is not due to starvation. It is a secondary result of inhibition of the photochemical reaction. Ashton suggested (*2*) that the action spectrum of the acute response is similar to the absorption spectrum of the chlorophylls. Apparently a vital cellular component may be destroyed by photooxidation in sensitive plants when the Hill reaction is blocked. This component may be replaced by synthesis if the plants are supplied a sugar or an amino acid. Although sorghum, *Coix*, and corn may show severe growth inhibition following treatment with atrazine, no acute toxicity symptoms ever become apparent.

Since barley, oats, and sorghum roots appear to contain water-soluble metabolites other than hydroxysimazine, the possibility of other degradation mechanisms must be considered. Also short-term studies with excised roots may not preclude the ability of the intact plant to degrade simazine to hydroxysimazine over longer time intervals.

Another kind of selectivity mechanism would be the differential ability of the several species to take up the herbicide and translocate it to chloroplast-containing leaf mesophyll tissue. Even within a mesophyll cell, and especially along the transport route, binding or deposition at inactive sites may take place. In the case of excised roots, there was no consistent difference in the ability of different species of Gramineae to take up C^{14}-simazine. There were also no evident trends in the amount of C^{14} found in the 80% ethanol-insoluble residue.

Acknowledgment

The author thanks the Geigy Agricultural Chemicals Co., Ardsley, N. Y., for the atrazine and C^{14}-simazine.

Literature Cited

(1) Allen, W. S., Palmer, R. D., *Weeds* **11,** 27 (1962).
(2) Ashton, F. M., *Plant Physiol. Suppl.* **37,** XXV (1962).
(3) Castelfranco, P., Foy, C. L., Deutsch, D. B., *Weeds* **9,** 580 (1961).
(4) Exer, B., *Experientia* **14,** 136 (1958).
(5) Gysin, H., Knüsli, E., *Advan. Pest Control Res.* **3,** 289 (1960).
(6) Hamilton, R. H., Ph.D. thesis, Michigan State University, 1960.
(7) Hamilton, R. H., Bandurski, R. S., Reusch, W. H., *Cereal Chem.* **39,** 107 (1962).
(8) Hamilton, R. H., Moreland, D. E., *Science* **135,** 373 (1962).
(9) Hamilton, R. H., Moreland, D. E., *Weeds* **11,** 213 (1963).
(10) Hoagland, D. R., Arnon, D. I., *Calif. Univ. Agr. Expt. Sta. Circ.* **347,** (1950).
(11) Honkanen, E., Virtanen, A. I., *Acta Chem. Scand.* **19,** 504 (1960).
(12) Koyama, T., Yamato, M., Kubota, K., *J. Pharm. Soc. Japan* **77,** 989 (1957).
(13) Moreland, D. E., Gentner, W. A., Hilton, J. L., *Abstracts, Weed Soc. Amer.* **40** (1958).
(14) Moreland, D. E., Gentner, W. A., Hilton, J. L., Hill, K. L., *Plant Physiol.* **34,** 432 (1959).
(15) Moreland, D. E., Hill, K. L., *Weeds* **10,** 229 (1962).
(16) Roth, W., *Compt. Rend.* **245,** 942 (1957).
(17) Roth, W., Knüsli, E., *Experientia* **17,** 312 (1961).
(18) Virtanen, A. I., Hietala, P. K., *Acta Chem. Scand.* **14,** 499 (1960).
(19) Wahlroos, Ö., Virtanen, A. I., *Ibid.*, **13,** 1906 (1959).

Received for review May 15, 1963. Accepted September 27, 1963. Division of Agricultural and Food Chemistry, 144th Meeting, ACS, Los Angeles, Calif., April, 1963. Contribution No. 307 from the Dept. of Botany, and Journal series paper No. 2763 from the Pennsylvania Agricultural Experiment Station.

METABOLISM OF HERBICIDES

The Metabolism and Translocation of 3-Amino-1,2,4-triazole by Canada Thistle

RICHARD A. HERRETT and WILLIAM P. BAGLEY

Agricultural Research Station, Chemicals Division, Union Carbide Corp., Clayton, N. C.

