THE EFFECT OF VARIATIONS IN CARBON AND NITROGEN CONCENTRATIONS ON PHENOLICS FORMATION IN PLANT CELL SUSPENSION CULTURES

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ABSTRACT.—Plant cells produce a wide variety of natural products of potential commercial importance. The production of secondary metabolites is normally very low in plant cell suspension cultures. Phenolics are important representatives of secondary metabolites. Their metabolic regulation provides a useful model system to test schemes to increase production rates for secondary metabolites. In this series of experiments we have examined the effect of carbon-nitrogen ratios (C/N) on extracellular phenolics production from Pauls Scarlet Rose (*Rosa* sp.) and soybean (*Glycine max* L.) cells in suspension culture. The maximum specific productivity of the culture (maximum g. phenolics produced per g. maximum cellular dry weight) varied by an order of magnitude as the initial C/N was varied by a factor of two to three. This study indicates the possibility of obtaining increased phenolics production by manipulating medium constituents.

Plant cell medium is usually developed to achieve maximum biomass by varying carbohydrate, nitrogen, or hormonal concentrations. The same constituents, however, may also be used to enhance natural product formation. Increased yields of secondary products will be a prerequisite to the commercial exploitation of plant cell suspension cultures for the "factory production" of such chemicals. This paper will examine the effects of manipulation of the initial carbon/nitrogen ratio in the medium on the formation of phenolics.

Much of the current research on the regulation of secondary products has centered on the metabolic control of the enzyme phenylalanine ammonium-lyase (PAL). Certainly PAL is one of the key enzymes in the regulation of phenolic metabolism. The synthesis of the more complex phenolics, such as the flavonoids, involves coordination of the above pathway with another major pathway to supply malonyl-CoA. Reviews of possible regulatory mechanisms are given by Creasy and Zucker (1) and by Grisebach and Hahlbrock (2). Phenolic metabolism provides a good model system for studies on the metabolic control of secondary product formation.

Phenolic compounds also are commercially important because of the role they may play in plant disease resistance as well as their role in imparting certain desirable tastes, smells, or colors to food products.

Several studies have examined the effects of nutrient changes on the formation of extractable phenolics from cell tissue or on total phenolics production in plant cell suspensions (3, 4, 5). From a practical viewpoint, certain systems for the production of secondary products can be best designed for the recovery of extracellular products with the cells retained as active catalysts (see Kan and Shuler (6)). Such a viewpoint requires an understanding of how environmental manipulations effect phenolic metabolism and extracellular accumulation of phenolics. In this paper we explore the effect of initial C/N ratio on extracellular phenolics production over a wider range of C/N values than previously reported.

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MATERIALS AND METHODS

Callus cultures of Paul's Scarlet Rose (*Rosa* sp.) were obtained from Dr. D. K. Dougall of the W. Alton Jones Cell Science Center, Lake Placid, N.Y., in June 1975. Soybean cells (*Glycine max* L.) were obtained from Dr. J. Thompson, Federal Nutrition Laboratory, U.S.D.A., Ithaca, N.Y., in May 1975. These cell lines have been lost due to a temperature regulator failure on the incubator. The soybean cells were maintained on B5-1 medium-solidified agar (1.5%), while the rose cells were maintained on modified MPR medium (MPR-1). The MPR-1 medium and conditions for the maintenance of the stock cultures have been described previously (8). Stock cultures were maintained on solid medium and were used to initiate suspension cultures.

The growth experiments were conducted at 26° in a New Brunswick Psychrotherm Shaker (New Brunswick Scientific Co., Inc.) with 150 ml of medium and 10 ml of inoculum in a 500 ml Erlenmeyer flask. The inoculum was prepared by placing a callus in a shake flask, allowing a suspension culture to develop, then transferring a 15% inoculum to other flasks. These second flasks provided the inoculum for the growth experiments. The growth flask experiment was initiated when the cells in the source flask had just entered the stationary phase of growth. Transfers and sampling were accomplished using de-tipped 10 ml pipettes. Transfers for the preparation of inoculum flasks were accomplished in such a way as to exclude the transfer of large lumps or aggregates. The flasks were agitated by shaking at 150 rpm. The cultures were maintained in the dark to prevent formation and accumulation of phenolic oxidation products which are known to inhibit growth (4).

