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Trisulfation of Hexoses by Means of Concentrated Sulfuric Acid[1]

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Sulfation of D-glucose, D-galactose, D-mannose and D-fructose with concentrated sulfuric acid yielded pure trisulfates of these hexoses. The situation of the sulfate groups in these trisulfates was decided on the basis of the behavior to oxidizing agents, methylation analyses and periodate oxidation. 1,3,6-Substitution for the aldohexoses and 1,2,4-substitution for the ketohexose were established.

The direct sulfation of monosaccharides has been carried out by using chlorosulfonic acid[3–6] or sulfur trioxide–pyridine complex.[7,8] The reactivity of these sulfating agents, however, is non-selective and the reaction products are usually mixtures composed of monosaccharide sulfates of different sulfation stage. Although the separation of the mixtures has been achieved by either chromatographic[7,9] or electrophoretic[8] technique, the procedure is laborious and unsuitable for preparative work. In the preceding paper[10] it was reported that the selective monosulfation of the primary hydroxyl group of monosaccharide was realized by the use of arysulfonyl chloride as sulfating agent. In this paper we shall report on the trisulfation of hexoses with concentrated sulfuric acid. The trisulfates resulted were single species and their structures were established on the basis of non-reducibility aganist Ag^{+}, Cu^{2+}, as well as $Fe(CN)_6^{3-}$, and by identification of hydrolyzate of the methylated products, and further by determination of periodate consumption.

Saito and co-workers reported on the monosulfation of D-glucose[11] and 2-amino-2-deoxy-D-glucose[12] with chlorosulfonic acid using concentrated sulfuric acid as solvent. But after repeating the experiments described, we found that the sulfation product was not a single monosulfate, but a mixture of sulfates of various sulfation stage if ether extraction was applied as described to eliminate sulfuric acid from the reaction mixture. We found also that the similar reaction resulted in without chlorosulfonic acid, *i.e.*, that concentrated sulfuric acid itself could act as sulfating agent as well as solvent, which coincides with the earlier work by Peligot[13] though his product was not well characterized. For example, D-glucose was dissolved in tenfold volumes of concentrated sulfuric acid at −5°. The reaction mixture was neutralized with barium hydroxide and examined by paper electrophoresis. Three components, A, B and C, corresponding to tri-, di- and monosulfate of D-glucose, respectively, were

1) A part of this work was presented at the 88th Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April 1968.
2) Location: *6–5 Toneyama, Toyonaka, Osaka.*
3) P. Claesson, *J. Prak. Chem.*, **2**, 20 (1879).
4) N. Neuberg and L. Liebermann, *Biochem. Z.*, **121**, 326 (1921).
5) T. Soda, *Biochem. Z.*, **135**, 623 (1923).
6) H. Ohle, *Biochem. Z.*, **136**, 446 (1923).
7) S. Peat, J.R. Turvey, M.C. Clancy, and T.P. Williams, *J. Chem. Soc.*, **1960**, 4761.
8) A.G. Lloyd, *Biochem. J.*, **75**, 478 (1960).
9) A.G. Lloyd, *Biochem. J.*, **83**, 445 (1962).
10) K. Takiura and S. Honda, *Yakugaku Zasshi*, **87**, 1248 (1967).
11) T. Saito and J. Noguchi, *Nippon Kagaku Zasshi*, **82**, 471 (1961).
12) K. Komatsu, T. Saito, and J. Noguchi, *Nippon Kagaku Zasshi*, **82**, 472 (1961).
13) E. Peligot, *Ann.*, **30**, 69 (1939).

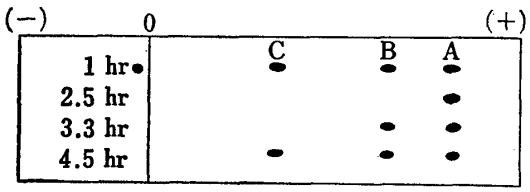

Fig. 1. Paper Electrophoresis of the Reaction Mixture of D-Glucose with Concentrated Sulfuric Acid

detected after reaction for one hour besides unreacted D-glucose at the starting line (Fig. 1). The components, B and C disappeared after 2.5 hours to give a single product, A. Prolonged reaction restored the electrophoretic pattern; namely, component B and C appeared again after 3.3 hours and 4.5 hours, respectively, presumably due to the partial hydrolysis caused by water formed. Thus, the appropriate reaction time for the preparation of pure sulfate A was to be said 2.5 hours. Shorter or longer reaction time did not favour the trisulfation. In consequence, D-glucose was sulfated under this condition, and the reaction mixture was neutralized with barium hydroxide. From the supernatant the product, sulfate A, was obtained as barium salt, which was converted into brucine salt and also into potassium salt for analysis and structural studies.

