Inhibitory Effects of Glycyrrhetic Acid Derivatives on 11β- and 3α-Hydroxysteroid Dehydrogenases of Rat Liver

Teruaki AKAO,[a] Tadao TERASAWA,[c] Susumu HIAI,[b] and Kyoichi KOBASHI*,[a]

Faculty of Pharmaceutical Sciences[a] and Research Institute for Wakan-Yaku (Traditional Sino-Japanese Medicines),[b] Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-01, Japan and Shionogi Research Laboratories,[c] Shionogi & Co., Ltd., Fukushima-ku, Osaka 553, Japan.
Received April 22, 1992

Glycyrrhetic acid (GA), an aglycone of glycyrrhizin (GL), is a potent inhibitor of 11β- and 3α-hydroxysteroid dehydrogenases. 11β-Hydroxysteroid dehydrogenase activity of rat liver microsomes was potently inhibited by GA, 3-deoxyglycyrrhetic acid (3-deoxyGA), 3-ketoglycyrrhetic acid (3-ketoGA), 3-epiglycyrrhetic acid (3-epiGA) and 11-deoxoglycyrrhetic acid (11-deoxoGA), with I_{50} values of $2—4 \times 10^{-7}$ M. However, 18α-stereoisomers ($I_{50} = 3—7 \times 10^{-6}$ M) of GA, 3-deoxyGA and 11-deoxoGA were one tenth less inhibitory on the enzyme activity than the corresponding 18β-isomers. On the other hand, 18α-stereoisomers of GA, 3-deoxyGA and 11-deoxoGA inhibited 3α-hydroxysteroid dehydrogenase activity of rat liver cytosol more potently than the corresponding 18β-isomers. I_{50} values of 18α- and 18β-isomers were 2 and 7×10^{-6} M, respectively, in the case of GA, 8 and 20×10^{-6} M in 3-deoxyGA, 3 and 20×10^{-6} M in 11-deoxoGA. These results indicate that the 18β-conformation of oleanane is important for the inhibition of 11β-hydroxysteroid dehydrogenase but on the contrary the 18α-conformation is important for the inhibition of 3α-hydroxysteroid dehydrogenase.

Keywords glycyrrhetic acid; 18α-glycyrrhetic acid; 11β-hydroxysteroid dehydrogenase; 3α-hydroxysteroid dehydrogenase; inhibition

Introduction

Glycyrrhizin (GL), an active component of licorice, *Glycyrrhiza glabra*, is ingested orally as a component of oriental medicine and as a sweetener. GL shows various pharmacological effects including a steroid-like action,[1] anti-viral action[2] and interferon-inducing activity,[3] but has a side effect such as pseudoaldosteronism.[4,5] On the other hand, GL is hydrolyzed to 18β-glycyrrhetic acid (GA), an aglycone of GL, by human intestinal bacteria,[6,7] though GL is not hydrolyzed to GA, but to GA monoglucuronide, by human liver β-D-glucuronidase.[8] Moreover, when GL is administered orally to human beings, GL is not detected in their sera but GA is detected.[9] Accordingly, these results suggest that GL administered orally is not absorbed from the gastrointestine, but GA is absorbed after the hydrolysis of GL to GA by intestinal bacteria and shows various pharmacological effects. Several studies have been reported on the inhibitory effects of GL and GA on steroid-metabolizing enzymes such as 5α-reductase,[10,11] 5β-reductase,[10,11] 3α-hydroxysteroid dehydrogenase (3α-HSD),[12,13] 3β-hydroxysteroid dehydrogenase (3β-HSD)[13] and 11β-hydroxysteroid dehydrogenase (11β-HSD)[14] and revealed that GA, not GL, potently ibhibited the activities of 5β-reductase, 3α-HSD, 3β-HSD and 11β-HSD. Therefore, it was postulated that the inhibition of 5β-reductase, 3α-HSD, and 11β-HSD resulted in steroid-like action, anti-inflammatory action, and pseudoaldosteronism, respectively.

