Glucose Catabolism in Normal and Autonomous Tobacco Tissue Cultures [1, 2]

J. Eugene Fox, Chong-maw Chen, and Ian Gillam [3]
Departments of Botany and Comparative Biochemistry and Physiology,
University of Kansas, Lawrence, Kansas

Callus tissue derived from the stem pith of *Nicotiana tabacum* L. cv. Wisconsin no. 38 invariably requires a medium containing an auxin and a kinin or the naturally occurring equivalent of these for growth in vitro (e.g. 2, 7). In recent years, however, strains which have lost their requirement for one or both of these plant growth regulators have appeared spontaneously in culture. One of these, a strain derived from normal tobacco callus but now completely autonomous for kinins and auxins, has been described previously (5) and its ability to synthesize auxin-like and kinetin-like materials noted (4, 14).

Strains with drastically reduced requirements for exogenously supplied growth substances are valuable since they provide a convenient means to investigate normal and neoplastic growth in plant tissues and to assess the role played by kinins and auxins in growth processes. Consequently, a study of the growth control mechanisms associated with the appearance of autonomy in plant tissues has been initiated; the work reported here is one aspect of that study.

Methods and Materials

The origin, growth habit, and the media used for culture of the normal, kinin-auxin requiring tissue (strain KX-1) and its fully autonomous derivative (strain O–1) have been previously described (5). O_2 uptake was measured in a Warburg respirometer at 25°. The gas phase was air. Tissues were sectioned free-hand into small slices 0.5 to 1.0 mm thick and 0.3 to 0.8 cm in diameter. The slices were washed for 2 hours in running tap water, rinsed 3 times in distilled water, blotted with cheese cloth, and suspended in 0.03 M potassium phosphate buffer, pH 6.0. Each Warburg flask contained approximately 35 mg (dry wt) tissue in 3.0 ml of buffer in the main compartment and 0.3 ml 4 N KOH in the center well.

Studies on the respiration of the C^{14} labeled substrates were conducted using the radiorespirometric technique of Wang et al. (13) modified in certain respects. Glucose-1-C^{14} (G-1-C^{14}), glucose-2-C^{14} (G-2-C^{14}), glucose-3, 4-C^{14} (G-3, 4-C^{14}), glucose-6-C^{14} (G-6-C^{14}), and uniformly labeled glucose (G-U-C^{14}) were purchased from New England Nuclear Corporation. Approximately 35 mg (dry wt) of either O–1 or KX–1 washed tissue slices were suspended in 4.5 ml of the tissue culture medium on which they had been growing (minus the organic components) at pH 6.0 in the main compartment of each modified Warburg flask; 0.5 ml of the C^{14} labeled substrate (20 μmoles, specific activity 0.05 mc/mM) was placed in the side arm. After passing air through the flask for 10 minutes to equilibrate the gas phase and the liquid, the contents of the side arm were poured into the tissue suspension in the main compartment of the flask. Throughout the incubation period at 25° the respirometer system was flushed with water-saturated air at 20 ml/minute. The air stream was allowed to escape through a glass sinter into a CO_2 trapping solution containing 2.0 ml of a mixture of ethanolamine: absolute ethanol, 1 : 2 v/v. At the end of each hour the trap solution was collected, rinsed out of the container with absolute ethanol, diluted to 5 ml, and added to 10 ml of a solution for scintillation counting [50 mg of 1, 4-bis-2-(4-methyl-5-phenyloxazoly) benzene and 3 g of *p*-terphenyl per liter of toluene].

After an incubation period of 5 hours, the medium was collected by filtration through coarse fritted glass, the tissues rinsed with an additional 10 ml of medium and the whole made up to 15 ml with water. A portion (50 μl) of this solution was added to a counting vial which contained 2 ml trap solution, 3 ml absolute ethanol and 10 ml of the counting solution. Samples were assayed for radioactivity in a Packard Tri-Carb model 334 liquid scintillation spectrometer.

Results

O_2 Consumption. O_2 uptake by autonomous and normal tissue slices over an experimental period of 3 hours is shown in table I. The time course of O_2 consumption in a typical experiment is demonstrated in figure 1. These results indicate that O_2 uptake in the autonomous strain of tobacco tissue is about 35 % greater on a dry weight basis than in the normal strain.