Analysis of the brucine salt agreed well with the calculated value of the brucine salt of D-glucose trisulfate trihydrate (Table I). A proof of trisulfation was also provided by de-

Table I. D-Glucose Trisulfate

	Analysis (%)								mp (decomp.)	$[\alpha]_D^{21}$ in water	$M_{D\text{-glucose 6-sulfate}}$
	Calcd.				Found						
	C	H	N	S	C	H	N	S			
Brucine salt $3H_2O$	54.33	5.84	5.07	5.80	53.99	5.76	5.04	5.76	220°	—	—
Potassium salt	—	—	—	17.99	—	—	—	17.93	—	+48.1°	2.18

termining the sulfur content of the potassium salt. Electrophoretic data, *i.e.*, $M_{D\text{-glucose 6-sulfate}}$ value (2.18) was also reasonable for trisulfate. Recently there has been a conflicting report by Nagasawa and co-workers[14] that oligosaccharide polysulfates were formed by sulfation of monosaccharides with concentrated sulfuric acid, due to simultaneous polymerization. However, the molecular weight of the brucine salt of D-glucose trisulfate determined by vapour pressure method was in good agreement with the calculated value. This evidence was supported by dialysis of the potassium salt of D-glucose trisulfate, since the greater part was dialyzed during 50 hours. Consequently, at least the fraction in our discussion, which was not precipitated with barium hydroxide, should be pure D-glucose trisulfate. Even if oligosaccharide sulfates are formed, they should remain in the precipitate of barium sulfate, as suggested by carbonization of the precipitate on ignition test as well as by the low yield (36%) of D-glucose trisulfate.

The behavior of D-glucose trisulfate to oxidizing agents, such as Fehling's reagent, Tollen's reagent or ferricyanide was deliverately investigated and it was found that no reduction occurred against such reagents. This was furthermore proved by the borohydride reduction of the barium salt in such a condition that did not cause hydrolysis of sulfate, which was checked by precipitation of barium sulfate. D-Glucose was the only product and no detectable amount of D-glucitol was found by gas liquid chromatography in the hydrolyzate of the reduction product. Non-reducibility may be attributed to C-1 substitution by a sulfate group. But at the same time the shielding effect caused by a 2-sulfate group must be took into consideration. However, the former case takes precedence from the following reasons; First, no mutaro-

14) K. Nagasawa, Y. Inoue, N. Tanoura, Y. Shinkai, and H. Yoshidome, Abstr. Papers of the 89 th Annual Meeting of the Pharmaceutical Society of Japan, Nagoya, April 1969, p. 612.

tation was observed with D-glucose trisulfate. Second, D-glucose 2-sulfate and D-galactose 2-sulfate are readily coloured by spraying with diphenylamine–aniline which is specific for reducing sugars,[15] while D-glucose trisulfate was detected only weakly with aniline hydrogen phthalate after heating over 150° for 20 minutes. Third, repeated methylation, followed by hydrolysis, gave a tailing chromatogram, indicating the difficulty of permethylation due to the steric effect of sulfate groups. The detection of a methylated D-glucose whose R_{TMG} (0.5) lies in the range of dimethyl-D-glucose (0.45—0.60), however, corresponds to C-1 substitution, rather than C-2 substitution; D-glucose trisulfate having a 2-sulfate group should give monomethyl-D-glucose as the most highly methylated product.

Partial hydrolysis of D-glucose trisulfate recovered the reducibility and gave D-glucose disulfate ($M_{\text{D-glucose 6-sulfate}}$1.72), which was unstable and immediately degraded to D-glucose monosulfate ($M_{\text{D-glucose 6-sulfate}}$1.00) and finally to D-glucose. Some attempts to isolate D-glucose disulfate were unsuccessful.