The flasks were generally sampled daily until the third to fifth day after the beginning of the stationary growth phase (based on mass). Glucose concentrations were measured with an enzymatic assay (Calbiochem, Glucose-S.V.R., La Jolla, Ca.). Ammonia nitrogen was assayed with a specific ion electrode (Orion Research, Inc., Cambridge, Ma.). Nitrate was measured using standard techniques (9). In all experiments the ammonia nitrogen was rapidly removed from the medium (in two or three days) independent of the growth-limiting nutrient. Consequently, ammonia nitrogen measurements are not included in the batch growth plots; nitrate-nitrogen was 97% of the total inorganic nitrogen in MPR-1 medium and 93% of that in B5-1 medium and was the controlling nitrogen source. Media are denoted as 1N if they contain the normal complement of inorganic nitrogen sources, ½N if they have one-half of the normal concentrations, and ¼N is it is one-fourth.

Dry weight was measured by filtering (under vacuum) a 3 to 4 ml sample from the flask through a 1.2 μm Millipore filter. The cells were washed with two aliquots of distilled water equal in volume to the sample size. The cell mass was dried at 70° to constant weight for the dry weight determination. A second sample was centrifuged and the supernatant liquid was frozen (−20°) for the subsequent measurement of phenolics, glucose, ammonia nitrogen, and nitrate. Total phenolics in the filtrate were measured using the technique described by Swain and Hillis (10), without modification. Gallic acid was the standard.

RESULTS AND DISCUSSION

Examples of the growth response of rose cells in batch culture to variations in medium composition are given in figs. 1 to 4. Soluble phenolics formation can be characterized as mixed primary-secondary production, since product formation begins in the growth phase but continues well into the stationary growth phase, often with an increased rate in the stationary phase. A significant external supply of carbon (glucose) must exist to support phenolic production during the stationary growth phase. However, an external supply of carbon is not sufficient to insure strong secondary production of phenolics as can be observed in fig. 4 for the experiment with 8% glucose in the medium. High glucose concentrations decreased growth rates as can be summarized from the data in table 1. The lower phenolics production at these high levels reflects significant changes in overall cellular metabolism.

The results from the fourteen experiments with rose cells are summarized in figs. 5 and 6. The plot of maximum specific productivity (maximum grams of phenolics formed during the batch cycle divided by the maximum dry weight achieved in the batch culture) versus initial C/N shows that the biomass has a maximum specific productivity at a C/N value of 30. The information from fig. 6 shows that the maximum potential concentration of phenolics is achieved at a C/N ratio of 35 (3% glucose and the normal complement of ammonia and nitrate-