The effect of the age of the culture on respiratory

[1] Received November 24, 1964.
[2] Supported in part by National Institutes of Health Grant (RG-9902).
[3] Present address: Department of Biochemistry, University of British Columbia, Vancouver.

Table I. *O_2 Consumption in Normal and Autonomous Tobacco Tissue Cultures*

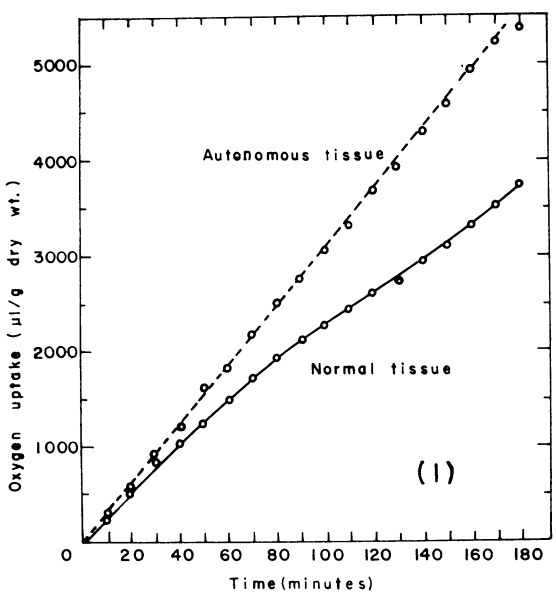
Expt. No.	Autonomous (strain O-1) *O_2 uptake (μl/g dry wt)	RQ	Tissue age (days)	Normal (strain KX-4) *O_2 uptake (μl/g dry wt)	RQ	Tissue age (days)
1	5210	1.12	16	3485	0.96	16
2	5499	0.94	21	3734	0.94	21
3	5585	1.01	26	4121	1.09	38
4	6191	0.92	28	4440	0.93	28
5	5680	0.97	34	4560	0.90	34
6	3154	0.91	45	2850	0.93	45
Avg	5219	0.98	28.3	3865	0.96	30.3

* Length of each experiment was 3 hours.

activities is shown in table I and figure 2. The O_2 uptake of the KX-1 tissue shows an increase from the sixteenth day reaching its maximum in the neighborhood of the thirty-fourth day of culture, after which it declines rapidly. In the autonomous strain the ability to take up O_2 seems to decline several days earlier. This difference may be more apparent than real since O-1 tissue grows at a faster rate than KX-1 (5) and therefore probably uses up the available nutrients sooner. The respiratory quotient (RQ) remained relatively constant throughout the experimental period (table I).

Glucose Uptake. Glucose uptake was measured by determining the radioactivity left in the medium after a 5-hour incubation period on C^{14} labeled glucose. The data presented here are based on the assumption that leakage of C^{14} labeled metabolites back into the medium does not occur to any appreciable extent, an assumption which was confirmed by chromatographic studies of the medium after incubation. Tobacco tissue slices of O-1 and KX-1 take up glucose from the incubation media at about the same rate (fig 3). While the age of the culture obviously has a pronounced effect on the rate of glucose uptake, there appears to be no great difference between the 2 strains in this respect.

Glucose Catabolism. In order to investigate the relative contribution of the catabolic pathways of glucose metabolism in O-1 and KX-1 tissues, the utilization of specifically labeled glucoses was examined by radiorespirometry. Most of the experiments discussed in this section were run for 5 hours and all were repeated from 2 to 4 times. The data shown in figures 4 and 5 demonstrate that the rate of $C^{14}O_2$ output in both O-1 and KX-1 tissues reaches a maximum by the end of the third or fourth hour and then levels off. Studies of glucose depletion show that only about half of the glucose has been removed from the medium after 5 hours. Age of the tissue is a variable, the effect of which was



