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STIMULATION OF [^{14}C] GLUCOSE UPTAKE AND METABOLISM IN BEAN ROOT TIPS BY NAPHTHENATES

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Abstract—Potassium naphthenates (KNap) and especially potassium cyclohexanecarboxylate (KCHC) stimulated the uptake and metabolism of [^{14}C-UL] glucose in excised bush bean root tips Not only was CO_2 production increased, but also amino acids containing glucose carbon having passed through soluble pools in root tissues, were more rapidly fixed into protein The conjugated form of naphthenate, rather than the free acid, may have been responsible for the observed stimulation of glucose assimilation

INTRODUCTION

THE CONVENTIONAL VIEW of the naphthenic acids in petroleum being primarily mononaphthenic and alkanoic acids recently has been expanded [1,2] In addition to compounds with molecular weights greater than 1000,[3] Eider[4] has confirmed the presence of several low molecular weight acids, including cyclohexanecarboxylic acid, in the naphthenic acid fraction from an Aruba crude oil

Data obtained in our laboratory[5–8] have shown that, in biological systems, these petrochemicals, including cyclopentane- and cyclohexanecarboxylic acids are conjugated. Two of the conjugates present in naphthenate-treated tissues were the glucose ester and the aspartic acid amide The naphthenic acid mixture[9] and cyclohexanecarboxylic acid[10] have plant growth-promoting properties

The earliest report of the use of naphthenates in a biological experiment dates back to 1921 In this experiment[11] 1 hr after the addition of 50 mg of sodium naphthenates to 10 ml of a 2·5% glucose solution which contained 250 mg of yeast, CO_2 production was 140 per cent of the control value In 1966, Bazanova and Akopova[12] reported that a 0·01% naphthenate solution stimulated respiratory rates of cotton plants when applied as a foliar

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[1] W K SEIFERT and R M TEETER, *Analyt Chem* **41**, 786 (1969)
[2] W K SEIFERT and R M TEETER, *Analyt Chem* **42**, 180 (1970)
[3] R F GOLDSTEIN and A L WADDAMS, *Petroleum Chemicals Industry* (3rd Edition) p 441, E & F N Spon, London (1967)
[4] N G EIDER, *J Paint Technol* **42**, 504 (1970)
[5] J G SEVERSON, JR, B A BOHM and C E SEAFORTH, *Phytochem* **9**, 107 (1970)
[6] J G SEVERSON, JR, *Canad Soc of Plant Physiol, Western Section Meetings, Vancouver, B C*, 18–19 February 1971 (Abstract)
[7] U PADMANABHAN, *Northwest Sci* **44**, 67 (1970)
[8] C E SEAFORTH, J G SEVERSON, JR and D J WORT Unpublished data
[9] D J WORT, *Can J Plant Sci* **49**, 791 (1969)
[10] D J WORT and K M PATEL, *Agron J* **62**, 644 (1970)
[11] C NEUBERG and M SANDBERG, *Biochem Z* **126**, 153 (1921)
[12] T B BAZANOVA and K M AKOPOVA, *Izv Akad Nauk Turkm SSR, Ser Biol Nauk* **5**, 53 (1966), *idem Biol Abs* **49**, 4685 (1968)

spray Abolina and Ataullaev[13] and Ladygina[14] also observed an increase in respiration in potato leaves following naphthenate treatment Fattah and Wort[15] obtained a significant increase in the rate of dark respiration in intact bean plants 7, 14 and 21 days following a foliar application of 0·5% potassium naphthenates (KNap) to 14-day-old plants. Peterburgsky and Karamete[16] observed that a foliar 0 007% naphthenate treatment increased the synthesis of ten amino acids at the beginning and at the end of the vegetative period in maize, and increased the synthesis of protein and non-protein nitrogenous substances in the leaves and roots Concentrations of naphthenates ranging from 0·005 to 0 01% considerably increased the total and protein nitrogen in the leaves of tomato, carrot, and beet following treatment [17] The application of 2×10^{-2} M KNap to 14-day-old bean plants resulted in increases in the specific activity of several leaf enzymes involved in nitrogen assimilation measured seven days after treatment [18] The treatment also increased the content of enzyme protein, and stimulated the incorporation of L-[^{14}C]leucine into protein

The basic objective of the work to be reported was to augment our understanding of how naphthenate compounds stimulate the growth of bean plants More specifically, the purpose was to determine if the complex naphthenate mixture, or an individual naphthenic acid, cyclohexanecarboxylic acid, affects the uptake and subsequent metabolism of labelled glucose in bean root tips

RESULTS AND DISCUSSION

As seen in Table 1, both naphthenate treatments significantly increased the ^{14}C activity in the ethanol-soluble, ethanol-insoluble and the respired $^{14}CO_2$ fractions. Using total