18α-Stereoisomer of GA (18α-glycyrrhetic acid, 18α-GA) had more potent inhibitory effects on 5β-reductase[11] and 3α-HSD[12] activities than GA, and also showed stronger anti-inflammatory action than GA,[11,15] suggesting that the 18α-planar conformation of GA is important for these effects. On the other hand, the 11-oxo group in ring C of GA was found to be essential for the inhibition of 5β-reductase activity because 11-deoxo-derivatives had little inhibitory effects on the enzyme activity.[16] Moreover, the 3β-hydroxyl group in ring A of GA seemed to be related to the inhibition of 3α-HSD activity because the activity was less inhibited by 3-epi-18β-glycyrrhetic acid (3-epiGA).[12] However, the inhibitory effects of various GA derivatives on 11β-HSD activity, which give rise to the pseudoaldosteronism, have not been studied previously.

In the present paper, inhibitory effects against 11β- and 3α-HSD activities were examined with 18α- and 18β-stereoisomers of GA, 3-deoxyglycyrrhetic acid (3-deoxyGA) and 11-deoxoglycyrrhetic acid (11-deoxoGA) (Fig. 1).

Materials and Methods

Animals and Preparation of Hepatic Microsomes and Cytosol Wistar-strain male rats, 8—12 weeks old, were purchased from Japan SLC Co. (Shizuoka). After sacrifice of the rats by decapitation, livers perfused with saline were homogenized in 4 volumes of 0.15 M KCl solution, and

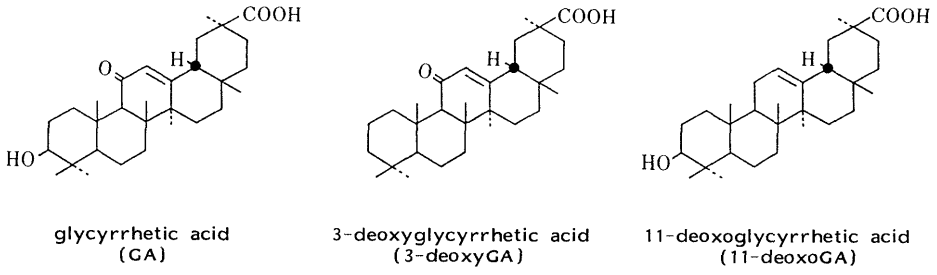

Fig. 1. Structure of GA, 3-DeoxyGA and 11-DeoxoGA
Symbol (●) shows 18-position of carbon.

then centrifuged at 10000 × *g* for 20 min. The supernatant was further centrifuged at 105000 × *g* for 90 min to yield the microsomal and the cytosol fractions. The microsomes were washed once with 0.15 M KCl containing 10 mM ethylenediaminetetraacetic acid (EDTA) and suspended with 50 mM potassium phosphate buffer (pH 7.2) containing 20% glycerol, 1 mM dithiothreitol and 1 mM EDTA. The cytosol fraction was further fractionated with 45—70% saturation of ammonium sulfate and then dialyzed overnight against 20 mM potassium phosphate buffer (pH 7.2). The specific activity of microsomal 11β-HSD was 1.1 nmol/min mg as $NADP^+$-dependent 11β-hydroxyprogesterone-oxidizing activity and that of cytosol 3α-HSD was 43 nmol/min mg as NAD^+-dependent androsterone (5α-androstan-3α-ol-17-one)-oxidizing activity.

Assay of Enzyme Activities The 11β-HSD activity was determined by measuring the oxidation rate of 11β-hydroxyprogesterone according to the method of Bush *et al.*[17] as follows: the reaction mixture contained 50 nmol of 11β-hydroxyprogesterone in 10 μl of ethanol, 0.4 μmol of $NADP^+$ and microsomal protein (0.42 μg) in 0.5 ml of 0.1 M potassium phosphate buffer (pH 8.0). The reaction was carried out for 10—30 min at 37 °C, and stopped with 100 μl of 1 N HCl. After being extracted twice with 2 ml of ethyl acetate, the extract was dried under vacuum, and chromatographed on silica gel plates (Merck, silica gel 60F-254, layer thickness 0.25 mm) with the solvent system of petroleum ether–chloroform–methanol (50 : 50 : 4, by vol.). 11β-Hydroxyprogesterone and its product, 11-ketoprogesterone, were detected on TLC plates under UV light as shown in Fig. 2. The quantity was analyzed with a TLC scanner (Shimadzu CS-910, $\lambda_s = 260$ nm, $\lambda_r = 400$ nm) using calibration lines obtained with authentic samples.