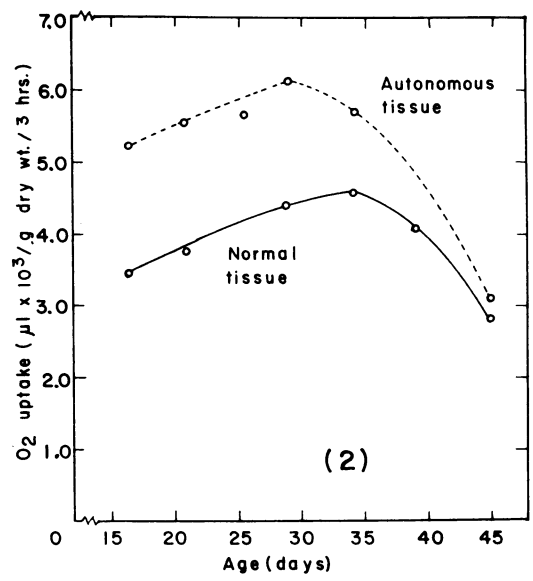


FIG. 1. Time course of O_2 consumption in a typical experiment (see table I, expt no. 2).
FIG. 2. O_2 consumption of normal and autonomous tobacco tissue slices as a function of age.

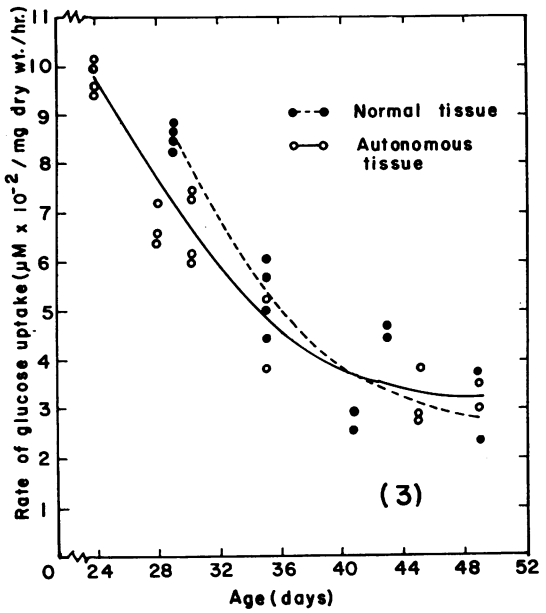


FIG 3. Effect of age on glucose uptake by normal and autonomous tobacco tissue slices. Each point represents an experiment involving 20 to 50 mg dry weight of tissue run for 5 hours. Glucose solutions were initially 20 μM.

not fully appreciated until a number of experiments had been run. Glucose uptake (and therefore CO_2 output) is greatly modified by the age of the tissue (fig 3). Since the experiments described here were run with tissue from cultures of a variety of ages (from 24–48 days), meaningful comparisons cannot be made on the basis of total CO_2 output per unit dry weight. It was found, however, that another measure of respiratory activity, the ratio cpm ($C^{14}O_2$) produced/cpm (glucose-C^{14}) taken up, was relatively independent of age and quite consistent from one experiment to the next. Such a finding very likely means that the older the tissue, the greater the proportion of nonviable cells included in the slices. The metabolic machinery in the living cells presumably does not change greatly during the period between 24 and 48 days.

Table II illustrates the recovery of $C^{14}O_2$ in a 5-hour period as a percent of the C^{14} taken up by the tissue as glucose. The most striking dissimilarity between the 2 strains is with respect to the recovery of $C^{14}O_2$ originally supplied in carbon atoms 3 and 4; in 5 hours approximately twice as much of the label taken up as glucose-3, 4-C^{14} is released as CO_2 by the autonomous tissue as by the normal strain. A smaller difference exists in the recovery of CO_2 from uniformly labeled glucose (28 % greater recovery in the O–1 strain), and this is probably due for the most part to the C-3, 4 difference. There may also be a slightly greater $C^{14}O_2$ recovery from glucose-2-C^{14} in the autonomous strain.

Table II. *Percent of Radiochemical Taken up by Tissues Appearing as $C^{14}O_2$ in 5 Hours*

Substrate	Normal tissue (KX-1)	Autonomous tissue (O-1)
	% recovery*	
Glucose-1-C^{14}	23.3 ± 2.69**	25.9 ± 1.32
Glucose-2-C^{14}	14.4 ± 2.66	17.6 ± 0.10
Glucose-3, 4-C^{14}	19.8 ± 2.00	40.6 ± 3.00
Glucose-6-C^{14}	11.9 ± 1.35	13.7 ± 0.25
Glucose-U-C^{14}	17.4 ± 1.91	22.3 ± 0.83

* Average of 2 to 4 replicate experiments.
** SE of the mean.