When the aqueous solution of the free acid of D-glucose trisulfate, which was prepared by treatment of the salt with cation exchange resin (H form), was heated under reflux, for 2 hours, mild hydrolysis took place to give a mixture of D-glucose and D-glucose monosulfate. The monosulfate fraction was isolated by anion exchange resin and subjected to the following structural studies.

(1) The methylation product of the monosulfate fraction was hydrolyzed to give 2,3,4-tri-O-methyl-D-glucopyranose (major) and 2,4,6-tri-O-methyl-D-glucopyranose (minor), indicating that the monosulfate was D-glucopyranose 6-sulfate contaminated with D-glucopyranose 3-sulfate (Table II).

Table II. Methylation Analysis of D-Glucose Monosulfate derived from D-Glucose Trisulfate

Partially methylated D-glucose		R_{TMG}
Reference	2,3,4-tri-O-methyl-D-glucopyranose	0.86
	2,4,6-tri-O-methyl-D-glucopyranose	0.81
Hydrolyzate of the permethylated monosulfate derived from D-glucose trisulfate		0.86 (major)
		0.81 (minor)

TMG represents 2,3,4,6-tetra-O-methyl-D-glucopyranose

(2) The optical rotation of the barium salt was +29.4°, which is close to that of D-glucose 6-sulfate (+29.0°) but is a little higher, possibly due to the contamination of D-glucose 3-sulfate (Table III).

Table III. Optical Rotation of D-Glucose Monosulfate (Ba Salt)

D-Glucose monosulfate		$[\alpha]_D^{18}$ in water
Reference	D-glucose 2-sulfate	+40.5°[15]
	3-sulfate	+33.0°[a,7]
	6-sulfate	+29.0°[a,7]
Monosulfate derived from D-glucose trisulfate		+29.4°

a) Temperature is not described.

(3) Periodate oxidation in phosphate buffer (pH 7.0) consumed 2.6 moles of periodate after 2 hours. This provides an additional evidence that the monosulfate is 6-sulfate contaminated possibly with 3-sulfate (Table IV).

15) S. Peat, D.M. Bowker, and J.R. Turvey, *Carbohyd. Res.*, 7, 225 (1968).

Table IV. Periodate Oxidation of D-Glucose Trisulfate and D-Glucose Monosulfate derived from D-Glucose Trisulfate (Theoretical consumption of periodate refers to Table VII and Table VIII.)

D-Glucose sulfate	Periodate consumption (mole)
D-Glucose trisulfate	0.0
D-Glucose monosulfate derived from D-glucose trisulfate	2.6

These experimental results showed that the monosulfate isolated from the partial hydrolyzate of D-glucose trisulfate was a mixture of D-glucose 3-sulfate and 6-sulfate. The predominance of 6-sulfate in the monosulfate mixture may be attributed to the greater stability of

Table V. Brucine Salts of D-Galactose Trisulfate, D-Mannose Trisulfate and D-Fructose Trisulfate

Hexose trisulfate	Analysis (%) Calcd. C	H	N	S	Found C	H	N	S	mp (decomp.)
D-Galactose 1,3,6-trisulfate $3H_2O$	54.33	5.84	5.07	5.80	54.11	5.86	5.26	5.92	222°
D-Mannose 1,3,6-trisulfate $3H_2O$	54.33	5.84	5.07	5.80	54.49	5.84	5.30	5.78	215°
D-Fructose 1,2,4-trisulfate $3H_2O$	54.33	5.84	5.07	5.80	53.85	5.86	4.97	6.06	245°

Table VI. Potassium Salts of D-Galactose Trisulfate, D-Mannose Trisulfate and D-Fructose Trisulfate

Hexose trisulfate	Analysis of S (%) Calcd.	Found	$[\alpha]_D^{24}$ in water	$M_{D\text{-glucose 6-sulfate}}$
D-Galactose trisulfate	17.99	17.90	+51.8° (c=1.33)	2.18
D-Mannose trisulfate	17.99	18.05	+16.7° (c=0.90)	2.18
D-Fructose trisulfate H_2O	17.41	17.15	− 4.0° (c=1.00)	1.92

Table VII. Periodate Oxidation of D-Galactose Trisulfate, D-Mannose Trisulfate and D-Fructose Trisulfate

