Question of the Ribosyl Moiety in the Promotion of Callus Growth by Exogenously Added Cytokinins*

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ABSTRACT: We have examined the role of the 9 position and the influence of the 9-substituents on the cytokinin activity of N^6-(isopent(en)yl)adenine derivatives. Five new cytokinin analogs have been employed in this study: 7-(3-methyl-2-butenylamino)pyrazolo[4,3-*d*]pyrimidine, 3-methyl-7-(3-methyl-2-butenylamino)pyrazolo[4,3-*d*]pyrimidine, 7-(3-methyl-2-butenylamino)-3-β-D-ribofuranosylpyrazolo[4,3-*d*]pyrimidine, 7-(3-methylbutylamino)pyrazolo[4,5-*d*]pyrimidine, and 3-methyl-7-(3-methylbutylamino)pyrazolo[4,3-*d*]pyrimidine. Their activities have been compared with those of the corresponding purine derivatives. The relative biological activities in the tobacco bioassay suggested that the addition of a ribosyl moiety in the 9 position of exogenously supplied cytokinins is not a prerequisite for their promotion of cell division and growth of plant tissue.

Among the large number of compounds that promote cell division and growth of plant tissue (cytokinin activity) (Bruce and Zwar, 1966; Skoog *et al.*, 1967; Dyson *et al.*, 1970; Skoog and Armstrong, 1970; McDonald *et al.*, 1971), the N^6-substituted adenines are the most active, followed by the corresponding ribonucleosides (Leonard *et al.*, 1969). The modified adenines include three cytokinins—6-(3-methyl-2-butenylamino)purine (N^6-isopentenyladenine) (**1**, *ex* ribose), 6-(4-hydroxy-3-methyl-2-butenylamino)purine (**2**, *ex* ribose, trans isomer), and 6-(4-hydroxy-3-methylbutylamino)purine (**5**) (Koshimizu *et al.*, 1967; Matsubara *et al.*, 1968)—which have been found to be naturally occurring, while the corresponding ribonucleosides include four compounds (**1**–**4**) which occur as components of tRNA (Burrows *et al.*, 1970). The relationship between the biological activity induced by the cytokinins when added to plant tissue under defined conditions and the presence of the cytokinins as naturally occurring compounds in plants and animals have received considerable attention, especially with regard to the role that these compounds may play in tRNAs (Skoog and Armstrong, 1970; Hall, 1970; Burrows *et al.*, 1971).

Basic to the concern over the relationship between physiological activity and natural occurrence is the variation in cytokinin activity with the level of modification. Thus 6-(3-methyl-2-butenylamino)purine is considerably more active in the tobacco bioassay than its corresponding ribonucleoside (Skoog *et al.*, 1967), while the corresponding ribonucleotide might be expected to be intermediate in activity between the two, based on the relative activities of 6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)purine, its corresponding ribonucleotide, and 6-furfurylaminopurine in the carrot phloem bioassay (Letham, 1966a,b).

In this regard, it would be desirable to ascertain whether or not the ribosyl moieties of exogenously added cytokinins have any importance in the promotion of cell division and growth. Kende and Tavares (1968) attempted to determine the need for ribosidation by the simple expedient of studying the relative cytokinin activities of 6-benzylaminopurine and 6-benzylamino-9-methylpurine. It was thought that the methyl group in the 9 position would preclude any possibility of ribosidation at that position, thereby indicating whether or not the ability of 6-benzylaminopurine to be ribosidated would enhance its function in the promotion of cell division and growth. The finding that the two compounds had the same activity did not conclusively show that the ribosyl moiety was unnecessary to the development of cell division and growth, however, since later experiments (Fox, 1969; Fox *et al.*, 1969, 1971) demonstrated by the use of labeled 6-benzylamino-9-methylpurine that the methyl group was rapidly lost in contact with cytokinin-requiring plant tissue, thus rendering the two compounds equivalent.