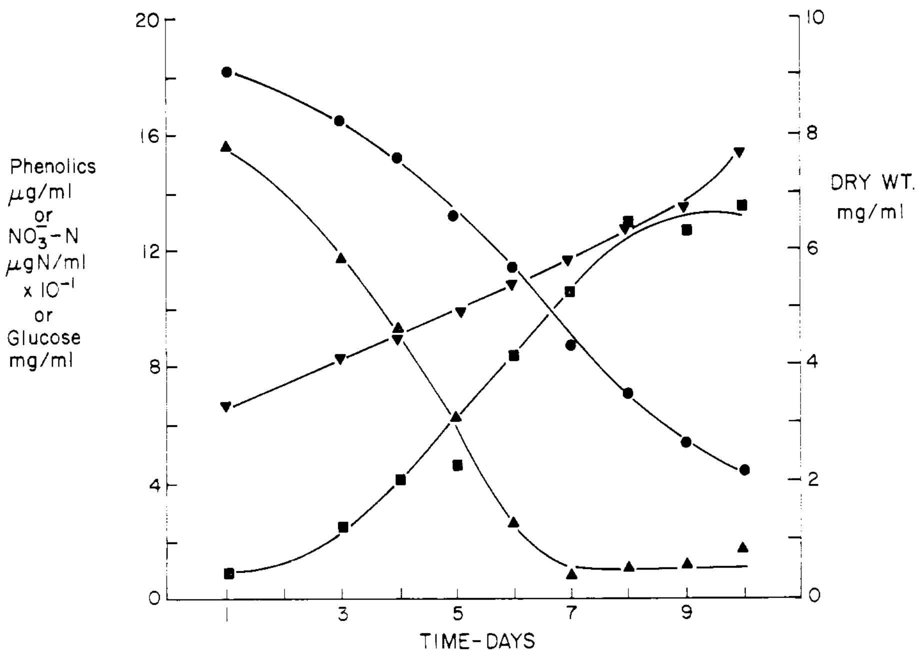

FIG. 1. Phenolics production, growth, and carbon and nitrate-nitrogen utilization for rose cells cultured in 2% glucose and ½N medium (172 ppm NO_3^--N). Data for each constituent given by: ● for glucose, mg/ml; ▲ for NO_3^--N, μgN/ml x 10^{-1}; ■ for dry weight, mg/ml; and ▼ for phenolics, μg gallic acid/ml.

nitrogen). The curve in fig. 6 has a shape similar to that in fig. 5 but is shifted to slightly higher C/N values.

In table 2 the possibility of a shift in mechanisms for the uptake of glucose and nitrate is indicated for rose cultures with both a high C/N value and the normal complement of nitrogen. For such cultures C/N values in excess of 23 result in a significant and abrupt change during the batch cycle in the ratio of uptake rates for C and nitrate-N. This change in uptake occurs when 200–250 mg/liter of nitrate-N remains and is approximately coincident with the initiation of an increasing rate of accumulation of extracellular phenolics. The cultures giving the greatest rates of phenolic production are those showing an abrupt increase in carbon uptake and exhibiting ultimately consumption of all carbon with nitrate still present. If the cultures ultimately have excess carbon after the complete exhaustion of all the nitrate, then the increased relative rate of carbon uptake is not directed strongly toward the net extracellular accumulation of phenolics. For those cultures with a high glucose level (8% and 10%) a third region of increased relative carbon uptake may exist at nitrate-nitrogen values of 50 to 100 mg/liter. No obvious correlation of this uptake region with phenolics production was evident.

For soybean cultures, growth curves were obtained that are similar to that for rose cells (i.e. figs. 1 to 4) except for a burst of phenolics production as the cells left the lag phase. This burst of activity was observed only in those flasks with the normal level of inorganic nitrogen. A typical batch growth curve is given

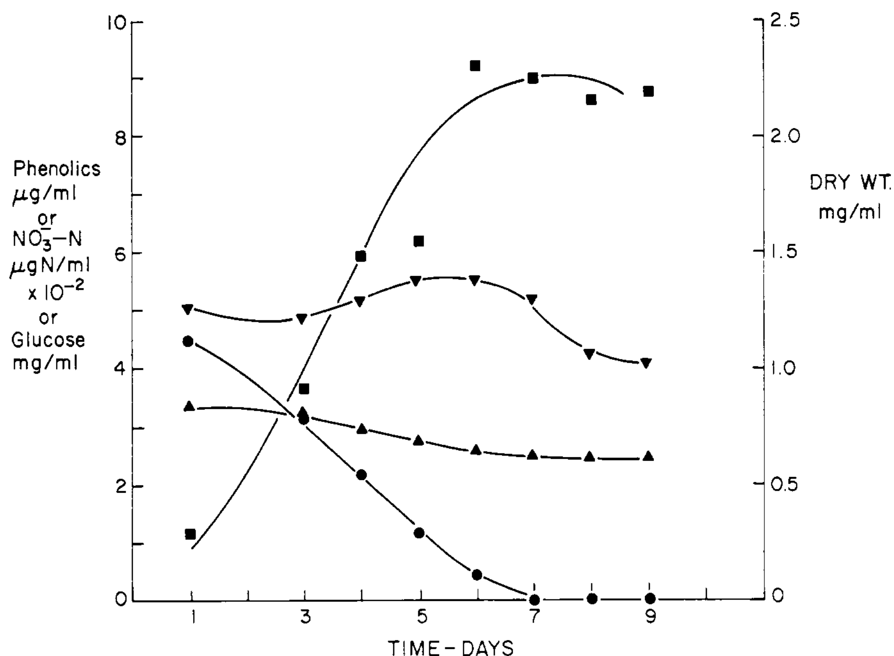

FIG. 2. Phenolics production, growth, and carbon and nitrate-nitrogen utilization for rose cells cultured in 0.5% glucose and 1N medium (344 ppm NO_3^--N). Data for each constituent given by: ● for glucose, mg/ml; ▲ for NO_3^--N, μgN/ml x 10^{-2}; ■ for dry weight, mg/ml; and ▼ for phenolics, μg gallic acid/ml.