TABLE 1 TOTAL RADIOACTIVITY, AS mμC, IN THE EtOH-SOLUBLE, EtOH-INSOLUBLE, AND RESPIRED CO_2 FRACTIONS OF CONTROL AND NAPHTHENATE-TREATED BEAN ROOT TIPS AFTER SUPPLYING [^{14}C] GLUCOSE

	Treatments		
	Control	KNap	KCHC
EtOH-soluble	67 2	81 2*	83 6*
EtOH-insoluble	61 1	63 1*	68 5*
Respired CO_2	53 9	63 0†	68 3†
Total radioactivity	182 2	209 0*	220 4*

Incubation sequence 6 hr in a [^{12}C] glucose medium, or in one containing 10^{-5} M KNap or 10^{-5} M KCHC, then 3 hr in a [^{14}C] glucose medium Each value represents the mean of four measurements

* Significantly different from the control value at the 0 05 level

† Significantly different from the control value at the 0 01 level

[13] G ABOLINA and N ATAULLAEV, in *Plant Stimulation A Symposium* (edited by K L POPOFF), p. 919, Bulg Acad of Sciences Press, Sofia (1969)

[14] E A LADYGINA, *Dokl, Vses Soveshch Primen Neft Rostovogo Veshchestva Sel Khoz*, 2nd, *Baku* 162 (1963), *Chem Abs* 20833k (1967)

[15] Q A FATTAH and D J WORT, *Can J Bot* **48**, 861 (1970)

[16] A V PETERBURGSKY and K I KARAMETE, in *Plant Stimulation A Symposium* (edited by K L POPOFF), p 978, Bulg Acad of Sciences Press, Sofia (1969)

[17] P S JUKOVA, in *Plant Stimulation A Symposium* (edited by K L POPOFF), p 1025, Bulg Acad of Sciences Press, Sofia (1969)

[18] D J WORT, J G SEVERSON, JR and D R PEIRSON, *Can Soc of Plant Physiol, Western Section Meetings, Vancouver, B C*, 18–19 February 1971 (Abstract)

radioactivity as a basis for comparison, ^{14}C activity in root tips treated with KCHC was greater than the control value by 20%; the increase observed in KNap-treated root tips was 13%. The data of Table 1 indicate that both compounds had a statistically significant stimulative effect on the uptake of labelled glucose from the incubation media The greater effect was obtained by the use of KCHC

TABLE 2 TOTAL RADIOACTIVITY, AS mμC, FOUND IN INDIVIDUAL EtOH-SOLUBLE AMINO ACIDS AND IN GLUCOSE FROM CONTROL AND NAPHTHENATE-TREATED ROOT TIPS AFTER SUPPLYING [^{14}C] GLUCOSE

	Treatments		
	Control	KNap	KCHC
Aspartic acid	2 22	2 25^{ns}	2 68^{ns}
Serine	5·12	9 08*	7 42*
Glutamic acid	1 47	1 68^{ns}	1·76^{ns}
Threonine	10 21	11 40^{ns}	13 03^{ns}
Alanine	10 10	10 25^{ns}	12 52^{ns}
Glutamine	1 45	1 47^{ns}	1 61^{ns}
γ-Aminobutyric acid	2 10	1 65^{ns}	2 14^{ns}
Valine	4 91	5 75†	7 32†
Isoleucine/leucine	3 54	4 38*	3 35^{ns}
Glucose	17 48	25 34^{ns}	23 17^{ns}
Total	58 68	72 79	75 54

Incubation sequence as in Table 1. Each value represents the mean of four measurements

^{ns} Not significantly different from the control value at the 0 05 level

* Significantly different from the control value at the 0 05 level

† Significantly different from the control value at the 0 01 level.

Each naphthenate treatment increased the ^{14}C activity in all ethanol-soluble amino acids, except one. ^{14}C activity in serine was significantly increased following KCHC and KNap treatments, while the increase observed in the case of valine was highly significant. Only the KNap treatment significantly increased the ^{14}C activity in isoleucine/leucine (Table 2) Because of variability in individual values, increases of 51 and 40% in the level of ^{14}C glucose in the ethanol-soluble fraction from KCHC- and KNap-treated tissues, respectively, were not quite statistically significant at the 0·05 level.