We wish to report an experiment using modified adenine analogs, actually 8-aza-9-deaza compounds, which demonstrates that, regardless of the mode of action of exogenous cytokinin, the ribose group in N^6-substituted adenosines does not enhance the cytokinin activity of these molecules. The experiment was based on determining the relative cytokinin activities of five compounds in the tobacco callus bioassay. These were 7-(3-methyl-2-butenylamino)pyrazolo[4,3-*d*]-pyrimidine (**7**), 3-methyl-7-(3-methyl-2-butenylamino)pyrazolo[4,3-*d*]pyrimidine (**8**), 7-(3-methyl-2-butenylamino)-3-β-D-ribofuranosylpyrazolo[4,3-*d*]pyrimidine (**9**), 7-(3-methylbutylamino)pyrazolo[4,3-*d*]pyrimidine (**10**), and 3-methyl-7-(3-methylbutylamino)pyrazolo[4,3-*d*]pyrimidine (**11**). In this series of compounds the 3 position, analogous to the 9 position in the purine series, which would have to be ribosidated to produce cytokinins if such a transformation were prerequisite to the development of cell division and growth, con-

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TABLE I: Data Pertinent to the Synthesis of **7**, **8**, **10**, and **11**.

Compound	Reaction Time (hr)	Solvent[a]	Yield (%)	Mp (°C)	M+
3-Methyl-7-methylthiopyrazolo-[4,3-*d*]pyrimidine (**7**)	5		43	143–145	$C_{10}H_{14}N_5$ Calcd: 203.1170 Found: 203.115
7-(3-Methylbutylamino)-pyrazolo[4,3-*d*]pyrimidine (**10**)	5		47	123–144.5	$C_{10}H_{15}N_5$ Calcd: 205.1327 Found: 205.134
3-Methyl-7-(3-methyl-2-butenyl-amino)pyrazolo[4,3-*d*]pyrimi-dine (**8**)	1.5	Ethanol–water	53	209.5–211.5	$C_{11}H_{15}N_5$ Calcd: 217.1327 Found: 217.132
3-Methyl-7-(3-methylbutyl-amino)pyrazolo[4,3-*d*]-pyrimidine (**11**)	5	Ethanol–petroleum ether	75	184–185	$C_{11}H_{17}N_5$ Calcd: 219.1483 Found: 219.148

[a] For recrystallization following chromatography.

TABLE II: Ultraviolet Spectral Data.

	pH 1				pH 7				pH 12			
Compound	λ_{max}^{EtOH} (nm)	$\epsilon \times 10^{-3}$	λ_{min} (nm)	$\epsilon \times 10^{-3}$	λ_{max}^{EtOH} (nm)	$\epsilon \times 10^{-3}$	λ_{min} (nm)	$\epsilon \times 10^{-3}$	λ_{max}^{EtOH} (nm)	$\epsilon \times 10^{-3}$	λ_{min} (nm)	$\epsilon \times 10^{-3}$
3-Methyl-7-methylthio-pyrazolo[4,3-*d*]pyrimidine	324 313 256	9.5 10.3 4.3	320 270 239	9.1 3.9 2.5	324 313 256	9.3 10.4 4.6	320 270 237	9.0 4.1 2.4	340 286 258	5.9 8.0 8.6	307 269 243	2.5 5.4 4.6
7-(3-Methyl-2-butenylamino)-pyrazolo[4,3-*d*]pyrimidine(**7**)	319 307	sh 15.5	227	4.4	293 231	12.6 6.1	251	4.1	302 262 240	9.0 7.0 13.1	277 257 229	5.2 6.2 10.3
7-(3-Methylbutylamino)pyra-zolo[4,3-*d*]pyrimidine (**10**)	319 306	sh 16.6	226	3.2	293 232	13.1 5.6	250	3.8	302 262 240	9.9 7.0 13.1	277 257 229	5.7 6.8 11.3
3-Methyl-7-(3-methyl-2-butenylamino)pyrazolo-[4,3-*d*]pyrimidine (**8**)	327 312 263	sh 13.0 6.3	281 228	5.6 3.8	319 307 296 288 238	sh 9.8 12.6 sh 6.7	305 258 226	9.5 4.1 5.2	310 265 245	7.8 sh 15.1	282 230	3.7 9.1
3-Methyl-7-(3-methylbutyl-amino)pyrazolo[4,3-*d*]-pyridine (**11**)	327 311 263	sh 12.7 6.3	281 227	5.0 3.0	319 307 295 288 237	sh 9.6 12.1 sh 6.1	304 257 225	9.5 3.8 4.7	308 265 245	7.6 sh 14.7	282 230	3.7 9.0
7-(3-Methyl-2-butylenamino)-3-β-D-ribofuranosyl)pyra-zolo[4,3-*d*]pyrimidine (**9**)	327 310 301 267	sh	308 278 230		319 305 295 287 235	sh sh sh	253 226		305 263 241		278 229	