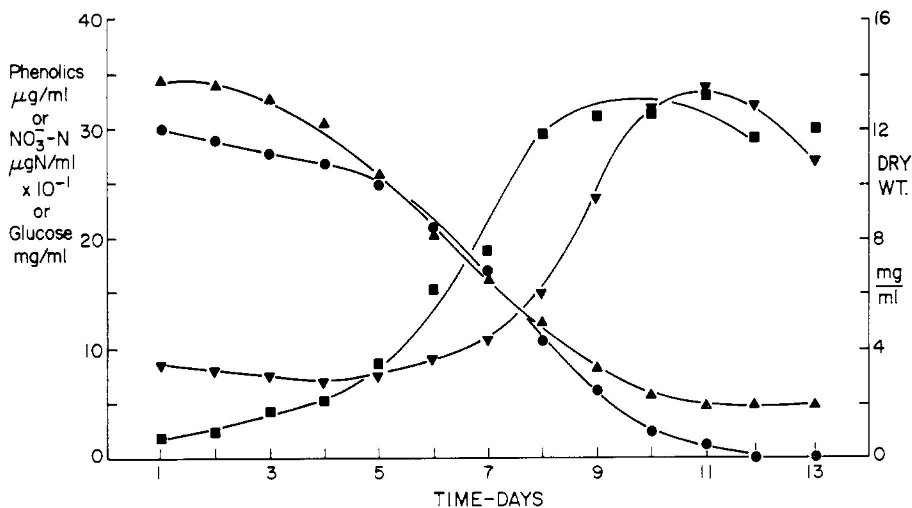

FIG. 3. Phenolics production, growth, and carbon and nitrate-nitrogen utilization for rose cells cultured in 3% glucose and 1N medium (344 ppm NO_3^--N). Data for each constituent given by: ● for glucose, mg/ml; ▲ for NO_3^--N, μgN/ml x 10^{-1}; ■ for dry weight, mg/ml; and ▼ for phenolics, μg gallic acid/ml.

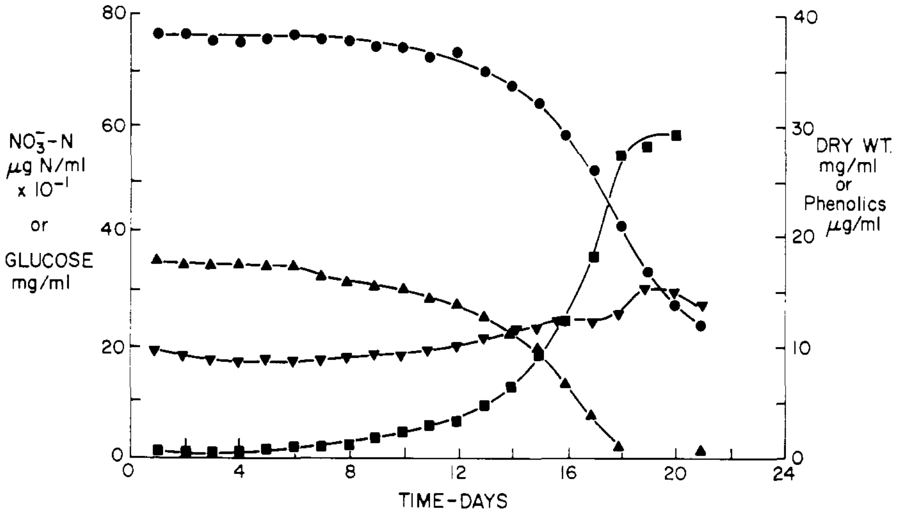

FIG. 4. Phenolics production, growth, and carbon and nitrate-nitrogen utilization for rose cells cultured in 8% glucose and 1N medium (344 ppm NO_3^--N). Data for each constituent given by ● for glucose, mg/ml; ▲ for NO_3^--N, μgN/ml x 10^{-1}; ■ for dry weight, mg/ml; and ▼ for phenolics, μg gallic acid/ml.