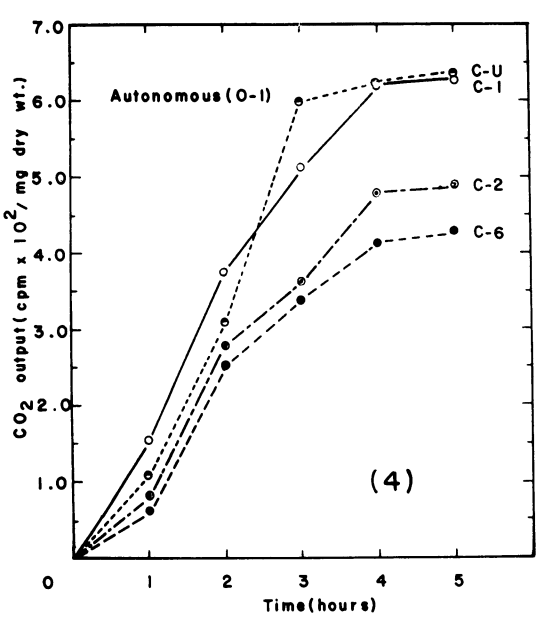
This difference in the way the tissues handle carbon atoms 3 and 4 of glucose was first suggested by studies in which the recovery of $C^{14}O_2$ was ascertained only for G-1-C^{14}, G-2-C^{14}, G-6-C^{14} and G-U-C^{14}. Since the compounds were supplied at the same specific activity (except for G-U-C^{14} which was supplied at the same total activity but having, therefore, only a sixth of the specific activity per carbon), a value for CO_2 recovery from carbon atoms 3 and 4 could be calculated by assuming that CO_2 recovery from carbon 2 equals that from carbon 5 and by using the following formula: $6(C\text{-}U)-[C\text{-}1+2(C\text{-}2)+(C\text{-}6)] = (C\text{-}3)+(C\text{-}4)$.

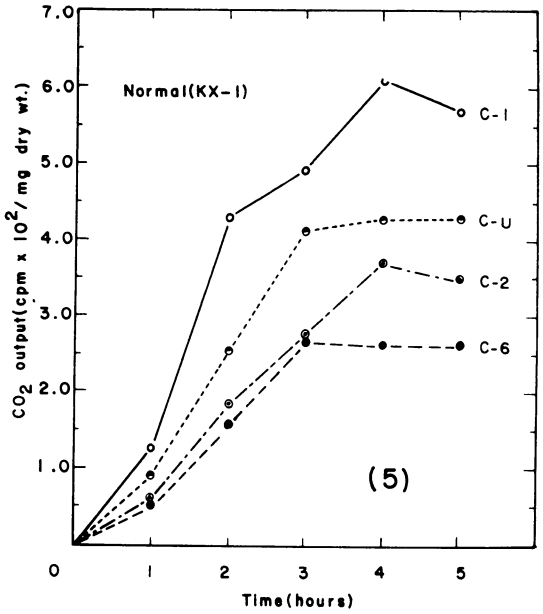
Making the further assumption that (C-3) = (C-4), we calculated that CO_2 recovery from carbon 3 of glucose in normal tobacco tissue was about 82 % of the observed $C^{14}O_2$ recovery from G-1-C^{14}. In the autonomous strain this figure was 140 %. Since $C^{14}O_2$ recovery from G-1-C^{14} is approximately equal in the 2 strains (table II), it would appear that CO_2 recovery from carbon 3 or 4 of glucose in 0–1 tissue is about 170 % of that in KX–1.