sists of a carbon rather than a nitrogen atom since the imidazole ring has been formally modified to a pyrazole ring. Ribosidation, or deribosidation, should be considerably less likely in this series than in the corresponding purine series (Suhadolnik, 1970). As a result, the presence or absence of a ribose group on the cytokinin added exogenously to the growth medium should specify exactly the nature of the cytokinin active compound or complex with respect to the ribose group, thereby permitting a determination of the necessity of the ribosyl moiety in the expression of cytokinin activity.

The system, then, consisted of four cytokinins which could not be easily ribosidated (**7**, **8**, **10**, and **11**) and one (**9**) which could not be as easily deribosidated as **1**. Data were gathered which compared growth response of tobacco tissue to this series of compounds with the response to the purine series, including the natural cytokinins, 6-(3-methyl-2-butenyl-

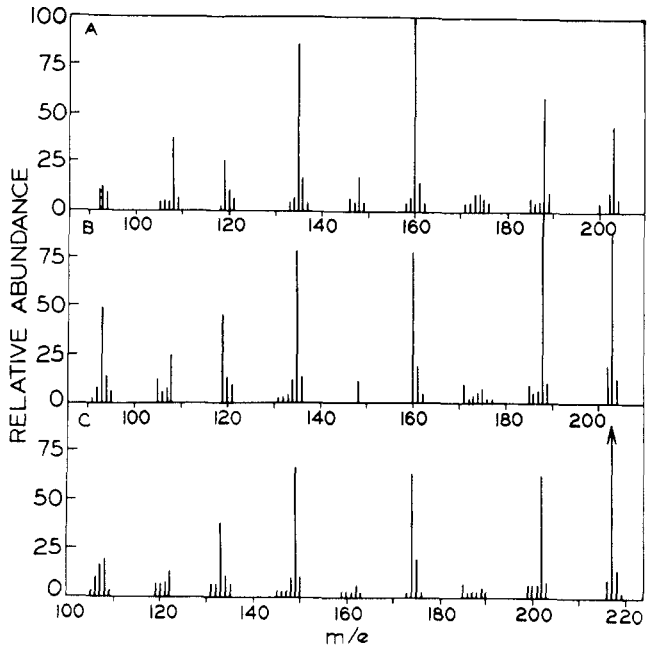


FIGURE 1: Mass spectral comparison at 70 eV of (A) 6-(3-methyl-2-butenylamino)purine (**1**, *ex* ribose), (B) 7-(3-methyl-2-butenylamino)pyrazolo[4,3-*d*]pyrimidine (**7**), and (C) 3-methyl-7-(3-methyl-2-butenylamino)pyrazolo[4,3-*d*]pyrimidine (**8**).

amino)purine (**1a** = **1**, *ex* ribose) and its ribosyl derivative **1**, and 6-(3-methylbutylamino)purine (**6**).

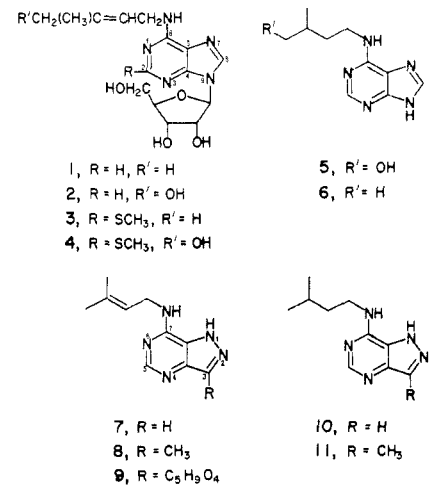
Experimental Section

Synthesis of Test Substances. The syntheses of **7**, **8**, **10**, and **11** are presented in terms of the representative synthesis of **7**. The synthesis of the precursor of **10** and **11**, 3-methyl-7-methylthiopyrazolo[4,3-*d*]pyrimidine, is given and modifications in reaction conditions for syntheses of **8**, **10**, and **11** are presented in Table I. Tables I and II contain data corresponding to the physical and spectral properties of all new compounds.