	Hexose trisulfate		Periodate consumption (mole)
Theoretical	aldohexopyranose	1,2,3-trisulfate	0
		1,2,4-trisulfate	0
		1,2,6-trisulfate	1
		1,3,4-trisulfate	0
		1,3,6-trisulfate	0
		1,4,6-trisulfate	1
	ketohexopyranose	1,2,3-trisulfate	1
		1,2,4-trisulfate	0
		1,2,5-trisulfate	1
		2,3,4-trisulfate	0
		2,3,5-trisulfate	0
		2,4,5-trisulfate	0
Found	D-galactose trisulfate		0.0
	D-mannose trisulfate		0.0
	D-fructose trisulfate		0.0

Table VIII. Periodate Oxidation of D-Galactose Monosulfate, D-Mannose Monosulfate and D-Fructose Monosulfate

Hexose monosulfate			Periodate consumption (mole)
Theoretical	aldohexopyranose	1-sulfate	2
		2-sulfate	1
		3-sulfate	1
		4-sulfate	2
		6-sulfate	3
	ketohexopyranose	1-sulfate	3
		2-sulfate	2
		3-sulfate	2
		4-sulfate	2
		5-sulfate	3
Found	monosulfate derived from D-galactose trisulfate		2.6
	monosulfate derived from D-mannose trisulfate		2.6
	monosulfate derived from D-fructose trisulfate		2.9

6-sulfate than 3-sulfate. The fact that the trisulfate did not consume periodate even after 2 hours, also suggests the structure of 1,3,6-trisulfate.

With D-galactose and D-mannose similar reaction occurred under the same condition, and respective trisulfates were obtained (Table V, VI).

The position of sulfate groups of these compounds was determined in the similar manner as described above. Since non-reducibility was observed, the 1-position is to be sulfated in each case. These compounds consumed no periodate (Table VII), while each of the monosulfates obtained by partial hydrolysis of these compounds consumed 2.6 moles of periodate (Table VIII). These results show that the other sulfate groups are on the 3- and 6-positions. Thus, it is concluded that the 1-, 3- and 6-positions of these aldohexoses are sulfated.

D-Fructose trisulfate obtained in the similar manner possessed no reducibility, too. Periodate consumption of the trisulfate and the monosulfate was 0.0 and 2.9 moles, respectively (Table VII, VIII). Methylation, followed by hydrolysis, of the monosulfate gave exclusively 3,4,5-tri-O-methyl-D-fructopyranose. Since the pyranose conformation was also demonstrated from the fact that the pyranoid absorption mixima at 700—900 cm^{-1} of D-fructose were retained in D-fructose trisulfate, these data indicate that the sulfate groups in D-fructose trisulfate should be placed on the 1-, 2- and 4-positions.

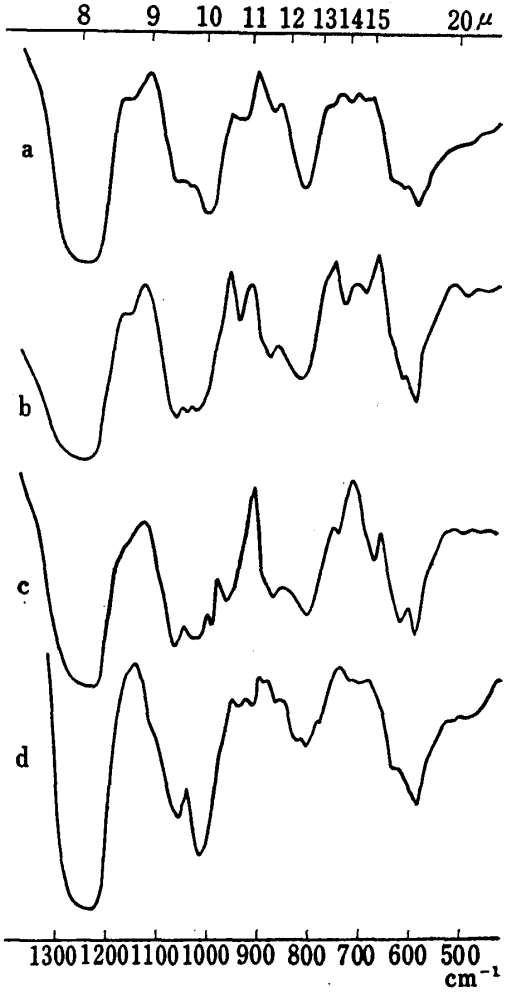

Fig. 2. Infrared Spectra of Hexose Trisulfates (K Salts) in KBr Discs

a : D-glucose 1,3,6-trisulfate
b : D-galactose 1,3,6-trisulfate
c : D-mannose 1,3,6-trisulfate
d : D-fructose 1,2,4-trisulfate

