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Chemical Reduction of Cycasin, the Toxic Glycoside of Cycad

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Introduction

Cycasin, methylazoxymethyl-β-D-glucoside, was first isolated and elucidated in our laboratory as the toxic principle of Japanese cycad, *Cycas revoluta* Thunb.[9] It was later shown to be a potent carcinogen for experimental animals[5], and thereafter, many studies on this compound were published[12]. The aglycone of cycasin was isolated as a colorless liquid from its enzymatic hydrolyzate with β-glucosidase and was proved to degrade spontaneously[3].

Reduction of this unusual aliphatic azoxy group must be of much interest in both organic and biological chemistry. Chemical reduction of cycasin with stannous cholride[9] and electrochemical reduction in polarography[10] were previously studied in this laboratory. A possible relationship was suggested recently[4] between the carcinogenic activitiy of aglycone of cycasin and its reduction with nicotinamide adenine dinucleotide-dependent dehydrogenases. In this paper we report on reduction of cycasin with sodium borohydride, and with hydrogen and palladium carbon as a catalyst.

Materials and Methods

Materials

Cycasin was isolated from the seeds of cycad, *Cycas revoluta* Thunb., in this laboratory. Plates of TLC were prepared with Silica-layer G from Nakarai Co. or Avicel from Funakoshi Co. Palladium carbon, 5% palladium, was from Wako Co. All chemicals used were of the purest grade.

Analysis

Cycasin was determined from its characteristic absorption at 215 nm. Analysis of sugars and cycasin by GC was carried out on trimethylsiliated[4] samples by Yanagimoto G-8 chromatograph equipped with a digital integrator, GPI-200. The reaction products were analyzed by PC and TLC. Spray reagents used were alkaline permanganate, alkaline silver nitrate, α-naphthol-sulfuric acid, and ninhydrin. DNP-derivatives were detected under UV-light. Commercial solution of formalin was assayed volumetrically[2], and was used as the standard for colorimetry by the method of Nash[6].

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The following abbreviations are used in this paper; PC, paper chromatography TLC, thin-layer chromatography GC, gas chromatography UV, ultra-violet IR, infrared NMR, nuclear magnetic resonance DNP, 2,4-dinitrophenyl.

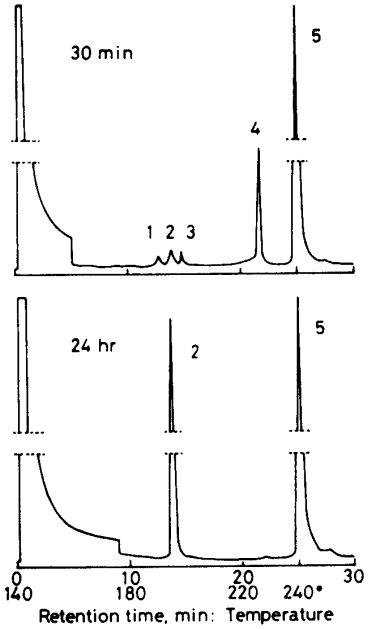

Fig. 1. Gas chromatogram of the products from cycasin reduced with $NaBH_4$. Samples: at 30 min- and 24 hr-reaction. Column: SE-52, 3 mm × 1.5 m. Program: 140° – 240°, 4°/min. Carrier: N_2. Peaks: 1, α-glucose 2, sorbitol 3, β-glucose 4, cycasin 5, androsterone (inner standard).

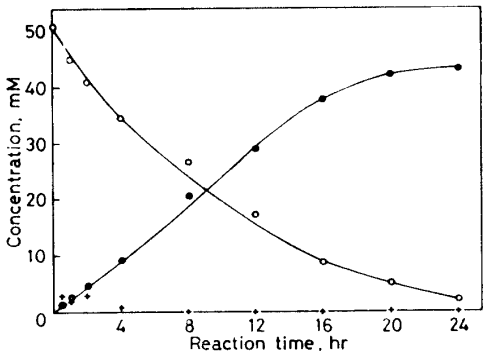

Fig. 2. Development of reduction of cycasin with $NaBH_4$, analyzed by GC. Open circle: cycasin. Closed circle: sorbitol. Cross: glucose.