3-Methyl-7-methylthiopyrazolo[4,3-d]pyrimidine. To 166 mg (1.0 mmole) of 3-methyl-7-thiopyrazolo[4,3-d]pyrimidine (Robins *et al.*, 1956a), dissolved in 2.0 ml of 1 N potassium hydroxide solution, was added 142 mg (1.0 mmole) of methyl iodide. The mixture was shaken until a single phase was present. The solution was neutralized and filtered, and the residue was purified by chromatography over silica gel, elution with ethyl acetate, to afford a white solid: yield 150 mg (83%), mp 229–232°. *Anal.* Calcd for $C_7H_8N_4S$ (M^+): 180.0469. Found: 180.047.

7-(3-Methyl-2-butenylamino)pyrazolo[4,3-d]pyrimidine (**7**). To 70 mg (0.42 mmole) of 7-methylthiopyrazolo[4,3-*d*]pyrimidine (Robins *et al.*, 1956b) was added 800 mg (0.94 mmole) of 3-methyl-2-butenylamine. The solution was heated at reflux under nitrogen for 5 hr, cooled, and purified by chromatography over 30 g of Sephadex LH-20, elution with 35% ethanol, followed by chromatography of the appropriate fractions over 30 g of Sephadex LH-20 elution with water Concentration of the appropriate fractions afforded a white solid (see Table I).

7-(3-Methyl-2-butenylamino)-3-β-D-ribofuranosylpyrazolo[4,3-d]pyrimidine (**9**). To a solution of 141 mg (*ca.* 0.5 mmole) of formycin (7-amino-3-β-D-ribofuranosylpyrazolo[4,3-*d*]-pyrimidine) in 5 ml of *N*,*N*-dimethylformamide was added 112 mg (0.75 mmole) of 3-methyl-2-butenyl bromide. The reaction mixture was maintained at room temperature in the dark for 24 hr and was then evaporated to dryness under diminished pressure to leave a liquid residue. The residue was dissolved in 5 ml of water and the resulting solution was heated on a steam bath for 2.5 hr, during which time the pH was maintained at 7.5 by the periodic addition of portions of 1 N sodium hydroxide solution. The cooled solution was concentrated and the residue was purified by chromatography over 30 g of Sephadex LH-20, elution with 35% ethanol, followed by chromatography of the appropriate fractions over 30 g of Sephadex LH-20, elution with water. Concentration of the appropriate fractions afforded **9** in low yield: mp 120–122°; mass spectrum, *m/e* 335.156, 320.133, 292.103, 246.135, 232.119, 203.115, 188.092, 160.062, 148.063, 135.056 (*cf.* Hecht *et al.*, 1969, 1970). *Anal.* Calcd for $C_{15}H_{27}N_5O_4$ (M^+): 335.1594. Found: 335.156.



Mass Spectral Determinations. The low-resolution mass spectra and verification of the molecular ion by peak matching were carried out for each of the new compounds except **9**, on the MS-9 spectrometer. The high- and low-resolution spectra corresponding to **9** were determined on a CEC-21-110 mass spectrometer.

Bioassay Procedures. The cytokinin activities of the compounds presented in Figures 2 and 3 were determined in the tobacco bioassay (Skoog *et al.*, 1967). The compounds were dissolved in a small volume of dimethyl sulfoxide and added in serial dilutions to the cooling agar media so as to ensure a constant dosage of carrier. The final concentration of dimethyl sulfoxide did not exceed 0.02% (v/v). This level of dimethyl sulfoxide was well below the concentration which would affect tissue growth (Schmitz and Skoog, 1970).