The initial velocity of NAD^+-dependent 3α-HSD activity was determined by measuring the oxidation of androsterone spectrophotometrically at 340 nm ($\varepsilon = 6270\ M^{-1}\ cm^{-1}$ for NADH) at 25 °C as reported in the previous paper.[12]

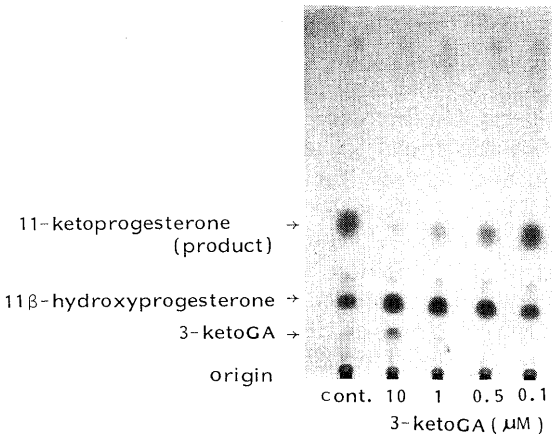

Fig. 2. Inhibition of 11β-Hydroxyprogesterone-Oxidizing Activity by 3-KetoGA

Experimental procedure was carried out by the addition of 0.1, 0.5, 1 and 10 μM 3-ketoGA as described in Materials and Methods. After development of TLC, the plate was photographed under UV.

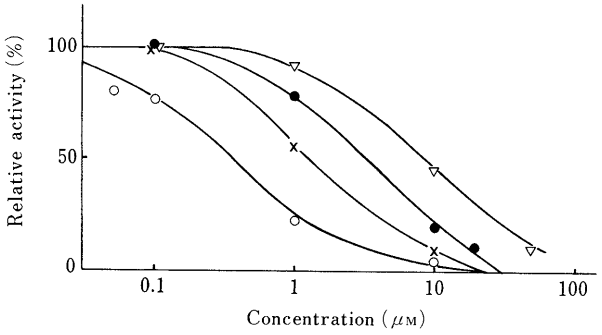

Fig. 3. Inhibitory Effects of GA, 18α-GA, Carbenoxolone and GAMG on 11β-HSD Activity

GA (○), 18α-GA (●), carbenoxolone (×), GAMG (▽).

Inhibitors at varying concentrations were added in 5—20 μl of methanol or acetonitrile. Control velocity without inhibitors was determined in the presence of the corresponding quantities of organic solvent. I_{50} values were obtained from linear-regression lines as the final concentrations of inhibitors that gave 50% inhibition.

Determination of Protein Protein was determined by the method of Lowry *et al.*[18] using bovine serum albumin as the standard.

Chemicals GA was purchased from Nacalai Tesque Inc. (Kyoto), and purified by repeated crystallization. 3-EpiGA and 3-keto-18β-glycyrrhetic acid (3-ketoGA) were prepared according to the method reported previously.[6] 18α- And 18β-stereoisomers of 3-deoxyGA and 11-deoxoGA were synthesized as reported previously.[19] 18β-Glycyrrhetyl mono-β-D-glucuronide (GAMG) was donated by Dr. M. Kanaoka (Research Institute for Wakan-Yaku, Toyama Medical and Pharmaceutical University). 18α-GA, androsterone, 11β-hydroxyprogesterone and 11-ketoprogesterone were purchased from Sigma Chemical Co. (MO, U.S.A.). All other reagents were of the best commercial quality available.

Results

Inhibition of 11β-HSD Activity by GA Derivatives Figure 3 shows the effects of GA, 18α-GA, carbenoxolone and GAMG on $NADP^+$-dependent 11β-hydroxyprogesterone-oxidizing activity of rat liver microsomes. The enzyme activity was inhibited dose-dependently and potently with GA ($I_{50} = 0.3$ μM) and carbenoxolone ($I_{50} = 1$ μM) as reported by Monder *et al.*[14] Although 18α-GA and GAMG also inhibited the enzyme activity, their inhibitions ($I_{50} = 3$ and 10 μM) were one tenth weaker, respectively, than those of GA and carbenoxolone. GL at the concentration of 50 μM slightly inhibited the activity as reported by Monder *et al.*[14]

3-DeoxyGA and 11-deoxoGA potently inhibited 11β-HSD activity at a concentration similar to GA (Fig. 4). Their I_{50} values were 0.2 and 0.3 μM, respectively. Moreover, the inhibitory activities of their 18α-stereoisomers were one tenth of those of 18β-isomers, as was the case of 18α- and 18β-isomers of GA. 3-KetoGA and 3-epiGA were also potently inhibitory ($I_{50} = 0.4$ μM) against the enzyme activity similar to GA (data not shown).