The metabolism of 3-amino-1,2,4-triazole (amitrole) by Canada thistle results in the formation of at least three chromatographically distinct derivatives. Two compounds, (Unknowns I and II) are similar to metabolites reported by other workers and relatively inactive. A third metabolite (Unknown III) is herbicidally more active than amitrole. Furthermore, Unknown III will translocate out of leaves under a condition (light starvation) unfavorable for the translocation of amitrole. The evidence is consistent with the hypothesis that amitrole must undergo metabolism to an active form prior to translocation within the phloem.

Previous studies (*3, 8, 11, 13, 15, 17*) have indicated a rapid metabolism of 3-amino-1,2,4-triazole (amitrole) in a wide variety of species. Two metabolites, Unknowns I and II, were observed (*8*) in Canada thistle (*Cirsium arvense* L.). One of these metabolites was isolated and reapplied to thistle and found to be inactive.

These same studies with thistle (*8*) demonstrated the existence of a lag between penetration of amitrole into the treated leaf and its subsequent translocation from that leaf. Under normal conditions, amitrole applied to a leaf is translocated via the phloem (*1, 9, 12*) to aerial and subterranean portions of the plant. In the absence of light, however, translocation of amitrole is inhibited (*1, 9, 12*). It has been considered that translocation of amitrole (*1*) and other synthetic plant growth regulators, such as 2,4-D (2,4-dichlorophenoxyacetic acid) (*14*), is associated with the photosynthetic food stream.

It was hypothesized (*8*) that for amitrole to be transported in the phloem it must undergo a chemical reaction to an active transport form. The present communication provides additional evidence in support of this hypothesis.

Materials and Methods

Canada thistle (*Cirsium arvense* L.) was grown under controlled environmental conditions (1500 foot-candles supplied by incandescent and fluorescent lamps) with alternating 12-hour light

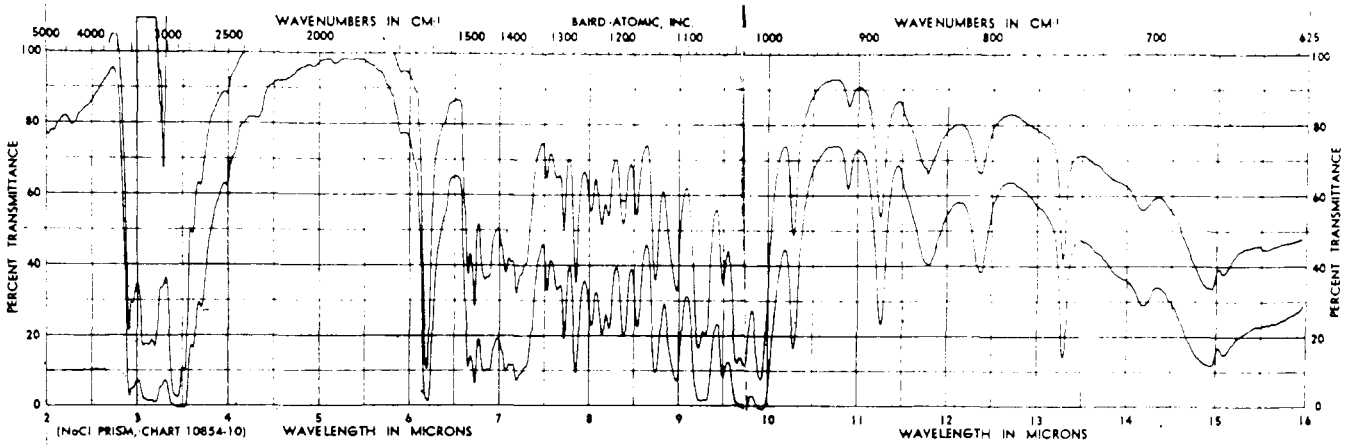

Figure 1. Infrared spectrum of the synthetic glucose-amitrole adduct at two concentrations prepared as Nujol mulls

Table I. R_f Values of Unknown I from Thistle as Compared with the R_f Values of a Similar Compound Obtained from Other Plants in Various Solvent Systems

Plant	Metabolite	R_f Value	Ref.
Isopropanol–NH_4OH–H_2O (80:5:15)			
Cotton	Metabolite Y	0.18	(*13*)
Thistle	Unknown I	0.14	
Isopropanol–NH_4OH–H_2O (6:2:2)			
Bean	Compound Y	0.63	(*15*)
Thistle	Unknown I	0.63	
Ethanol–*n*-Butanol–H_2O (4:1:1)			
Cotton	Metabolite Y	0.20	(*13*)
Thistle	Unknown I	0.24	
Methanol–HCOOH–H_2O (80:15:5)			
Cotton	Metabolite Y	0.61	(*13*)
Thistle	Unknown I	0.66	