Results and Discussion

Compounds **7**, **8**, **10**, and **11** were synthesized by condensation of 3-methylbut(en)ylamine with the appropriate 7-methylthio-3-substituted pyrazolo[4,3-*d*]pyrimidine. The low-resolution mass spectra of **7** and **8** showed fragmentation patterns identical with the patterns arising from the corresponding purine. This is illustrated in Figure 1, which depicts the spectra of 6-(3-methyl-2-butenylamino)purine (**1**, *ex* ribose), 7-(3-methyl-2-butenylamino)pyrazolo[4,3-*d*]pyrimidine (**7**), and 3-methyl-7-(3-methyl-2-butenylamino)pyrazolo[4,3-*d*]-pyrimidine (**8**). The spectrum of **8** has been shifted 14 *m/e* units to correct for the difference in its molecular weight

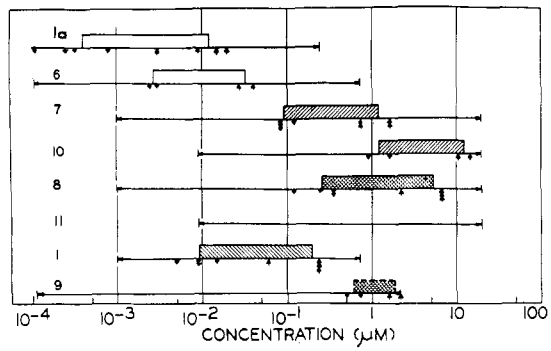


FIGURE 2: Relative cytokinin activities of analogs of N^6-isopent(en)yladenine and adenosine derivatives. The compounds are numbered as in the text; **1a** is **1**, *ex* ribose. The base lines represent the tested concentration ranges, and the arrows under the base lines represent the start and end points, in individual experiments, of the concentration range over which growth increases as a nearly linear function of the log of concentration of added cytokinin. Bars represent the average range of the linear growth response. A bar outlined by a dashed line indicates that the compound became toxic to callus growth at concentrations which were too low to support maximal callus growth; absence of a bar indicates that the compound failed to support any callus growth.

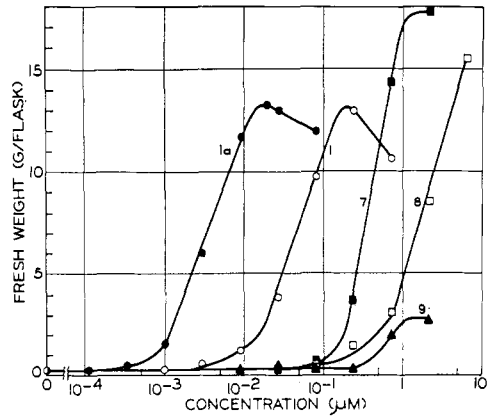


FIGURE 3: Fresh weight yield of tobacco callus tissue cultured on three cytokinin analogs and on N^6-isopentenyladenine and adenosine derivatives as determined in a single experiment (C 115, 10-8-70 to 11-11-70). The compounds are numbered as in the text; **1a** is **1**, *ex* ribose.

from those of the other two, thereby facilitating the parallel comparison of fragmentation patterns. A generalized scheme corresponding to this type of fragmentation pattern has been published for compounds in the purine series (Hecht *et al.*, 1970). Although not explicitly presented here, the mass spectra of **6**, **10**, and **11**, and those of the precursors, 6-methylthiopurine, 7-methylthiopyrazolo[4,3-*d*]pyrimidine, and 3-methyl-7-methylthiopyrazolo[4,3-*d*]pyrimidine, have the same relationship to each other as do **1**(*ex* ribose), **7**, and **8**, in agreement with the structural assignments for these compounds.

Compound **9** was synthesized by alkylation of formycin (7-amino-3-β-D-ribofuranosylpyrazolo[4,3-*d*]pyrimidine) with 3-methyl-2-butenyl bromide, followed by rearrangement of the intermediate adduct to **9** in aqueous solution. This was analogous to the synthesis of the isomeric 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (N^6-isopentenyladenosine) (**1**) (Leonard *et al.*, 1966; Leonard and Grimm, 1967).