These results suggest that 3-hydroxyl and 11-oxo groups of GA do not have an important role in the inhibition of 11β-HSD activity but the 18β-configuration of GA derivatives contributes to the inhibition.

Inhibitory Effects of 18α- and 18β-Stereoisomers of 3-Deoxy- and 11-DeoxoGAs on NAD^+-Dependent 3α-HSD Activity In the previous paper,[12] we showed that the

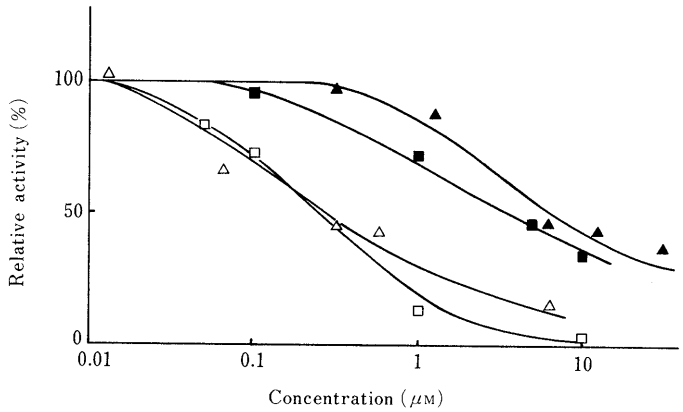

Fig. 4. Inhibition of 11β-HSD Activity by 18α- and 18β-Stereoisomers of 3-DeoxyGA and 11-DeoxoGA

3-DeoxyGA (△), 3-deoxy-18α-GA (▲), 11-deoxoGA (□), 11-deoxo-18α-GA (■).

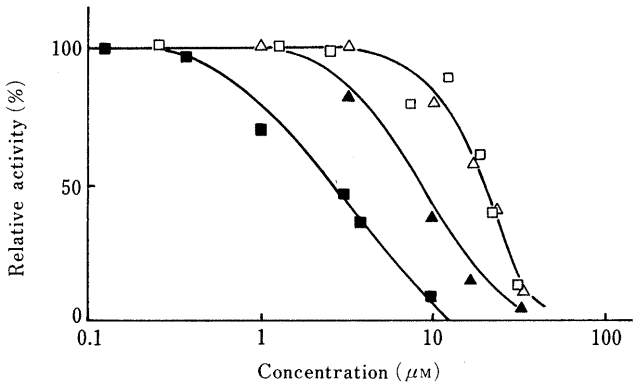

Fig. 5. Inhibition of 3α-HSD Activity by 18α- and 18β-Stereoisomers of 3-DeoxyGA and 11-DeoxoGA

3-DeoxyGA (△), 3-deoxy-18α-GA (▲), 11-deoxoGA (□), 11-deoxo-18α-GA (■).

TABLE I. Inhibition of 11β-HSD and 3α-HSD by GA and the Related Compounds

Compound	I_{50} value (μM)	
	11β-HSD	3α-HSD
GA	0.3	7[a]
18α-GA	3	2[a]
3-DeoxyGA	0.2	20
3-Deoxy-18α-GA	7	8
11-DeoxoGA	0.3	20
11-Deoxo-18α-GA	4	3
3-KetoGA	0.4	6[a]
3-EpiGA	0.4	80[a]
Carbenoxolone	1	6[a]
GAMG	10	50[a]
GL	>50	50[a]

a) Reported in the previous paper.[12]

18α-isomer of GA inhibited 3α-HSD activity more potently than the 18β-isomer (GA). Although both 3-deoxy- and 11-deoxoGAs had less inhibitory effects ($I_{50}=20\,\mu M$) on the enzyme activity than GA ($I_{50}=7\,\mu M$), their 18α-isomers inhibited 3 and 8 fold more potently than the corresponding 18β-isomers as shown in Fig. 5. These facts indicate that the 18α-configuration of GA derivatives is important for the inhibition of 3α-HSD activity, in reverse of the case of the inhibition of 11β-HSD activity. Our previous and present results on the inhibition of 11β- and 3α-HSD activities by various GA derivatives are summarized in Table I.