The data in table II, however, (based on measurements of $C^{14}O_2$ output from tissues respiring glucose-3(4)-C^{14}) indicate that this method of calculation somewhat underestimates the actual value. As shown in table III the ratio 3, 4/1 is 0.85 for KX–1 tissue and 1.57 for O–1. The discrepancy could be explained if the distribution of label in glucose-U-C^{14} was, in fact, not uniform. Our data suggest the possibility that the specific activity of carbons 3 and 4 is somewhat less than the average specific activity of carbons 1, 2, 5 and 6 in uniformly labeled glucose. An alternate explanation for the discrepancy involves the assumption previously made that CO_2 recovery from carbon 2 is equal to that from carbon 5. Such an assumption is valid only

Table III. *Ratio Values Derived from the Percent Recoveries of $C^{14}O_2$ Shown in Table II*

Position of label	Normal tissue KX-1	Autonomous tissue O–1
C_6/C_1	0.51	0.53
$C_{3,4}/C_1$	0.85	1.57
C_6/C_2	0.83	0.78
C_1/C_2	1.62	1.47





FIGS. 4, 5. Interval recovery of $C^{14}O_2$ from normal and autonomous tobacco tissues respiring variously labeled glucose substrates. Values are corrected for slight variations in the specific activities of the samples of glucose used.

if the fraction of glucose catabolized by way of the pentose pathway with a corresponding recycling of the pentose so derived through the EMP (Emden-Meyerhof-Parnas) pathway is small in relation to total glucose catabolism. This, in fact, may not be true in the tissues dealt with in this study, and the formula could, by overestimating CO_2 recovery from carbon 5, underestimate that from carbons 3 and 4.

The preferential oxidation of carbon 1 to CO_2 over that of carbon 6 (figs 4, 5, table III) in both strains provides evidence for the operation of the pentose pathway in these tissues (1). Since C_6/C_1 ratios are by themselves inadequate for an estimation of the exact proportion of the glucose metabolized by the pentose pathway (8), no statement can be made as to the relative importance of this pathway of glucose degradation in normal and autonomous tissues. The C_6/C_1 as well as the C_6/C_2 and C_1/C_2 ratios observed here, however, suggests no great difference between the 2 strains in non-EMP pathways.

Table IV shows the distribution of C^{14} after normal and autonomous tissues were incubated for 5 hours in the radiorespirometer in the presence of uniformly labeled glucose. CO_2 recovery from glucose was 28 % greater in O-1 tissue than in KX-1 (table IV). These results are in reasonably good agreement with those from the Warburg respirometer studies described in preceding paragraphs in which O_2 consumption (equal to CO_2 evolution in these tissues respiring sucrose) was about 35 % greater in the autonomous strain.

Seventy percent ethanol extracts from tissues which had been metabolizing variously labeled glucose substrates were examined by paper chromotography and autoradiography. While differences in the pattern of radioactive spots appeared depending upon the original position of the label, O-1 and

Table IV. *Distribution of Radioactivity of Uniformly Labeled Glucose Metabolized for 5 Hours*

Glucose -U-C^{14} 525.000 cpm was supplied. The oven-dried tissues were ground in a mortar and the powder extracted for 24 hours at room temperature with 5 % trichloroacetic acid (10 ml per 30 mg of dried tissue). The solvent was decanted and the tissues reextracted in fresh trichloroacetic acid for another 24 hours and the extracts combined (cold trichloroacetic acid fraction). The residue was then extracted for 15 minutes in boiling 70 % ethanol followed by a solution containing 3 parts 95 % ethanol to 1 part diethyl ether (v/v). Finally the tissues were air dried and extracted for 15 minutes with 5 % trichloroacetic acid at 90° (hot trichloroacetic acid fraction).

	Normal tissue (KX-1)	Autonomous tissue (O-1)
	% of radioactivity taken up by tissue	
Recovered as CO_2*	15.50*	21.90
Cold trichloroacetic acid soluble	39.24	34.43
Ethanol-diethyl ether soluble	2.14	2.23
Hot trichloroacetic acid soluble	0.85	0.24
Not extracted (by difference)	42.27	41.20

* The figures in table II differ from these since they are an average of four experiments while these data are for a single experiment.