Infrared spectra of these hexose trisulfates in 800—860 cm^{-1} region resembled each other. They all gave an intense band at 800—810 cm^{-1}, which was a characteristic of these sulfates, not encountered in hexose sulfates of lower degree of sulfation. Absorption at 810—820 cm^{-1}, 830 cm^{-1} and 850—860 cm^{-1}, corresponding to the sulfate C–O–S bond vibration of primary, equatorial secondary, and axial secondary Cl conformation,[16] respectively, seems to have been united to give an intense band at 800—810 cm^{-1}. This shift might be attributed to the strong interaction between the highly anionic group, giving deformed pyranose conformation.

Experimental

General Procedures——All evaporations were carried out under diminished pressure below 40°.

(1) Determination of N and S: Nitrogen and sulfur of the brucine salts were determined by micro-Kjeldahl method and Ag_2SO_4 method, respectively. Sulfur of the potassium salts was determined by the benzidine titrimetric procedure described in British Pharmacopoeia.[17]

(2) Molecular Weight: Molecular weight was determined by vapour pressure method using Hitachi Perkin-Elmer 115 Molecular Weight Apparatus. Brucinium sulfate was employed as a standard and ΔR was measured in methanolic medium at 39.6°.

(3) Paper Chromatography: Descending paper chromatography was carried out on Toyo Roshi No. 51 filter paper using the solvent system of butan-1-ol–ethanol–water–ammonia (40:10:49:1). Methylated sugars were detected with aniline hydrogen phthalate.

(4) Paper Electrophoresis: Potential of 50 V/cm was applied for 30 min on Toyo Roshi No. 51 filter paper. The buffer used was acetic acid–pyridine (pH 6.5), prepared by mixing 0.1 N acetic acid with pyridine. Hexose sulfates were detected with aniline hydrogen phthalate after heating over 150° for 20 min. For trisulfates toluidine blue was also employed.

(5) Gas Liquid Chromatography: The hydrolyzate of the borohydride reduction product of D-glucose trisulfate was trimethylsilylated according to the procedure described by Sweeley and co-workers[18] and then chromatographed on SE-52 column (3 mm × 2 m) using Shimazu GC-1C instrument equipped with a hydrogen flame ionization detector. The retention time of α-D-glucose, β-D-glucose and D-glucitol was 5.55, 8.37 and 7.03, respectively, when chromatographed at 170° and at the flow rate of 60 ml/min.

Barium Salt of Hexose Trisulfate——To 20 ml of vigorously stirred concentrated sulfuric acid (96.0%, *d*, 1.84) cooled at −5° was added portion by portion 2.0 g of anhydrous hexose. Stirring was continued for 2.5 hr at −5°. The viscous solution was poured slowly onto 200 g of ice water and neutralized with cold saturated aqueous solution of barium hydroxide. The precipitate of barium sulfate was removed by filtration with Hiflo Super Cell and the filtrate was concentrated to dryness to give amorphous barium salt of hexose trisulfate, the yield ranging from 2.5 g to 2.7 g. For purification the product was dissolved in 10 ml of water and ten fold volumes of ethanol was added gradually with constant stirring. Purified barium salt of hexose trisulfate was precipitated as amorphous powder. Yield: barium D-glucose 1,3,6-trisulfate, 2.5 g (36%); barium D-galactose 1,3,6-trisulfate, 2.1 g (32%); barium D-mannose trisulfate 1,3,6-trisulfate, 2.4 g (35%); barium D-fructose 1,2,4-trisulfate, 2.3 g (34%).