Three types of photometers, Hitachi 101, EPU-2A, and EPS-3T, were used. IR spectra were measured on KBr tablets by the apparatus of Hitachi EPI-G2. Ammonia and amines were determined byYanagimoto amino acid analyzer SLC-5N. NMR spectra, by Nihon-Denshi JEOL MH-100 type, were measured on a solution in deuterium cholroform, and trimethylsilane was used as the internal standard.

Results and Discussion

1. Reduction with sodium borohydride

(1) Reaction

Two ml each of aqueous solution of 50 mM cycasin was added to test tubes containing sodium borohydride, and was incubated at 30°. The initial concentration of borohydride was made as 1 M. The reaction was sustained by adding dilute acetic acid at predetermined periods for the analysis of products.

The absorption at 215 nm showed a rapid decrease in the early period of reaction. In accordance with this, the spot of cycasin in PC and TLC reduced its size and became negligible after 20 hr-reaction. Several spots of reaction products were detected on the chromatograms. Coloration of the reaction mixture toward Nessler's reagent showed formation of either ammonia or amines or both. Formaldehyde was also detected qualitatively.

(2) Cycasin and sorbitol

The reaction mixture was treated on a small column of Dowex 50 (H^+ form) in order to remove the interfering cations for the analysis of sugars. Residual borate in the passes and washes of the column was removed by repeated eveporation *in vacuo* with added methanol.

Fig. 1 shows an example of results in GC. Four peaks observed in the chart of early stage of reaction were determined to be of α- and β-glucose, cycasin, and sorbitol from their

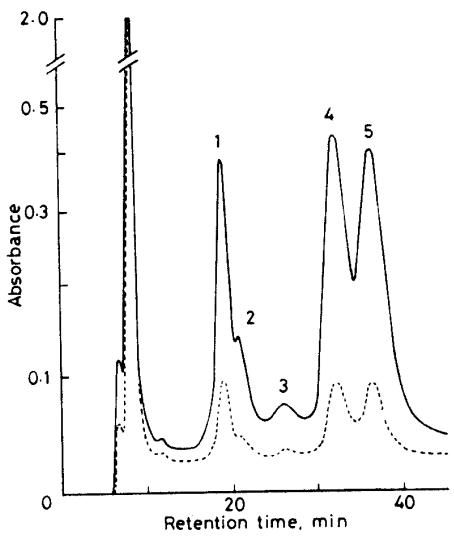

Fig. 3. Amines produced in reduction of cycasin with $NaBH_4$, analyzed by amino acid analyzer. Solid line: 570 nm. Broken line: 440 nm. Peaks: 1, 2, 3, unknowns 4, ammonia 5, methylamine.

values of retention time. The former 3 peaks were not detected in the final stage, but only one peak due to sorbitol was found. The whole time-course was as shown in Fig. 2.

Sorbitol was further identified as follows. Rf values of the spot detected on silica-gel plates were identical with those of authentic specimen, 0.65, 0.43, and 0.63, in 3 solvent systems; ethylacetate-benzene-methanol (1 : 1 : 3), ethylacetate-methanol (3 : 1) and (1 : 3), respectively. A part of deionized sample of the final stage was purified by gel-filtration through Sephadex G-10, and was analyzed by IR. The spectra of sample and sorbitol-specimen coincided completely.

(3) Formaldehyde and amines

The amount of produced formaldehyde was small and no more than 1 %, in mole of originally used cycasin, at the final period.

Formation of some ninhydrin-positive substances was observed in TLC. Analysis by amino acid analyzer exhibited, as shown in Fig. 3, five peaks included those corresponded to ammonia and methylamine. To isolate these substances, cations adsorbed on the column of Dowex 50 were eluted with 1 N hydrochloric acid. The eluate was made alkaline and steam distilled to collect volatile amines as their hydrochlorides.