TABLE 1. The effect of nutrient concentrations on growth rates.

Medium	Cell	Doubling Time-t_d hr	Specific growth rate-μ hr^{-1}
MPR-1-2% glucose-¼N	Rose	46	0.015
MPR-1-2% glucose-½N	Rose	38	0.018
MPR-1-0.5% glucose-1N	Rose	38	0.018
MPR-1-1.0% glucose-1N	Rose	38	0.018
MPR-1-1.5% glucose-1N	Rose	37	0.019
MPR-1-2.0% glucose-1N	Rose	37[b]	0.019
MPR-1-2.5% glucose-1N	Rose	40	0.017
MPR-1-3.0% glucose-1N	Rose	38	0.018
MPR-1-5.0% glucose-1N	Rose	50	0.014
MPR-1-8.0% glucose-1N	Rose	56[b]	0.012
MPR-1-10% glucose-1N	Rose	70	0.010
B5-1-2% glucose-¼N	Soybean	29	0.024
B5-1-2% glucose-½N	Soybean	29	0.024
B5-1-0.5% glucose-1N	Soybean	31	0.022
B5-1-1% glucose-1N	Soybean	31	0.022
B5-1-2% glucose-1N	Soybean	30	0.023

[a]μ equals ln2/t_d.
[b]Average of duplicate runs.

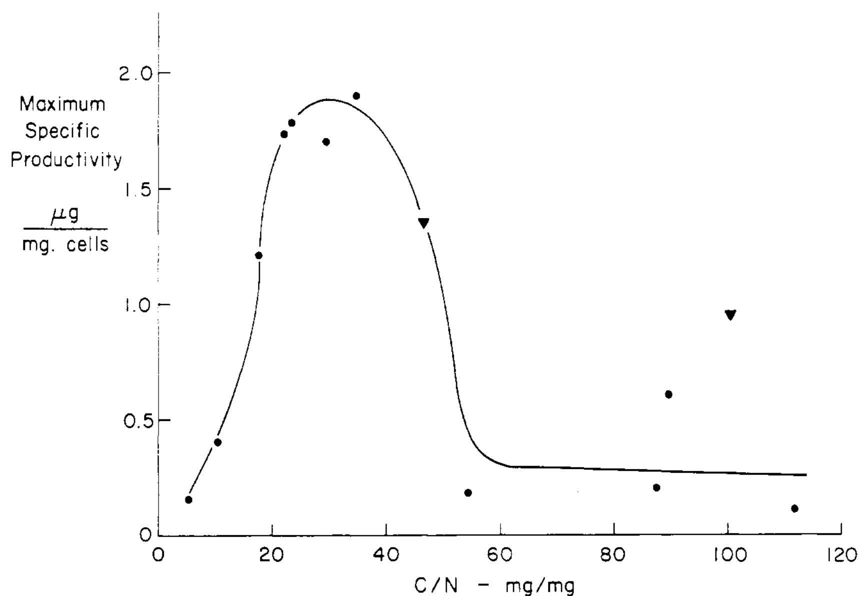

FIG. 5. The relationship of maximum specific productivity to carbon to nitrogen ratio (C/N) for rose cells is shown. Data from each experiment with the normal complement of nitrogen (i.e., 1N) are denoted by ● while those with the normal glucose concentration (2%) but reduced nitrogen are denoted by ▼. Replicate flasks were used at C/N = 23 and C/N = 88.