(80° F.) and dark (70° F.) periods. Amitrole was applied to the upper surface of the leaf with a micropipet and a Hamilton screw-type syringe. Volumes between 10 and 100 μl. were applied as several droplets spread out over the distal third of the leaf. Following application, all plants were observed closely until the droplets dried to ensure that no surface run-off to the stem occurred. The typical response—the achlorophyllous new growth—induced by amitrole is termed discoloration. Necrosis refers to the localized burning response observed at the point of application, generally observed following application of excessive amounts of amitrole. The leaf infiltration methods used to obtain two metabolites, Unknowns I and II, were essentially those previously reported (*8*, *15*). Elution of amitrole and its metabolites from the IR-120 cation exchange resin with NH_4OH (100 ml., 4*N*) through the resin, and concentrations of this eluate in vacuo to remove ammonia were the only modifications in the previous methods (*8*).

Amitrole and Unknown I were analyzed as previously described (*7*). Unknown I was isolated free of Unknown II by preparative paper chromatography in *n*-butanol–95% ethanol–H_2O (55:30:15) followed by elution and rechromatography in methanol–formic acid–H_2O (80:15:5). Unknown I (ca. 50 μg. amitrole equivalents) was applied to thistle and found to be incapable of inducing discoloration of the new growth. This compound has R_f values comparable to those reported by other workers for a similar metabolite (Table I).

Bioassay

Solutions containing the test chemical were placed in Petri plates containing 25 lettuce (*Lactuca sativa*, var. Great Lakes) and tomato (*Lycopersicum esculentum*, var. Bonny Best) seeds each. Seeds were allowed to germinate at 80° F. for 5 days. At the end of this period root lengths were measured and the growth was expressed as a per cent inhibition of a deionized-water control. Probit transformations of the per cent inhibition were plotted against the log of the concentration in parts per million. From these curves, the concentration required to inhibit root growth 50% of the control (I_{50}) can be derived. All treatments were duplicated in each experiment, and each experiment was repeated at least three times.

Translocation

Thistles were maintained in complete darkness for 66 hours (light starved). The compounds to be tested were applied to the light-starved plants and to corresponding plants maintained in the light at comparable air temperatures. Twenty-four and 48 hours after application treated leaves of a portion of the plants under the light and dark regimes were excised at the base of the leaf. The treated leaves of a second portion of the plants were kept intact but were washed with water to remove any amitrole remaining on the leaf surface. Additional treated plants were kept intact without washing. After leaf treatment, excision, or washing, plants were placed under the lights. The experiment was repeated twice with three replications each time.

Results

A third metabolite of amitrole previously unreported was obtained by modification of the original procedure (*8*). The concentrated aqueous extract from vacuum-infiltrated leaves was streaked directly on Whatman 3MM filter paper and developed in a descending manner with the *n*-butanol solvent. One-centimeter strips were cut perpendicular to the direction of solvent flow and eluted with water. The eluates were then concentrated (40° C. in vacuo) and reapplied to thistle.

Extremely rapid and effective responses, both discoloration and necrosis, were observed associated with a chromatogram area distinct from amitrole. This active area was designated Unknown III. If the distance amitrole moved is considered as unity, Unknown III moved consistently at an $R_{amitrole}$ of 0.60 to 0.68. Considerably less time was required for the appearance of localized necrosis and discoloration than the minimum times observed for amitrole when Unknown III was applied to thistle. Unknown III was obtained from intact treated plants and was not considered an artifact of the infiltration procedure.