Discussion

A side effect, pseudoaldosteronism, has been observed in patients given high doses of GL for prolonged periods.[4,5] Recently, this side effect was found to be characteristic of the syndrome of apparent mineralcorticoid excess with 11β-HSD deficiency,[20] and to be due to the potent inhibition of 11β-HSD by GA.[14,21] Thus, GA potently inhibited the 11β-HSD activity of rat kidney and liver *in vitro*, and orally administered GA inhibited the enzyme activity of rat kidney cortex and supressed the plasma corticosterone level *in vivo*. On the other hand, GL has little inhibitory effect on the enzyme activity, but carbenoxolone potently inhibited it.[14] In the present study, 18β-stereoisomers of GA derivatives such as 3-deoxy- and 11-deoxoGAs showed the potent inhibition of 11β-HSD activity similar to GA. However, 18α-stereoisomers of the corresponding compounds showed weaker inhibitions (Figs. 3 and 4), indicating that the 18β-configuration of oleanane is important for the inhibition of 11β-HSD. Moreover, GA is more cytotoxic against human fibroblasts than 18α-GA,[22] though GA is more effective than 18α-GA as an anti-mutagenic and anti-tumor initiating agent.[23] Penning and Talalay proposed a good correlation between the anti-inflammatory effects of drugs and their inhibitory effects on 3α-HSD activity.[24,25] In our previous report,[12] 18α-GA, GA and carbenoxolone, which have an anti-inflammatory action,[11,15,26-28] potently inhibited 3α-HSD activity. Moreover, 18α-GA inhibited the enzyme activity more potently than GA, parallel to the order of the anti-inflammatory actions of these agents.[11,15] For example, 18α-GA at a daily dose of 3 mg/kg *p.o.* exhibited a similar inhibitory effect on GA at a daily dose of 30 mg/kg *p.o.* on the formation of cotton pellet granuloma in mice. In the present study, 18α-isomers of 3-deoxy- and 11-deoxoGAs inhibited 3α-HSD activity more potently than the 18β-isomers (Fig. 5). Moreover, 5β-reductase activity of rat liver cytosol was also more potently inhibited by 18α-GA than by GA.[11] From these findings, the planar conformation of 18α-stereostructure of oleanane, similar to the conformation of predonisolone,[11] may be important for the anti-inflammatory action and the inhibition of 3α-HSD and 5β-reductase activities. Accordingly, 18α-stereoisomers such as 18α-GA are considered to be better drugs having higher anti-inflammatory properties but lower side effects.

Takahashi *et al.* reported that 11-deoxoGA and 11-deoxoglycyrrhetol had little inhibitory effect on 5β-reductase activity.[16] It seems plausible that the 11-oxo-$\Delta^{12(13)}$ system in ring C of GA is competitive with the 3-oxo-$\Delta^{4(5)}$ system in ring A of cortical steroids at the active site of the reducing enzyme. This hypothesis is compatible with the results that both 18α- and 18β-isomers of 11-deoxoGA had the same inhibitory effects on 11β-HSD activity as the corresponding GA isomers (Fig. 4), suggesting that the 11-oxo-group in ring C of GA is not competitive with the 11-oxo-group in ring C of cortical steroids at the active site. On the other hand, 11-deoxoGA showed less inhibitory effect on 3α-HSD activity than GA (Table I). Moreover, 3-epiGA and 3-deoxyGA were weaker inhibitors against 3α-HSD than GA, and 3-deoxy-18α-GA was also weaker than 18α-GA (Table I), though 3-ketoGA had the same inhibitory efficacy as GA. These results suggest that 3β-hydroxyl and 3-ketonic groups in ring A of GA play an important role in the inhibition of 3α-HSD.

Acknowledgments We thank Mrs. M. Tomita-Doki for her technical assistance, Miss S. Takayanagi for her secretarial assistance, and the Animal Laboratory Center of our university for breeding rats.