Potassium Salt of Hexose Trisulfate——An aqueous solution of the barium salt of hexose trisulfate was passed through an Amberlite IR-120 (K form) column and the effluent was concentrated to a small volume. Upon gradual addition of ethanol, amorphous potassium salt of hexose trisulfate was precipitated, which was collected by filtration, dried *in vacuo* on phosphorus pentoxide and analyzed. Potassium D-glucose trisulfate (78.2 mg) was dissolved in 20 ml of water and dialyzed in cellophane tube (Visking, 32/36) against running water. Recovery of the non-dialyzable material was 10.2 mg (13%) after 50 hr.

Brucine Salt of Hexose Trisulfate——An aqueous solution of the potassium salt of hexose trisulfate was passed through an Amberlite IR-120 (H form) column and the effluent was mixed with a methanolic solution containing equivalent amount of brucine. The solvent was evaporated to dryness and the crystalline residue was recrystallized from methanol. Analytical data are listed in Table I and V. Molecular weight of potassium D-glucose trisulfate:Calcd., 1.66×10^3; Found, 1.64×10^3.

Borohydride Reduction of D-Glucose Trisulfate——To 2.0 ml of a 5% solution of barium D-glucose trisulfate was added under stirring 10 mg of sodium borohydride, and stirring was continued for 20 min. No precipitation of barium sulfate was observed during the reaction. The reaction mixture was acidified with sulfuric acid to the acid concentration of 10%, and heated on a boiling water bath for 2.5 hr. The hydrolyzate was neutralized with saturated aqueous solution of barium hydroxide and the precipitate was removed

16) J.R. Turvey, *Advan. Carbohyd. Chem.*, **20**, 194 (1965).
17) "British Pharmacopeia," 1958, p. 207.
18) C.C. Sweeley, R. Bentley, M. Makita, and W.W. Wells, *J. Chem. Soc.*, **85**, 2497 (1963).

off by centrifugation. The supernatant was evaporated to dryness and the residue was dissolved in methanol. After the removal of boric acid by repeated evaporation of the methanolic solution, the syrupy residue was submitted to gas liquid chromatography.

Potassium Salt of Hexose Monosulfate——A 10% aqueous solution of hexose trisulfate (free acid form) was prepared by passage of the potassium salt through an Amberlite IR-120 (H form) column. The effluent was heated under reflux in nitrogen atmosphere for 2 hr. The hydrolyzate, which contained hexose and hexose monosulfate, was introduced onto a Dowex-1 (OH form, 100—200 mesh) column and the column was eluted with water and then with N sulfuric acid. The N sulfuric acid eluate was neutralized with saturated aqueous solution of barium hydroxide and the precipitate of barium sulfate was removed by centrifugation. The supernatant was passed through an Amberlite IR-120 (K form) column and evaporated to a small volume. Upon gradual addition of tenfold volumes of ethanol the patassium salt of hexose monosulfate was obtained as amorphous powder. *Anal.* Calcd. for $C_6H_{11}O_9SK$: S, 10.75. Found: potassium D-glucose monosulfate, 10.62; potassium D-galactose monosulfate, 10.84; potassium D-mannose monosulfate, 10.50; potassium D-fructose monosulfate, 10.79.

Methylation of the Potassium Salt of Hexose Sulfate——To 2 ml of cold anhydrous N,N-dimethylformamide solution containing 100 mg of the potassium salt of hexose sulfate was added 0.47 ml of methyl iodide and 0.36 g of freshly prepared silver oxide. The mixture was stirred for 24 hr at room temperature. Fivefold volumes of methanol was added to the reaction mixture and the precipitate was filtered off. The filtrate was evaporated to dryness and the residue was hydrolyzed in 1 ml of 5% hydrochloric acid for 4 hr under reflux. The hydrolyzate was evaporated to dryness and extracted with small amount of water. The extract was deionized by passing through an Amberlite IR-120 (H form)column, followed by an Amberlite IRA-400 (OH form), and analyzed by paper chromatography comparing with the authentic specimen of partially methylated hexoses.

Periodate Oxidation——The mixture of 1 ml of 10^{-3}M hexose sulfate and 10 ml of 10^{-3}M potassium periodate in phosphate buffer (pH 7.0) was kept at $25° \pm 1°$. The consumed periodate was measured after standing for 2 hr polarometrically according to the procedure established by Takiura and Koizumi.[19]

19) K. Takiura and K. Koizumi, *Yakugaku Zasshi*, **78**, 961 (1958).