A crystalline preparation, easily obtained by the addition of methanol, was identified as ammonium chloride from its IR spectrum. The fraction of methylamine was purified by gel-filtration through Sephadex G-10, and was identified from its IR spectrum compared with that of authentic specimen. The amount of methylamine, determined by amino acid analyzer, showed a gradual increase with the time-course of reaction, but remained in about 0.1 equivalent of cycasin in the final stage.

Another single component was obtained in silica-gel column chromatography with ethylacetate-benzene-methanol (1 : 1 : 3) as the developing solvent. It was identified as N-methylhydroxylamine hydrochloride from the same IR spectra of the sample and specimen.

2. Catalytic reduction of cycasin in neutral medium

(1) Hydrogenation

The apparatus was a type of catalytic hydrogenation for normal pressure and temperature, equipped with magnetic stirrer[8]. The reaction system was a solution of 2 mmoles, 504 mg, of cycasin and 100 mg of palladium carbon in 50 ml of water. The consumption of hydrogen was read from the gas buretts. Samples were withdrawn at an appropriate interval, freed from the catalyzer, and analyzed. The consumption of hydrogen and the changes in absroption at 215 nm during the reaction time were as shown in Fig. 4. The degradation of cycasin was assumed to be completed after 30 hr. Analyses by PC and TLC agreed with this assumption showing a gradual contraction of the spot of cycasin with an enlargement of that of glucose.

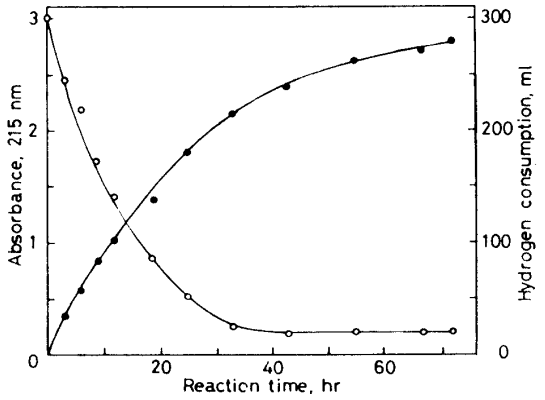

Fig. 4. Development of catalytic reduction of cycasin in neutral medium. Open circle: absorption at 215 nm. Closed circle: hydrogen-consumption.

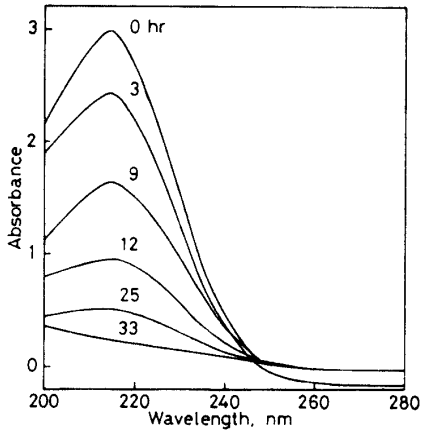

Fig. 5. Changes of UV spectrum in catalytic reduction of cycasin in neutral medium.

Production of ammonia or amines or both was proved by Nessler's reagent and that of formaldehyde by Nash's.

(2) UV spectra

The changes of spectra with time-course are shown in Fig. 5. The aglycone of cycasin shows just the same absorption spectrum as cycasin[3], but it decomposes easily by heat, accompanying disappearance of the absroption. Then, if free aglycone existed together with cycasin in the hydrogenation mixture, the spectrum should show some transformations before and after heating the sample solution. Such a change, however, was not observed on every spectrum shown in Fig. 5, and so free existence of the aglycone *per se* was denied.