Amitrole has been reported to be metabolized to a glucose adduct (*5*, *6*, *16*). One report (*16*) presents chromatographic evidence obtained with Canada thistle. Unknown III was compared with a synthetic glucose adduct of amitrole. The adduct was synthesized by refluxing amitrole (0.025 mole) dissolved in minimum 95%

ethanol with glucose (0.025 mole) dissolved in minimum hot methanol in the presence of trace amounts of HCl for 48 hours. The white crystals which appeared were filtered and washed with cold methanol and were then recrystallized twice from hot methanol: 204°–5° C. (dec.), lit. (*6*) 215° C. (dec.). Calculated for $C_6H_{14}O_5N_4$: C, 39.02%; H, 5.65; N, 22.76. Found: C, 38.64; H, 5.85; N, 21.61. The infrared spectrum obtained using a Nujol mull preparation (Figure 1) of this synthetic product is comparable to that reported for the amine glucoside (*5*). It is also comparable to the infrared spectrum (Sadtler Reference: 12539) of a product obtained by heating amitrole (0.3 mole) and glucose (0.1 mole) in water, 200°–201° C. (dec.) (*2*).

Neither the synthetic amine glucoside nor Unknown III reacts with *para*-anisidine, ninhydrin, or H-acid. Both compounds can be hydrolyzed to amitrole with cation exchange resin and dilute acid. The $R_{amitrole}$ values of the amine glucoside (0.24–0.28) and Unknown III (0.60–0.68) in the *n*-butanol solvent prove that these are dissimilar entities. The method used for the determination of the $R_{amitrole}$ values of the two compounds was as follows. Aliquots were spotted and developed in the *n*-butanol solvent. One-centimeter strips perpendicular to the direction of solvent flow were cut, eluted with water, hydrolyzed with HCl (0.43*N*) for 2 hours at 80° C., and then analyzed for amitrole. This procedure made it possible to obtain positive reaction for each compound whereas the unhydrolyzed material was undetectable.

Eluates of paper chromatograms containing Unknown III (cut from $R_{amitrole}$ 0.4 to 0.8) indicated the presence of small amounts of amitrole when analyzed directly. Repeated chromatography (3×) of this eluate continued to show the presence of amitrole, indicating continued breakdown of Unknown III during elution and concentration. Addition of HCl (1.71*N*) followed by heating (80° C.) resulted in the appearance of substantial quantities of a substance giving a positive color reaction (Table II). Maximum formation of amitrole occurred after heating for 2 to 4 hours. Additional heat reduced the amount obtained. Cochromatographic analyses with authentic amitrole indicated amitrole was present in the hydrolyzate.

An increase in detectable amitrole was obtained when an aliquot of Unknown III was treated with a cation exchange resin (10 grams of IR-120, H+ form). However, hydrolysis on the resin was not complete, and the addition of heat and HCl increased the detectable amitrole. Repeated treatment with cation exchange resin failed to increase the degree of hydrolysis significantly.

Table II. Hydrolysis of Unknown III

Treatment	Amitrole Formed (μg. per ml.)
Original eluate from chromatograms	4.7
HCl (1.71*N*) and heat 80° C. for	
1 Hour	77.0
3 Hours	86.0
4 Hours	101.0
6 Hours	101.0
7.5 Hours	88.0
24 Hours	49.0
IR-120 resin, no heat	28.7
IR-120 resin, no heat 2nd time	30.0
IR-120 resin HCl (1.71*N*) + heat (80° C.) 1 hour	70.7

Table III. Amount of Amitrole Contained in Various Derivatives Required to Inhibit the Growth of Tomato and Lettuce Roots by 50% (I_{50})

Compounds	I_{50} (P.P.M. of Amitrole) Tomato	Lettuce
Amitrole	4.4	15.6
Amine glucoside[a]	183.6	986.0
Unknown III[b]	0.9	1.9

[a] P.P.M. of amine glucoside × 0.34.
[b] Calculated on the basis of amitrole released by acid hydrolysis.

The amount of amitrole contained as Unknown III was determined by heating eluates containing Unknown III with HCl (1.71*N*).

Results of growth-inhibition studies with amitrole and its various derivatives are shown in Table III. Amitrole as Unknown III was more active as an inhibitor of lettuce and tomato root growth than amitrole itself. Amitrole when introduced as the amine glucoside was substantially less active than the original amitrole. This provides additional evidence for the dissimilarity of Unknown III and the synthetic amine glucoside.

Results of the translocation study are presented in Table IV. Based on the degree of discoloration, no detectable translocation of amitrole occurred under dark conditions when the treated leaf was excised 24 or 48 hours after treatment. No translocation occurred in 24 hours, and little translocation occurred in 48 hours after application of amitrole under light conditions. In both cases, sufficient penetration of amitrole occurred to induce discoloration as shown by these plants which had been washed free of surface amitrole and the treated leaf kept intact. Unknown III, on the other hand, translocated rapidly out of the treated leaf under both light and dark conditions. A smaller amount of Unknown III was used because of the extensive necrosis at the point of application in the dark.