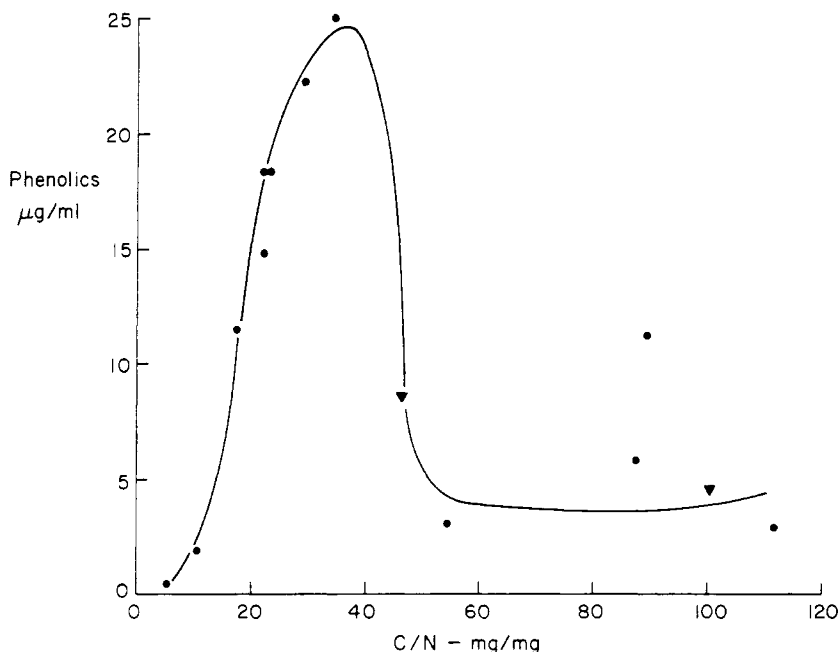

FIG. 6. The relationship of maximum phenolics produced (i.e., maximum concentration minus that initially present) to C/N ratio for rose cells cultured in shake flasks. Data from experiments with the normal complement of nitrogen (i.e., 1N) are denoted by ● while those with the normal glucose concentration (2%) but reduced nitrogen are denoted by ▼.

TABLE 2. The ratio of uptake rates for carbon and nitrate nitrogen for Paul's Scarlet Rose.

Medium	Initial C/N	Uptake Ratio[a] μgC/time ÷ μg NO_3^--N/time	First nutrient exhausted[b]
MPR-1-2% glucose-¼N	92.8	20.3	N
MPR-1-2% glucose-½N	46.4	21.5	N
MPR-1-½% glucose-1N	5.8	19.4	C
MPR-1-1% glucose-1N	11.6	20.8	C
MPR-1-1.5% glucose-1N	17.4	24.4	C
MPR-1-2% glucose-1N	23.2	27.3	C
MPR-1-2% glucose-1N	23.2	28.8	C
MPR-1-2.5% glucose-1N	29.0	20.8 & 41.4	C
MPR-1-3% glucose-1N	34.8	22.4 & 56.9	C
MPR-1-5% glucose-1N	58.0	25.5 & 41.6	N
MPR-1-8% glucose-1N	92.8	24.6 & 43.0 & 79.1	N
MPR-1-8% glucose-1N	92.8	24.0 & 36.9 & 65.1	N
MPR-1-10% glucose-1N	116.0	24.2 & 44.2 & 96.6	N

[a]If the data clearly indicates more than one slope, the slopes are listed in chronological order from the point of inoculation. The uptake ratio is conveniently obtained from the slope of a line on a plot of C versus nitrate-nitrogen concentrations.

[b]Only C and N were measured; other nutrients may have been exhausted first.