KX–1 were similar in this respect in each case. The methods used here were too crude to detect quantative differences; it is clear, however, that no major qualitative differences exist between the 2 strains with respect to glucose metabolites extractable in 70 % ethanol. For extracts from tissues metabolizing uniformly labeled glucose, radioactivity was noted in at least 2 reducing compounds and in several ninhydrin-positive compounds, the major ones having R_F values similar to alanine and valine. Because of the essential similarity between the 2 strains, this line of inquiry was not pursued further.

Discussion

In many ways the autonomous tobacco tissue (O–1 strain) used in this study is the physiological equivalent of tumorous or neoplastic plant tissues induced by bacterial or viral activities. In fact, this tissue differs in its biosynthetic capacities only in minor details from fully autonomous crown-gall tumor tissue described by Braun (2). O–1 is a typical neoplastic plant tissue in that, unlike the normal tobacco tissue from which it was derived, it no longer requires an exogenous supply of kinins and auxins. Autonomic capacity appeared spontaneously and there is no evidence of viral or bacterial involvement (5). It is of some interest, therefore, to compare the metabolism of this strain with that of tumor tissues initiated by bacteria or viruses.

Higher oxidative activity of autonomous tissue as compared to normal as found in this study has also been reported in *Datura* crown-gall tissue cultures by Brucker and Schmidt (3). Earlier studies have generally shown reduced respiratory levels in crown-gall tumor tissues when these were compared with normal strains. Klein (9) indicated that crown-gall tumor tissue of *Parthenocissus tricuspidatus* had a lower respiration rate than either habituated or normal tissue from the same organism even though the growth rate of the crown-gall strain was substantially higher than that of the other two. Tamaoki et al. (12) indicated that mitochondria from crown-gall tissues of tomato had lower oxidative activity and were lower in phosphorylating efficiency than mitochondria from normal callus tissue although the 2 strains grew at similar rates. Gentile (6) reported similar findings in mitochondria from normal and tumor tissues of *Rumex*.

Scott et al. (11) have suggested that an enhanced oxidative decarboxylation of glucose to ribose takes place in tumor tissues of *Beta vulgaris* induced by *Agrobacterium tumefasciens*. This conclusion is based primarily on their observation that certain enzymes of the pentose pathway were much more active in homogenates of tumor tissue as compared with normal. The relationship of the activity of extracted enzymes to their operation in vivo is, however, obscure as the authors pointed out.

The neoplastic tissue used in this study differs rather markedly from a number of crown-gall tumor tissues in its increased capacity for O_2 uptake (35 % greater on a dry wt basis than normal tobacco tissue). This increased O_2 consumption is paralleled by a greater CO_2 evolution as shown by manometric studies (table I). The RQ of tumor tissue is not significantly different from normal callus. Our data indicate that the difference in rate of O_2 consumption is probably not due to major differences between the 2 strains in pathways of carbohydrate catabolism.

The preferential conversion of carbon atom 1 to CO_2 compared to carbon 6 suggests that the pentose pathway is very likely operative in both strains of tobacco tissue, but the C_6/C_1 ratios observed (table III) suggest no great difference between the normal and autonomous tissue. It is recognized, however, that because of numerous side reactions it is not possible to use the ratio of CO_2 evolution from C_6 and C_1 labeled glucoses as a direct estimate of the amount of glucose catabolized by the pentose pathway (8). Consequently, there may be a quantitative difference in pentose pathway activity between O–1 and KX–1 which is obscured by side reactions affecting C_6/C_1 ratios, although if such a difference exists, it is not likely to be large since the C_6/C_2 and the C_1/C_2 ratios are also closely similar (table III).

The much more extensive conversion of carbon atoms 3 and 4 in glucose to CO_2 in the autonomous than in the normal strain (table II) suggests that the major metabolic difference between the 2 is the rate at which oxidative decarboxylation of pyruvate derived from glucose takes place; the increased capacity for O_2 uptake in the O–1 strain supports this conclusion. Presumably the acetate derived in this manner would support an increase in biosynthetic activities in the autonomous tissue. The relative similarity in the 2 strains with respect to recovery of $C^{14}O_2$ from C_2 and C_6 labeled glucose would argue against there being any great difference in tricarboxylic acid cycle activity unless a greater portion of the carbon were funneled off during the cycle for synthetic purposes in the O–1 strain.