Table IV. Response of Thistles to Unknown III and Amitrole under Light and Dark Conditions[a]

Time of Leaf Treatment,[b] Hours	Leaf Treatment	Relative Discoloration[c] Amitrole	Unknown III
	Light Conditions		
24	Washed	+++	+++
48	Washed	+++	+++
24	Excised	0	+++
48	Excised	+	+++
	Dark Conditions		
24	Washed	+++	+++
48	Washed	+++	+++
24	Excised	0	+++
48	Excised	0	+++

[a] Amitrole (100 μg. per plant) and Unknown III (8.6 μg. per plant-based acid hydrolysis) applied to leaves after preconditioning plants for 66 hours in light or dark.
[b] Following leaf treatment, those plants in the dark were placed under lights.
[c] 0 = no discoloration; + = slight discoloration; ++ = moderate discoloration; +++ = severe discoloration.

Discussion

Three distinct products of amitrole metabolism have been observed in thistle. One metabolite, Unknown II, was shown previously (*8*) to be incapable of inducing the typical amitrole response in thistle. This agrees with results obtained by other workers (*3*, *4*, *10*) with a similar metabolite. A second metabolite from thistle, Unknown I, was shown in the present study to be nontoxic when isolated and reapplied to thistle. Based on R_f values, Unknown I appears to be similar to Miller and Hall's Metabolite Y (*13*) and Racusen's Compound Y (*15*). Racusen (*15*) was unable to obtain any indication of phytotoxicity on *Lemna* by Compound Y.

A third metabolite, Unknown III, has been obtained from vacuum-infiltrated thistle leaves and intact plants. This metabolite is considerably more effective than amitrole in the induction of discoloration (8.6 μg. of Unknown III will induce severe discoloration; a minimum of 25 μg. of amitrole are required to give the same response). Unknown III is also a more effective inhibitor of seedling growth than amitrole. This essentially is a bioactivation. Racusen (*15*) pointed out that amitrole inhibition of both liver and leaf catalase is greater in vivo than can be accounted for by in vitro inhibition.

Translocation of synthetic growth regulators out of treated leaves is light dependent. Little or no amitrole will translocate from a light-starved leaf (*1*, *9*, *12*). This has been confirmed

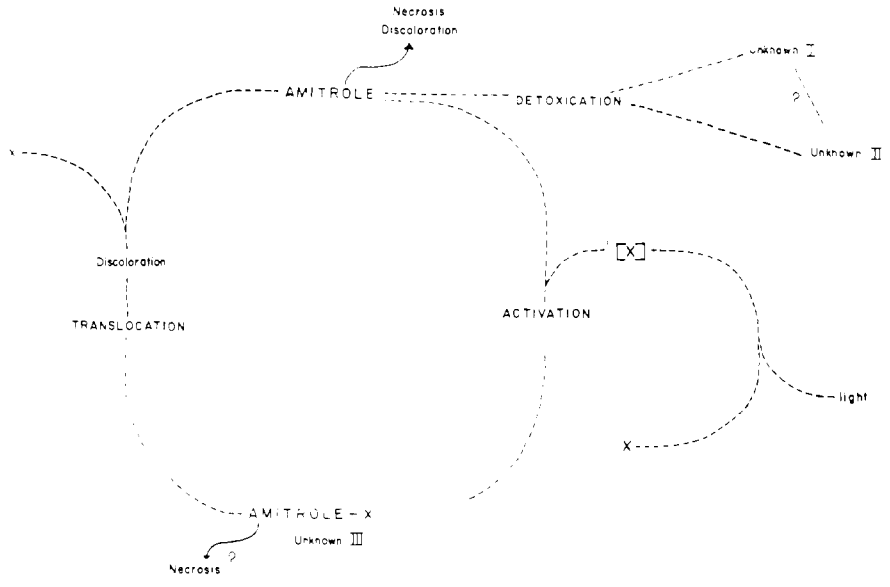

Figure 2. Proposed reaction sequences of amitrole metabolism in plants

with amitrole translocation in thistle in the present study. In the presence of light, no evidence of translocation is observed if the treated leaf is removed 24 hours after application. Slight activity is observed when the treated leaf is removed 48 hours after application of amitrole; however, it is considerably less than that obtained when the treated leaf is allowed to remain on the plant. This may be considered as additional support for the lag phenomena (*8*) associated with amitrole translocation.