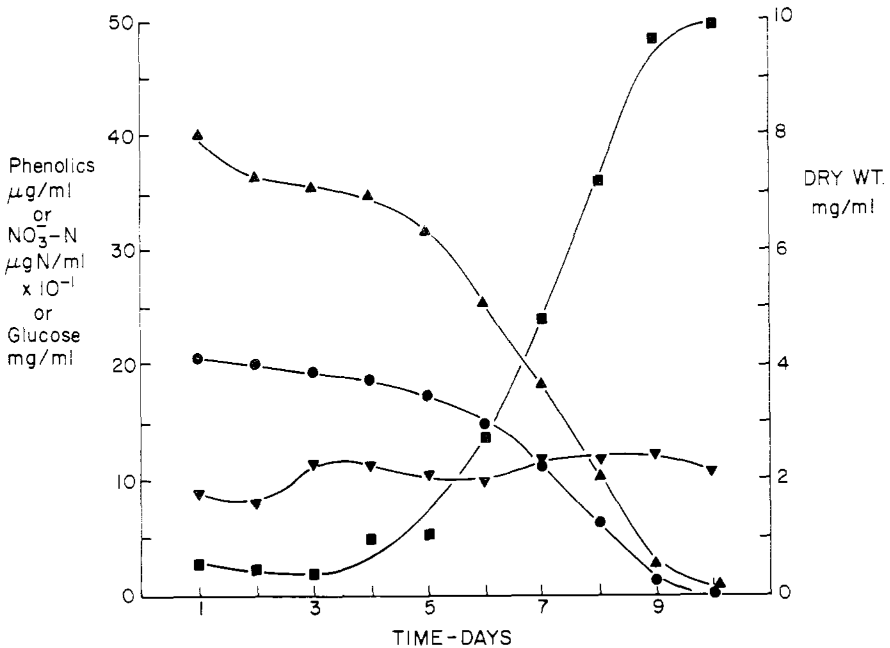

FIG. 7. Phenolics production, growth, and carbon and nitrate-nitrogen utilization for soybean cells in unmodified B5-1 medium (2% glucose, 344 ppm NO_3^--N). Data for each constituent given by: ● for glucose, mg/ml; ▲ for NO_3^--N, μgN/ml x 10^{-1}; ■ for dry weight, mg/ml; and ▼ for phenolics, μg gallic acid/ml.

in fig. 7. As shown in fig. 8, the maximum specific productivity of soybean cultures increases with C/N ratio; the true maximum point was not located but presumably would be at a C/N value greater than 85. The loss of the original cell line has precluded the continuation of the experiment to determine the exact location of the maximum. In contrast to rose cells a definite minimum value of the maximum specific productivity may occur at a C/N ratio of 20.

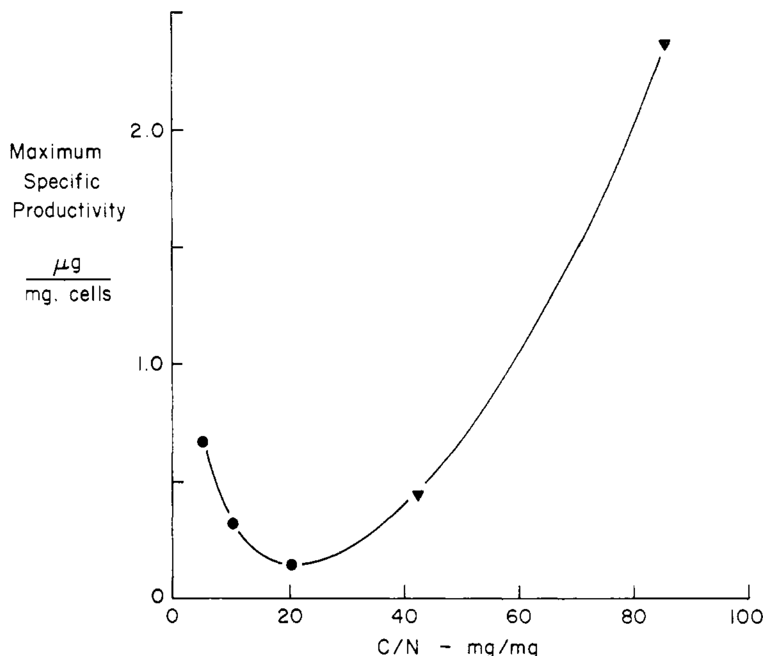

FIG. 8. The relationship of maximum specific productivity to carbon to nitrogen ratio (C/N) for soybean cells is shown. Data from each experiment with the normal complement of nitrogen (i.e., 1N) are denoted by ● while those with the normal glucose concentration (2%) but reduced nitrogen are denoted by ▼.

An attempt was made to fit these data on growth and product formation to the model suggested by Leudeking and Piret (11). Their model, originally derived for lactic acid production from bacteria, has been used successfully for a number of microbial products. The product formation pattern in these plant cells has proven to be more complex and cannot adequately be described by the model of Leudeking and Piret (11) or any other simple model which includes both primary and secondary production. The "quality" or "physiological state" of the biomass must be an important parameter. Consequently, phenolic production may depend upon the physiological history of the culture. The physiological history is used here to describe both the long term changes resulting for culture maintenance on artificial medium and the short term changes brought about by exposure to various sequences of environmental stimuli. The long term effects may be genetic in nature while the short term effects more metabolic.