The mass spectral fragmentation pattern of **9** was similar to the spectrum obtained for the adenosine derivative **1** (Hecht *et al.*, 1969, 1970), except for differing intensity in certain peaks due to *C*-ribosyl, rather than *N*-ribosyl linkage (S. M. Hecht, in preparation).

The cytokinin assays indicated that **7** was at least ten times as active as **10** (Figures 2 and 3) as had been found for the respective isomeric purines, 6-(3-methyl-2-butenylamino)purine (**1**, *ex* ribose) and 6-(3-methylbutylamino)purine (**6**). Thus the *relative* changes in activity within each of the two series were the same, although about 300 times as much of either **7** or **10** was required to elicit the same growth response as could be obtained with the corresponding purines. Compound **11** was also much less active than **8**, and the cumulative effect of the nonplanar side chain and methyl substituent rendered it so much less active that only a slight growth response was elicited by any concentration up to 20 μM.

The formal addition of a methyl group to the 3 position of the pyrazolo[4,3-*d*]pyrimidine nucleus, analogous to the 9 position of the purine nucleus, lowered activity slightly (Figure 2) as seen from the relative activities of **7** and **8**. This slight lowering might be attributed to a further "obstacle" to ribose-dependent cytokinin activity or, more probably, to a general diminution of cytokinin activity which tends to accompany multiple substitutions (Skoog *et al.*, 1967; Hecht *et al.*, 1970).

It could thus be shown that those structure–activity relationships which were valid in the purine series were also valid in the pyrazolo[4,3-*d*]pyrimidine series and that the presence of a similarly positioned methyl group lowered activity somewhat. The formal addition of a ribose group to the pyrazolo[4,3-*d*]pyrimidine ring system as in **9** resulted in a compound which was not only very low in activity in terms of the region of its linear growth response (Figure 2) but gave only slight growth response and only at concentrations above 0.5 μM. The comparison must be qualified by recognition of possible differences in membrane permeability, metabolism, etc.

Thus, not only did the addition of a "fixed" ribose group *not* aid in the development of cytokinin activity, but it practically eliminated activity altogether. The most likely interpretation of this experiment is that, regardless of the mode of action of cytokinins, or the nature of their function in tRNA, exogenously added cytokinins do not require the presence of a ribose group for the promotion of the activity that results. In mechanistic terms, *i.e.*, in terms of the mechanism of action of the cytokinins, the essential lack of activity of **9** and **11** is probably not as meaningful as the qualitative presence of activity in **7**, **8**, and **10**. The latter set of compounds is not likely to be ribosidated at the 3 position under the cytokinin test conditions, so the observation that they do show activity strongly suggests that the cytokinins may function without ribosidation of the exogenously added heterocyclic compound. The finding that **11**, three structural features removed from the natural cytokinin 6-(3-methyl-2-butenylamino)-purine, was devoid of growth-promoting activity proved important in that it led us to test for (and find) anticytokinin activity in this compound in appropriate tobacco bioassays (Hecht *et al.*, 1971).

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References

Bruce, M. T., and Zwar, J. A. (1966), *Proc. Roy Soc., Ser. B 165*, 245.

Burrows, W. J., Armstrong, D. J., Kaminek, M., Skoog, F., Bock, R. M., Hecht, S. M., Dammann, L. G., Leonard, N. J., and Occolowitz, J. (1970) *Biochemistry 9*, 1867, and references therein.

Burrows, W. J., Skoog, F., and Leonard, N. J. (1971), *Biochemistry 10*, 2189.

Dyson, W. H., Chen, C. M., Alam, S. N., Hall, R. H., Hong, C. I., and Chheda, G. B. (1970), *Science 170*, 328.

Fox, J. E. (1969), Conference on Cytokinins, University of Wisconsin, Madison, Wisconsin, April.

Fox, J. E., Sood, C. K., Buckwalter, B., and McChesney, J. D. (1971), *Plant Physiol. 47*, 275.

Fox, J. E., Sood, C. K., and McChesney, J. D. (1969), *Plant Physiol. Suppl. 44* (6), 2.