The amount of ^{14}C label in aspartic acid, glutamic acid, and alanine from the ethanol-insoluble hydrolysate from KCHC-treated root tips was significantly increased, and were 123, 126 and 162% of the control value, respectively However, in the hydrolysate from KNap-treated root tips only ^{14}C activity in alanine was significantly greater than the control value (Table 3)

Although the uptake and metabolism of carbohydrate and the effects of growth regulators on excised root tissue have been extensively reviewed by Butcher and Street,[19] the information relating to the effect of growth-promoting compounds on the uptake and metabolism of sugars is very limited Kim and Bidwell[20] showed that both indole-3-acetic

[19] D. N BUTCHER and H E STREET, *Bot Rev* **30**, 513 (1946)

[20] W K KIM and R. G S BIDWELL, *Can J Bot* **45**, 1751 (1967)

TABLE 3 TOTAL RADIOACTIVITY, AS mμC, FOUND IN AMINO ACIDS FROM THE EtOH-INSOLUBLE HYDROLYSATE FROM CONTROL AND NAPHTHENATE-TREATED BEAN ROOT TIPS AFTER SUPPLYING [^{14}C] GLUCOSE

	Treatments		
	Control	KNap	KCHC
Aspartic acid	3 76	3 58^{ns}	4 73*
Glutamic acid	3 35	3 54^{ns}	4 27*
Alanine	2 13	3 01*	3 58*

Incubation sequence as in Table 1 Each value represents the mean of four measurements

^{ns} Not significantly different from the control value at the 0 05 level

* Significantly different from the control value at the 0 05 level

acid (IAA) and 2,4-dichlorophenoxyacetic acid generally reduced the uptake and retarded the metabolism of labelled glucose by excised pea root tips Goldsworthy[21] demonstrated that mannose competitively inhibited the phosphorylation of glucose by hexokinase in root tissues, while higher concentrations of glucose inhibited the uptake of mannose by competing for the hexokinase enzyme Even though the effect of naphthenate compounds on this particular enzyme has not been studied, the significant increase in glucose uptake observed in naphthenate-treated root tissues suggests that hexokinase activity was also increased Fattah and Wort[15] and Wort *et al* [18] have shown that the activities of nine different enzymes in bean plants were increased following naphthenate treatment It was therefore suggested[18] that the stimulation of plant metabolism by naphthenates must be general

Even though the naphthenate treatments increased the ^{14}C activity in almost all ethanol-soluble amino acids, significant increases of glucose carbon in serine and valine suggest that certain metabolic pathways may be affected to a greater extent The increased level of ^{14}C in serine indicates that folate metabolism may be affected,[22] while in the case of valine the regulation of the transamination of α-ketoisovaleric acid may be implicated Glycine was not detected on the radiochromatograms, suggesting that serine was not rapidly converted to glycine

^{14}C activity in aspartic acid, glutamic acid, and alanine in the hydrolysate of the ethanol-insoluble fraction from root tips treated with KCHC was significantly increased The stimulated incorporation of these amino acids into protein, coupled with the lack of significance observed in the ^{14}C activity in the same three amino acids in the treated root tips from the ethanol-soluble fraction, suggests that protein synthesis was increased by naphthenate treatment This is supported by results obtained in our laboratory[18] which have shown that both the amount of protein, and the level of incorporation of L-[^{14}C] leucine into protein of leaf blades from bean plants was increased by KCHC and KNap treatments.

Zenk reported that in various plant tissues IAA[23] and α-naphthaleneacetic acid[24] were

[21] A GOLDSWORTHY, *Studies in the carbohydrate metabolism of excised roots* Ph D Thesis, University of Wales (1964)

[22] E A COSSINS and S K SINKA, *Biochem J* **101**, 542 (1966)

[23] M H ZENK, *Nature, Lond* **191**, 493 (1961)

[24] M H ZENK, *Planta* **58**, 75 (1962)

converted to a mixture of the glucose ester and the aspartic acid amide, and that these conjugated compounds were referred to as being 'true detoxication' products, i e not essential in the growth induction process Kim and Bidwell[20] also suggested that the aspartic acid conjugate of IAA was not responsible for the reduction of glucose uptake and the impairment of glucose metabolism in pea root tips Recently, it has been shown[25] that IAA can form a macromolecular conjugate with *t*RNA Even though the presence of this macromolecular conjugate could not be confirmed,[26] the existence of such a conjugate has interesting implications. Davis and Galston[26] also suggested the possibility of indoleacetyl aspartate (IAA-Asp) becoming attached to *t*RNA^{asp} yielding a compound similar to formylmethionine-*t*RNA which might serve as a protein chain initiator However, using ^{14}C labelled aspartate these authors could not demonstrate the presence of IAA-Asp-*t*RNA^{asp} in pea stem sections

Studies in our laboratory[5–8] have shown that in the roots and leaves of bean plants [7-^{14}C]KCHC was rapidly converted to a mixture of the glucose ester, the aspartic acid amide and several unknown metabolites In these experiments KCHC, and in the present experiment KCHC and KNap were not detected on the chromatograms As stated previously, Wort *et al.*[18] reported that the activity of several important enzymes was increased following naphthenate treatment However, no increase in enzyme activity was obtained following an *in vitro* addition of the free acid It would appear that naphthenate stimulation of glucose uptake and metabolism observed in this experiment may have been in some way associated with the presence of the conjugated form of naphthenate, rather than with the free acid Experiments are currently being designed which will test the validity of this hypothesis