(3) Cycasin and sugar component

Deionization procedure was omitted in this case, and the sample solution was directly trimethylsiliated for analysis by GC. Fig. 6 shows examples of 12 and 43 hr-hydrogenation. Not the peak due to sorbtol but due to

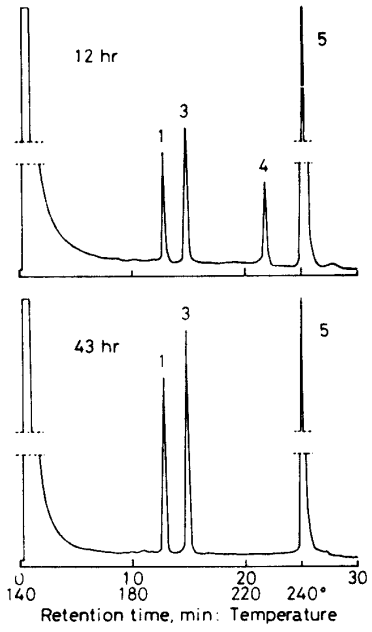

Fig. 6. Gas chromatogram of the products in catalytic reduction of cycasin. Samples: at 12 and 43 hr-reaction. Chromatography: same as in Fig. 1. Peaks: 1, α-glucose 3, β-glucose 4, cycasin 5, androsterone (inner standard).

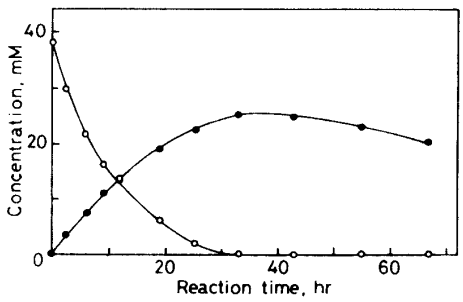

Fig. 7. Development of catalytic reduction of cycasin in neutral medium. Open circle: cycasin. Closed circle: glucose.

glucose and cycasin were observed. Cycasin was not detected in the final stage of reaction.

Quantitative development, shown in Fig. 7, agreed with the result of UV spectrophotometry. The yield of glucose from cycasin was not in an equimolar relationship.

(4) Formaldehyde and amines

The produced formaldehyde showed a higher yield than that in the reduction with borohydride. It was maximum at 24 hr, 15% of cycasin in mole, and gradually decreased to 7.5% after 50 hr.

The ninhydrin-positive substances were collected on a column of Dowex 50 (H^+ form) and eluted with acid. Four spots were detected on silica-gel TLC. One of them was assigned to be methylamine hydrochloride from its Rf values compared with those of authentic specimen, 0.48, 0.11, 0.10, and 0.10, in 4 solvent systems; n-butanol-acetic acid-water (4: 1: 2), ethylacetate-methanol (1: 3) and (3: 1), and ethylacetate-benzene-methanol (1: 1: 3), respectively. The amount of produced methylamine was up to 50% of the applied cycasin.

3. Catalytic reduction of cycasin in acidic medium

(1) Hydrogenation and development of the reaction

The reaction was carried out on the similar reaction system in the same apparatus as in the hydrogenation in neutral medium. The point of difference was that 0.05 N hydrochloric acid was used instead of water as solvent.

The most eminent discrepancy between the reactions in these two solvent systems was a very rapid progress in acid medium. The curve of hydrogen-consumption ascended linearly and reached a plateau after a distinct refraction at 3 hr-reaction. This point of refraction corresponded well to the point of disappearance of cycasin analyzed by UV spectrophotometry, TLC, and GC. The consumption of hydrogen at this point was 3.5 moles per mole of cycasin.

Liberation of glucose, proved by TLC, was in good correspondence with the degradation of cycasin, but the yield was about 50% of cycasin in mole. Formaldehyde in the reaction mixture reached the maximum, 8% of cycasin, after 30 min, and decreased to a very low value at the point of cycasin-disappearance. The final amount was only 0.2%.

(2) Ammonia and amines

Formation of at least 4 amines was recognized on the ninhydrin-developed TLC on silica-gel and Avicel. The former plate was developed with n-propanol-2% ammonia (7: 3), and the latter, with n-butanol-acetic acid-water (4: 1: 2). Two of the spots were deduced to be ammonia and methylamine, respectively, from their retention time on amino acid analyzer. The amount of ammonia ascended linearly up to the point of disappearance of cycasin, 3 hr, and then showed a plateau. Methylamine showed a constant increase to the maximum, 35% of cycasin, at 6 hr, and then slowly decreased.