The biochemical interpretation of the above results is complicated by probable changes in the growth-limiting substrate. For rose cells the media with reduced nitrogen inputs were nitrogen-limited. Those with glucose concentrations of

1.5% (w/v) or less and the normal level of nitrogen were carbon-energy limited. However, those cultures with glucose concentrations in excess of 1.5% and the normal complement of nitrogen may possibly have been limited by another nutrient, most probably phosphate (3).

Finally, these results must be interpreted remembering that light was excluded. Since PAL is under photo control, the exclusion of light has a significant effect on the net synthesis of phenolic compounds. The level of PAL activity can be increased markedly by exposure to light (1) and would be expected to increase the formation of phenolic compounds. However, the purpose of this study is not to maximize but rather to test the effect of the C/N ratio on phenolic's production.

These results can be compared to that of other workers. Nash and Davies (3) measured the total phenolic content within rose cells as a function of time in culture. They observed a significant net loss of phenolics during the lag phase and a cessation of phenolics production upon the exhaustion of carbohydrate from the medium which occurred at the onset of the stationary growth phase. In our studies rose cells did not produce high levels of extracellular phenolics until after the stationary phase. The early depletion of carbohydrate (fig. 2), however, did cause a cessation of phenolics production before the onset of the stationary phase. The decline that we observed in phenolics during the lag phase was very modest (~5 to 20%) rather than the 50% decline Nash and Davies (3) found. Otherwise our results on the extracellular accumulation of phenolics are consistent with that of Nash and Davies (3) and the intracellular accumulation of phenolics.

More recently Amorim, Dougall, and Sharp (4) have reported on a more extensive set of experiments to determine the effect of carbohydrate and nitrogen concentrations on total phenolic and leucoanthocyanins synthesis in Paul's Scarlet Rose cells. Total ethanol-extractable phenols from cell tissue plus total phenols in the culture medium were measured at days 4, 8, and 12 for batch-grown suspension cultures. Their cultures were grown in the presence of illumination (3.03 Wm^{-2}). They concluded that increasing exogenous glucose in the culture medium resulted in an increased synthesis of total phenols and leucoanthocyanins in stationary phase cells. Because of the growth inhibitory effects of high glucose concentrations, they employed a maximum glucose concentration of 0.2M (~3.6%). Their maximum C/N ratio was approximately 37, which nearly corresponds to the C/N value, giving the maximum productivity in our cultures. From their data a maximum specific productivity of 2.1 μg as chlorogenic acid/mg dry weight at a C/N value of 37 can be calculated, which is close to the value given in fig. 5 (1.9 μg as gallic acid/mg dry weight). Their cultures would be expected to have higher productivity since their cultures were illuminated and they measured both intracellular and extracellular total phenolics. By restricting themselves to moderately low values of glucose and moderately high concentrations of nitrate, they did not explore the region of high C/N values as reported on in this paper and did not observe the sharp maximum in phenolics production as shown in fig. 5.

Another recent contribution, by Phillips and Henshaw (5), has explored the regulation of synthesis of phenolics in stationary phase suspension cell cultures of *Acer pseudoplatanus* L. They postulate that protein synthesis partially regulates phenolics production through competition for common precursors, particularly phenylalanine. The addition of 2,4-D and urea were shown to increase

protein synthesis and inhibit phenolics production. The addition of sucrose to nitrogen- and phosphorous-depleted medium containing stationary phase cells led to the rapid intracellular accumulation of phenolics after a five-day lag period. Our results at C/N values of less than 40 are consistent with this hypothesis. For high C/N values, the low phenolic productivity observed in spite of high relative carbon uptake values possibly indicates the intervention of other regulatory processes. However, our studies may have terminated measurements on phenolic synthesis too soon, at least in some cases, to capture a phase of renewed phenolic biosynthesis after a substantial lag in the stationary phase. Also, the possibility that glucose and sucrose may give different patterns of polyphenol metabolism cannot be exluded (5).

ACKNOWLEDGMENTS

This work was supported in part by NSF Grant No. ENG 77-17855. We gratefully acknowledge the technical assistance of Mr. Alex Tannahill.

Received 5 March 1979.

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