References

1) A. Kumagai, S. Yano, and M. Otomo, *Endocrinol. Jpn.*, **4**, 17 (1957).
2) R. Pompei, O. Flore, M. A. Marccialis, A. Pani, and B. Loddo, *Nature* (London), **281**, 689 (1979).
3) N. Abe, T. Ebina, and N. Ishida, *Microbiol. Immunol.*, **26**, 535 (1982).

4) J. A. Molhuysen, J. Gerbrandy, L. A. deVries, J. C. deJong, J. B. Lenstra, K. P. Turner, and J. G. G. Borst, *Lancet*, ii, 135 (1950).
5) J. W. Conn, D. R. Rovner, and E. L. Cohen, *J. Am. Med. Assoc.*, **205**, 80 (1968).
6) M. Hattori, T. Sakamoto, K. Kobashi, and T. Namba, *Planta Med.*, **48**, 38 (1983).
7) Ta. Akao, Te. Akao, and K. Kobashi, *Chem. Pharm. Bull.*, **35**, 705 (1987).
8) Te. Akao, Ta. Akao, M. Hattori, M. Kanaoka, K. Yamamoto, T. Namba, and K. Kobashi, *Biochem. Pharmacol.*, **41**., 1025 (1991).
9) N. Nakano, H. Kato, H. Suzuki, N. Nakao, S. Yano, and M. Kanaoka, *Yakuri To Chiryo*, **8**, 4171 (1980).
10) Y. Tamura, T. Nishikawa, K. Yamada, M. Yamamoto, and A. Kumagai, *Arzneim.-Forsch.*, **29**, 647 (1979).
11) S. Amagaya, E. Sugishita, Y. Ogihara, S. Ogawa, K. Okada, and T. Aizawa, *J. Pharmacobio-Dyn.*, **7**, 923 (1984).
12) Te. Akao, Ta. Akao, M. Hattori, T. Namba, and K. Kobashi, *Chem. Pharm. Bull.*, **40**, 1208 (1992).
13) S. A. Latif, T. J. Conca, and D. J. Morris, *Steroids*, **55**, 52 (1990).
14) C. Monder, P. M. Stewart, V. Lakshmi, R. Valentino, D. Burt, and C. R. W. Edwards, *Endocrinology*, **125**, 1046 (1989).
15) E. Sugishita, S. Amagaya, and Y. Ogihara, *J. Pharmacobio-Dyn.*, **5**, 379 (1982).
16) K. Takahashi, S. Shibata, S. Yano, M. Harada, H. Saito, Y. Tamura, and A. Kumagai, *Chem. Pharm. Bull.*, **28**, 3449 (1980).
17) I. E. Bush, S. A. Hunter, and R. A. Meigs, *Biochem. J.*, **107**, 239 (1968).
18) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
19) T. Terasawa, T. Okada, T. Hara, and K. Itoh, *Eur. J. Med. Chem.*, **27**, 345 (1992).
20) C. Monder, C. H. Shackleton, H. L. Bradlow, M. I. New, E. Stoner, F. Iohan, and V. Lakshmi, *J. Clin. Endocrinol. Metab.*, **63**, 550 (1986).
21) B. B. Walker and R. W. Edwards, *Clin. Endocrinol.*, **35**, 281 (1991).
22) J. S. Davidson, I. M. Baumgarten, and E. H. Harley, *Biochem. Biophys. Res. Commun.*, **134**, 29 (1986).
23) Z. Y. Wang, R. Agarwal, Z. C. Zhou, D. R. Bickers, and H. Mukhtar, *Carcinogenesis*, **12**, 187 (1991).
24) T. M. Penning and P. Talalay, *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 4504 (1983).
25) T. M. Penning, I. Mukharji, S. Barrows, and P. Talalay, *Biochem. J.*, **222**, 601 (1984).
26) R. S. H. Finney and G. F. Somers, *J. Pharm. Pharmacol.*, **10**, 613 (1958).
27) K. K. Tamgri, P. K. Seth, S. S. Parmar, and K. P. Bhargava, *Biochem. Pharmacol.*, **14**, 1277 (1965).
28) R. S. H. Finney and A. L. Tárnoky, *J. Pharm. Pharmacol.*, **12**, 49 (1960).