Unknown III, on the other hand, will translocate readily either in the presence or absence of light, suggesting that the lag is actually a biosynthesis of Unknown III. Thus, translocation of amitrole is not directly associated with the movement of the photosynthetic food stream. Light provides the energy source or substrate for the synthesis of Unknown III, but once the compound is formed, translocation is no longer light-dependent.

The structure of Unknown III is presently unknown. It does, however, contain amitrole. Based upon paper chromatographic and biological evidence, Unknown III is not the amine glucoside which has been proposed (*5, 6, 16*) as a product of amitrole metabolism. The ion exchange resin data indicate there may be more than one component, one of which is resin-labile and the other resin-stable.

Figure 2 presents a possible scheme relating amitrole metabolism to its systemic herbicidal properties. Once amitrole has penetrated the leaf, the compound may be either detoxified to Unknowns I and II or activated to Unknown III. It is not known in the detoxication portion of the scheme whether Unknowns I and II arise from independent reactions or whether one might be an intermediate in the formation of the other. Neither metabolite can induce discoloration when applied at twice the amount of amitrole required.

In the activation portion, amitrole is metabolized to an active transport form. Translocation of amitrole requires both time and light, whereas translocation of Unknown III occurs very rapidly and in the absence of light. Possibly Unknown III breaks down to amitrole either prior to or during translocation; however, the apparent energy barrier limiting translocation of applied amitrole is overcome with the biosynthesis of Unknown III.

The observed increase in activity of Unknown III may be accounted for if we consider that the detoxication reactions occur on amitrole per se and not on the active form until cleavage has occurred.

As a working hypothesis, the authors should like to present the following. Amitrole must undergo metabolism to a chemically distinct entity prior to translocation. Systemic herbicidal activity of this compound is a direct consequence of this metabolism. It is only possible to speculate on the general relationship of bioactivation and phloem mobility; however, it has been well documented that light is required for the translocation of another phloem-mobile growth regulator, 2,4-D (*11*). The translocation of 2,4-D may also occur as a result of a similar biosynthetic activation.

Literature Cited

(1) Anderson, O., *Weeds* **6,** 370 (1958).
(2) Bishop, J. Russell, Amchem Products, Inc., Ambler, Pa., private communication, 1962.
(3) Carter, M. C., Naylor, A. W., *Botan. Gaz.* **122,** 138 (1960).
(4) Carter, M. C., Naylor, A. W., *Physiol. Plantarum* **14,** 20 (1961).
(5) Frederick, J. F., Gentile, A. C., *Arch. Biochem. Biophys.* **92,** 356 (1961).
(6) Gentile, A. C., Fredrick, J. F., *Physiol. Plantarum* **12,** 862 (1959).
(7) Herrett, R. A., Linck, A. J., J. Agr. Food Chem. **9,** 466 (1961).
(8) Herrett, R. A., Linck, A. J., *Physiol. Plantarum* **14,** 767 (1961).
(9) Massini, P., *Acta Botan. Neerl.* **10,** 99 (1961).
(10) *Ibid.*, **12,** 64 (1963).
(11) Massini, P., *Biochim. Biophys. Acta* **36,** 548 (1959).
(12) Massini, P., "Second U. N. International Conference on the Peaceful Uses of Atomic Energy," Pergamon Press, London, 1959.
(13) Miller, C. S., Hall, W. C., J. Agr. Food Chem. **9,** 210 (1961).
(14) Mitchell, J. W., Brown, J. W., *Botan. Gaz.* **107,** 393 (1946).
(15) Racusen, D., *Arch. Biochem. Biophys.* **74,** 109 (1958).
(16) Rogers, B. J., "The Hormolog," Amchem Products, Inc., Ambler, Pa., 1957.
(17) Rogers, B. J., *Weeds* **5,** 5 (1957).

Received for review September 4, 1963. Accepted December 2, 1963. Division of Agricultural and Food Chemistry, 144th Meeting, ACS, Los Angeles, Calif., April 1963.