " " " " " 4½ " " .

‡ Use of complete maintenance medium (serum and lactalbumin hydrolyzate) produced a virus titer of 7.1.

TABLE III. Interaction of Pyruvate and Glucose as Substrates for Normal and FMDV Infected Bovine-Kidney Culture Cells.

Substrate	Glucose used		Pyruvate used		Lactic acid produced	
	Normal*	Infected†	Normal*	Infected†	Normal*	Infected†
1×10^{-3} M pyruvate	—	—	.36	.45	.00	.02
Idem + 2.5×10^{-3} M glucose	1.71	1.92	.49	.42	2.70	2.92
" + 1×10^{-2} M glucose	2.76	3.17	.42	.27	4.89	5.64
1×10^{-2} M pyruvate	—	—	7.00	7.18	.24	.26
Idem + 2.5×10^{-3} M glucose	1.68	1.97	5.30	5.16	2.55	3.49
" + 1×10^{-2} M glucose	2.83	3.40	3.40	3.62	4.84	5.64
2.5×10^{-3} M glucose	1.54	1.69	—	—	2.56	2.89
1×10^{-2} M glucose	2.52	2.80	—	—	4.49	4.42

* μM/hr/culture based on 3 hr period. † μM/hr/culture based on 4½ hr period.

combined with glucose did stimulate glucose consumption with a corresponding increase in lactic acid production, but with no significant increase in virus production. Acetate and pyruvate did not contribute to virus production, as had been partially indicated in other work(4). Infection with FMDV did not alter the cellular response to any of these substrates, except for the known increase in glucose consumption(1).

Table III illustrates the interaction of glucose and pyruvate as substrates in normal and infected cells. Pyruvate utilization was 2½ to 3 times greater than that of glucose at the 1×10^{-2} M substrate level, and increased in normal cells from 3.40 μM/hour culture to 7.00 μM/hour/culture as glucose concentration decreased from 1×10^{-2} M to zero. On the other hand, glucose utilization seemed independent of pyruvate concentration, as data from other experiments did not support the slight indication given in Table III of increasing glucose utilization accompanying increasing pyruvate concentration. Pyruvate utilization showed similar dependence on its own concentration as did glucose over the range 1×10^{-2} to 1×10^{-3} M. Infected cells in presence or absence of glucose did not consistently change their rate of pyruvate consumption. Glucose consumption was increased as usual in infected cells using the defined medium.

Cultures treated with malonate to interfere with possible Krebs cycle metabolism of glucose and pyruvate did not produce additional lactic acid and consumption of each substrate was only decreased 7-10% (Table IV, V). In these cultures, pyruvate consumption was about 4-fold larger than that of glucose, as compared to the 2½-3-fold greater consumption shown for a different set of cultures in Table III. Endogenous lactate formation was unaffected by malonate.

The respiration results in Table V establish that pyruvate is largely metabolized through other than oxidative pathways, and that acetate does not function as an oxidative substrate. The low level functioning oxidative process that does use pyruvate is not poisoned by malonate. Pyruvate utilization of the concentrated cell suspensions was only about one-fifth of that for cell layers, possibly

TABLE IV. Effect of Malonate on Glucose and Pyruvate Utilization in Bovine Culture Cells.

Substrate	Glucose used*	Pyruvate used*	Lactic acid produced*
1×10^{-2} M glucose	2.39	—	3.82
Idem + 1×10^{-2} M malonate	2.14	—	3.30
1×10^{-2} M pyruvate	—	9.33	.51
Idem + 1×10^{-2} M malonate	—	8.47	.38
TB-II	—	—	.04
1×10^{-2} M malonate	—	—	.05

* μM/hr/culture based on 3 hr period.

TABLE V. Respiration of Bovine Kidney Culture Cells on Acetate and Pyruvate Substrates.*

Substrate	Amt of substrate used, μM	Oxygen uptake, μM†
1×10^{-2} M acetate	0	0
1×10^{-2} M pyruvate	9.63	1.40
Idem + 1×10^{-2}M malonate	8.92	1.44

* Values based on 10^7 cells for a 5 hr period.
† Corrected for endogenous respiration.

due to the extra handling received by the suspended cells.

Discussion. In agreement with findings for other cell systems, utilization of glucose by bovine kidney cultures is dependent on both concentration of glucose and materials forming the substrates. The glucose concentration needed to saturate the available enzymes is about 1×10^{-2} M, although the greatest efficiency of glucose uptake is at 5×10^{-4} M.

Lactic acid is produced from glucose only, and its production is proportional to glucose concentration. The amino acids in lactalbumin hydrolyzate, which might be expected to produce lactic acid from pyruvic acid through transamination reactions, increase lactic acid only by stimulation of glucose utilization. Acetate does not stimulate lactate production nor oxygen uptake; results expected from its lack of consumption.

Pyruvate utilization ranged up to 4-fold greater than that for glucose and was apparently metabolized largely by non-oxidative pathways but did not yield lactic acid. Increased quantities of glucose, whose breakdown *via* 3-phosphoglyceraldehyde* would be expected to increase the quantity of reduced diphosphopyridine nucleotide (DPNH) and thus stimulate reduction of pyruvate to lactate, decreased pyruvate utilization while maintaining the usual lactic acid production from glucose alone. Pyruvate utilization was unchanged by virus infection in contrast to glucose utilization which was increased to about the same extent as with a more complete maintenance medium containing serum and lactalbumin hydrolyzate(1).