It appears, therefore, that the greater growth rate and concomitant biosynthetic activities of the autonomous tobacco tissue used in this study could be explained on the basis of an increased rate of decarboxylation of pyruvate. Such an explanation of course, says nothing about the underlying mechanisms for this change. Furthermore, the relationship of the metabolic change discussed here to the activation of hormone synthesizing systems is not at all clear. It is anticipated that this study will be the groundwork for a more intensive treatment of these questions.

Summary

A pronounced difference in oxygen consumption and glucose catabolism exists between an auxin-kinin dependant tobacco tissue and a derived strain auton-

omous for these growth regulators. On a dry weight basis the latter takes up oxygen at a rate roughly half again as fast as the former although the rate of glucose uptake is about the same in the 2 strains.

By the use of glucose labeled with C^{14} in different positions and the recovery of CO_2 in a Warburg manometer modified for radiorespirometry, it was shown that pentose pathway activity is prominent in both kinds of tissue to about the same degree. However, when glucose-3, 4-C^{14} is respired, a much greater fraction of the radioactivity taken up per mg dry weight is recovered as $C^{14}O_2$ in the autonomous tissue than in the normal. The data suggest that the greater growth rate and increased oxygen consumption in the autonomous tissue are associated with an enhanced oxidative decarboxylation of pyruvate derived from glucose.

Acknowledgment

The authors express their deep appreciation to Dr. Paul Kitos for many suggestions and discussions and for assistance with the radiorespirometer.

Literature Cited

1. Bloom, B. and D. Stetten, Jr. 1953. Pathways of glucose catabolism. J. Am. Chem. Soc. 75: 5446.
2. Braun, A. C. 1962. Tumor inception and development in the crown-gall disease. Ann. Rev. Plant Physiol. 13: 533–58.
3. Brucker, W. and W. A. K. Schmidt. 1959. Zum Zuckerstoffwechsel des Kallus - und Crown-gall-Gewebes von *Datura* und *Daucus*. Ber. Deut. Botan. Ges. 72: 321–32.
4. Fox, J. E. 1962. A kinetin-like substance in habituated tobacco tissue. Plant Physiol. 37: xxxv.
5. Fox, J. E. 1963. Growth factor requirements and chromosome number in tobacco tissue cultures. Physiol. Plantarum 16: 793–803.
6. Gentile, A. C. 1963. Oxidative activities of mitochondria from callus and tumor tissues of *Rumex acetosa* L. J. Exptl. Botany 14: 412–18.
7. Jablonski, J. R. and F. Skoog. 1954. Cell enlargement and cell division in excised tobacco pith tissue. Physiol. Plantarum 7: 16–24.
8. Katz, J. and H. G. Wood. 1960. The use of glucose-C^{14} for the evaluation of the pathways of glucose metabolism. J. Biol. Chem. 235: 2165–77.
9. Klein, R. M. 1957. Growth and differentiation of plant tissue cultures. In: Rhythmic and Synthetic Processes in Growth. Princeton University Press, Princeton, New Jersey. p 31–58.
10. Lance, C. 1961. Etude comparative des systèmes déshydrogénasiques de mitochondries isolees de souches de tissus normaux et tumoraux de *Scorsonère* cultivés in vitro. Compt. Rend. 252: 933–35.
11. Scott, K. J., J. S. Craigie, and R. M. Smillie. 1964. Pathways of respiration in plant tumors. Plant Physiol. 39: 323–27.
12. Tamaoki, T., A. C. Hildebrandt, R. H. Burris, A. J. Riker, and B. Hagihara. 1960. Respiration and phosphorylation of mitochondria from normal and crown-gall tissue cultures of tomato. Plant Physiol. 35: 942–47.
13. Wang, C. H., I. Stern, C. M. Gilmour, Sigrid Klungsoyr, D. J. Reed, J. H. Bialy, B. E. Christensen, and V. H. Cheldelin. 1958. Comparative study of glucose catabolism by the radiorespirometric method. J. Bact. 76: 207–16.
14. Weis, J. S. and J. E. Fox. 1963. Materials with auxin-like activity in autonomous tobacco tissue. Plant Physiol. 38: xxxviii.