Hall, R. H. (1970), *Progr. Nucl. Acid Res. Mol Biol. 10*, 57.

Hecht, S. M., Bock, R. M., Schmitz, R. Y., Skoog, F., and Leonard, N. J. (1971), *Proc. Nat. Acad. Sci. U. S.* (in press).

Hecht, S. M., Leonard, N. J., Occolowitz, J., Burrows, W. J., Armstrong, D. J., Skoog, F., Bock, R. M., Gillam, I., and Tener, G. M. (1969), *Biochem. Biophys. Res. Commun. 35*, 205.

Hecht, S. M., Leonard, N. J., Schmitz, R. Y., and Skoog, F. (1970), *Phytochemistry 9*, 1173.

Kende, H., and Tavares, J. (1968), *Plant Physiol. 43*, 1244.

Koshimizu, K., Kusaki, T., Mitzui, T., and Matsubara, S. (1967), *Tetrahedron Lett.*, 1317.

Leonard, N. J., Achmatowicz, S., Loeppky, R. N., Carraway, K. L., Grimm, W. A. H., Szweykowska, A., Hamzi, H. Q., and Skoog, F. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 709.

Leonard, N. J., and Grimm, W. A. H. (1967), *Biochemistry 6*, 3625.

Leonard, N. J., Hecht, S. M., Skoog, F., and Schmitz, R. Y. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 175.

Letham, D. S. (1966a), *Life Sci. 5*, 1999.

Letham, D. S. (1966b), *Phytochemistry 5*, 269.

Matsubara, S., Koshimizu, K., and Nakahira, R. (1968), *Sci. Rep. Kyoto Pref. Univ. 19A*, 19.

McDonald, J. J., Leonard, N. J., Schmitz, R. Y., and Skoog, F. (1971), *Phytochemistry 10*, 1429.

Robins, R. K., Furcht, F. W., Grauer, A. D., and Jones, J. W. (1956b), *J. Amer. Chem. Soc. 78*, 2418.

Robins, R. K., Holum, L. B., and Furcht, F. W. (1956a), *J. Org. Chem. 21*, 833.

Schmitz, R. Y., and Skoog, F. (1970), *Plant Physiol. 45*, 537.

Skoog, F., and Armstrong, D. J. (1970), *Ann. Rev. Plant Physiol. 21*, 359.

Skoog, F., Hamzi, H. Q., Szweykowska, A. M., Leonard, N. J., Carraway, K. L., Fujii, T., Helgeson, J. P., and Loeppky, R. N. (1967), *Phytochemistry 6*, 1169.

Suhadolnik, R. J. (1970), Nucleoside Antibiotics, New York, N. Y., Wiley, pp 364–385.

Isolation, Identification, and Specific Localization of Di-2-ethylhexyl Phthalate in Bovine Heart Muscle Mitochondria*

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ABSTRACT: A component associated with the triglycerides of beef heart mitochondria has been shown to be identical with di-2-ethylhexyl phthalate. Studies on the intracellular distribution of di-2-ethylhexyl phthalate in heart muscle show that this compound is associated with mitochondrial fractions.

During the course of our studies on the fatty acids of several lipid classes from subcellular fractions of heart muscle, we observed an aberrant peak associated with the fatty acids of mitochondrial triglycerides. This component, which was originally suspected to be a highly unsaturated fatty acid, at times formed approximately 60% of the total esterified fraction derived from the triglycerides of mitochondria. The specific localization of this component prompted us to investigate its structure and distribution. This paper describes the isolation and characterization of di-2-ethylhexyl phthalate (DEHP)[1] in heart muscle mitochondria. The detection of DEHP is unique in that (a) this compound has not been reported previously in mammalian tissues; (b) it has been detected in heart muscle mitochondria from several other species and is not limited to the ruminants.

Experimental Section

Subcellular Fractionation. Beef hearts were obtained fresh from the abattoir and transported to the cold room packed in Dry Ice. All general procedures were carried out at the

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[1] Abbreviation used is: DEHP, di-2-ethylhexyl phthalate.