The pathways of pyruvate metabolism in bovine kidney cells are not clearly defined. Substrate pyruvate is not metabolized to lactic acid as shown by lactic acid analyses. Respiration data indicate that less than 6% of the consumed pyruvate can be oxidized completely to carbon dioxide and water, since 2.5 moles of oxygen are required for each mole of pyruvate. Oxidation to acetic acid can account for 29% of pyruvate consumption since here only ½ mole of oxygen would be required per mole of pyruvate. The oxidation that does occur is apparently not *via* the Krebs cycle since malonate has no effect on it, and carbon dioxide is not present for possible fixation with pyruvate to form the dicarboxylic acids catalyzing the cycle. Pyruvate does not apparently recycle to glycolytic intermediates or to hexose since glucose utilization is independent of pyruvate concentration. Transamination reactions producing alanine are not likely, since the reverse reaction starting with lactalbumin hydrolyzate apparently does not occur.

Microorganisms and mammalian tissues are known to utilize pyruvate in significant amounts, but no mammalian cell cultures have been described which utilize pyruvic acid to a greater extent than glucose. The reasons for bovine-kidney culture cells having this high pyruvate metabolism are not established, but presumably are linked to the high rate of aerobic glycolysis.

Summary. Normal primary bovine kidney cultures, partially depleted of endogenous nutrients, utilized pyruvate at a 2½- to 4-fold greater rate than glucose. The pathways of pyruvate metabolism were not clearly defined. Pyruvate was not appreciably metabolized oxidatively since oxygen uptake could account for only 6-29% of the total pyruvate consumed and malonate only slightly decreased its utilization. Pyruvate, singly, did not form lactate and was used to a greater extent as glucose concentration decreased. Glucose utilization, close to maximal at 1×10^{-2} M and dependent on glucose concentration, was independent of pyruvate concentration. Virus infection did not change the rate of pyruvate utilization in contrast to the increase found in glucose uptake. Lactalbumin hydrolyzate did not yield lactic acid of itself but did stimulate glucose uptake and corresponding lactate formation. Acetate was not oxidized nor produced lactate.

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Effect of Thyroid Hormone on the Pathways of Glucose Oxidation in the Intact Rat. (27119)

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An increase in metabolic activity of the phosphogluconate pathway occurs when human erythrocytes are incubated with physiological concentrations of triiodothyronine(1). This raises the possibility that thyroid hormone may stimulate the phosphogluconate pathway *in vivo*. Such a mechanism, if operative *in vivo*, would explain the uncoupling of oxidation from phosphorylation observed in the thyrotoxic animal, since increased metabolism *via* this pathway is accompanied by reduction of triphosphopyridine nucleotide (TPN), the reoxidation of which does not appear to be closely linked with phosphorylation(2). Thus it seems important to evaluate the activity of this pathway of carbohydrate metabolism in the intact animal, both when an excess and a deficit of the thyroid hormone is present.

A widely accepted, although frequently criticized(3,4), method for such an investigation is the measurement of the oxidation of the 1-carbon *vs* the 6-carbon of glucose by the monitoring of expired $C^{14}O_2$ following injection of specifically labeled glucose.

Method. Young (100 g) Wistar littermate female rats were either injected subcutaneously with 250 μg 3,3'5' triiodo-L-thyronine (Sigma Chemical Co.) daily for 7 days, or had the thyroid surgically removed 7-10 days prior to experiment. Oxygen consumption of animals in both experimental groups and in an untreated control group was measured(5) prior to injection of radioactive glucose. Since the average weight of rats in the 3 groups remained essentially similar (differences were not statistically significant) thyroidectomy apparently did not alter food intake and cannot be considered to influence the results.

One and five-tenths microcuries of specifically-labeled radioactive glucose (specific activity: glucose-1-C^{14}, 2.8 μc/mg; glucose-6-C^{14}, 2.0 μc/mg) (Volk Radiochemicals, Chicago) per 100 g rat was injected intravenously by the dorsal tail vein and the expired $C^{14}O_2$ radioactivity was continuously measured by a flow counter with associated graphic recording apparatus.‡ The resulting rate curves were manually integrated and the data are expressed as per cent of injected dose.

Results and discussion. Oxygen consumption, in milliliters O_2 consumed per minute per gram total body weight ($\pm$ SE), is as follows for the 3 groups; hyperthyroid (4 rats), 0.049 $\pm$ 0.005; euthyroid (6 rats), 0.026 $\pm$ 0.001; hypothyroid (6 rats), 0.021 $\pm$ 0.004.

Results from the tracer study, calculated as per cent of injected glucose-C^{14} appearing as $C^{14}O_2$, are presented in Fig. 1. Counting error was less than 5% at all ranges tabulated. In the hyperthyroid animals, rate of oxidation of both the glucose-1-C^{14} and glucose-6-C^{14} is increased (3.4- and 3.7-fold respectively); there being no significant difference between the 2 labeled positions. In the thyroidectomized rats, however, rate of oxi-

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