EXPERIMENTAL

The procedures were based on those described by Kim and Bidwell [20] Uniform seeds of the dwarf bush bean (*Phaseolus vulgaris* L c v Top Crop) were grown for 7 days in vermiculite in a growth room Root systems of uniform size were quickly rinsed with distilled water, blotted, and 5 mm terminal root sections were cut

Incubation The medium consisted of 20 μmoles $Ca(NO_3)_2$ $4H_2O$, 100 μmoles KH_2PO_4 and 280 μmoles sucrose in a total vol of 4 4 ml [27] To serve as a control, the complete medium was added to the main (lower) compartment of a glass bottle (capacity 55 ml), which contained an upper compartment, capacity 6 7 ml (a Skrip ink bottle) Two identical bottles also with the complete medium contained in addition the K salts of cyclohexanecarboxylic acid (KCHC) (1×10^{-5} M) or naphthenic acids (KNap) (1×10^{-5} M) The medium in each bottle was adjusted to pH 6 with 1 N NaOH, autoclaved for 20 min at 15 lb/in^2 and the K salt of penicillin G (15 μg/ml) and streptomycin sulfate (30 μg/ml) were added after cooling.

Three sets of root tips (40 tips each), from the root systems of eight different plants (42–47 mg fresh wt per set) were placed in the main compartment of three bottles, 1 5 ml of 1 N NaOH was added to the smaller (upper) compartment, and the bottles were tightly capped For the first phase of incubation the media also contained [^{12}C] glucose (0 5%) After 6 hr of incubation in the dark at 25° with constant shaking, each set of root tips was removed, rinsed with distilled H_2O and blotted Each set was then placed in separate bottles containing the complete medium in which [^{14}C] glucose (Amersham/Searle, Toronto, Ontario), 2 5 μmoles and 7 5 μc per set of root tips, replaced the [^{12}C] glucose of the first phase of incubation A fresh solution of NaOH was added to the upper compartment of each bottle The bottles were again tightly capped and incubation was continued for an additional 3 hr

Tissue analysis At the end of the second incubation period each set of root tips was removed, rinsed with distilled H_2O and blotted The root tips in each set were divided into two groups of 20 and each group transferred to boiling 75% EtOH After 2 hr the EtOH-soluble fractions were placed in separate vials The root tips in each set were rinsed twice with boiling 75% EtOH, and the rinsings combined with the EtOH-soluble fractions The EtOH-soluble fractions were evaporated to dryness in an air stream and 1 ml of 25%

[25] T Yamaki and K Kobayashi, *7th International Conference on Plant Growth Substances, Canberra, Australia*, December 1970 (Abstract)

[26] P J Davis and A W Galston, *Plant Physiol* **47**, 435 (1971)

[27] W. A Andreae, J R. Robinson and M W H van Ysselstein, *Plant Physiol.* **36**, 783 (1961)

MeOH was added to each fraction A 25-μl aliquot was withdrawn from each 1 ml sample and was applied to a 20 × 20 cm TLC plate coated with cellulose MN 300 HR (0 5 mm wet thickness) (Macherey & Nagel, Co) Chromatograms were developed in *n*-BuOH–glacial HOAc–H_2O (4 1 5, top phase) (BAW) and 80% phenol Total radioactivity of the EtOH-soluble fraction was determined on another 25-μl aliquot by liquid scintillation counting

Root tips which has been EtOH extracted were divided into two groups of 10 One group was transferred to 1 ml of formamide, the tissues solubilized by heating the mixture at 200° for 5 hr and, after mixing, the solution was diluted to 3 ml with distilled H_2O and total radioactivity determined on a 0 2-ml aliquot The other group was hydrolysed in 2 ml of 6 N HCl for 10 hr at 105° After evaporating to dryness, 0 5 ml of 25% MeOH was added and each fraction (35 μl) was applied to TLC plates of cellulose MN 300 HR and developed as before Following identification using standards, the amino acids were removed from the plate and radioactivity determined by scintillation counting The leucine and isoleucine standards did not separate from each other Radioactivity in the NaOH which contained the respired $^{14}CO_2$ was determined on 0 1 ml samples by scintillation counting

The data were subjected to an analysis of variance of a nested design, and a comparison of means by Duncan's New Multiple Range Test [28]

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[28] R G D STEEL and J H TORRIE, *Principles and Procedures of Statistics* McGraw-Hill, New York (1960)

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