Identification of amines was studied on their DNP-derivatives[7,11]. The hydrogenated solution, 50 ml, was added with 3 ml of 2,4-dinitrofluorobenzene, adjusted to pH 9.0 with 2 N sodium hydroxide, and incubated overnight at 40° in the dark with constant stirring. The reaction mixture was extracted 3 times with ether, and the extract was washed with water and evaporated. DNP-amines thus obtained were dissolved in methanol and applied for preparative TLC on silica-gel, 0.5 mm in thickness, developed with chloroform. Developed bands were scraped from the plates and eluted with methanol, evaporated, and recrystallized from methanol. Among several DNP-derivatives observed, six were obtained as single component and were designated as DNP-amine A – F. DNP-amine A, C, D, and

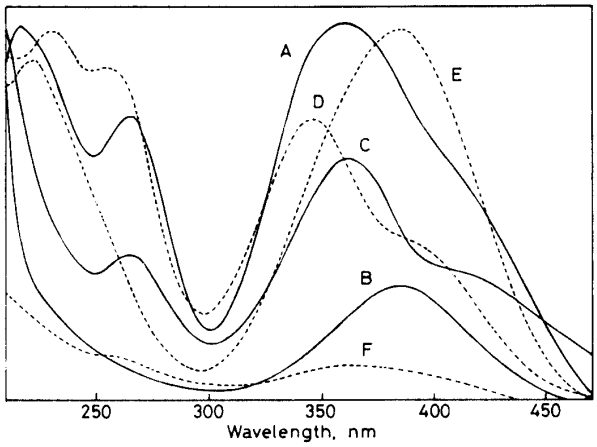

Fig. 8. UV and visible spectra of DNP-amine A – F, derived from the products in catalytic reduction of cycasin in acidic medium.

E were pale yellow needles, B was an oil, and F was a brown solid. The major products were C and D.

UV and visible spectra of DNP-A – F were as shown in Fig. 8. The absorption maxima at around 260 nm was attributed to the benzene ring, and that at 360 nm was known[11] to show a shift to the longer wavelength region according to the class of amines, 1ry, 2ry, and 3ry. Then, D was assumed to be 1ry amine, A, C, and F to be 2ry, and B and E to be 3ry.

IR spectra of A and B agreed with these assumptions. A showed the absorption of -NH- at 3300^{-1} and that of $-CH_3$ at 3000 – 2800. As for B, absorption of -NH- was not observed and that at 3030 – 2750 was characteristic.

DNP-C was identified as 2,4-dinitro-N-methylaniline from its IR and NMR spectra. The absorption of -NH- (3340) and $-CH_3$ (3000–2880) was observed in IR spectrum, which agreed with that of specimen synthesized from methylamine hydrochloride. NMR showed that δ 6.85 ppm (1H, doublet), 8.23 (1H, quartet), and 9.05 (1H, doublet) were assinged to protons of $=CH-$ of $C_{3,5,6}$ in the benzene ring, and 3.11 (3H, doublet) and 8.3 – 8.8 (1H, broad), to those of $-CH_3$ and -NH-, respectively.

DNP-D was identified as 2,4-dinitroaniline from its absorption of $-NH_2$ (3450, 3330) in IR and agreement with IR of synthesized specimen. The absorption due to -NH- in IR was not observed on DNP-E and F, and these results agreed with the assumption in UV and visible spectra that E was 3ry, but not with that F was 2ry.

Thus among the six produced amines, methylamine (C) and ammonia (D) were identified. Further elucidations on one 2ry amine, A, two 3ry, B and E, and one uncertain, F, were left.

4. Discussion

Chemical reduction of cycasin with stannous chloride was carried out in a strong acidic medium, and a stoichiometric production of methylamine, formaldehyde, ammonia, and glucose was observed[9]. Polarography of cycasin was studied on wide range of pH, and an electrode reaction, reduction of the azoxy group into hydrazo and hydrazo-radical, was assumed[10]. Cycasin was known to be unstable in an alkaline medium and degraded non-stoichiometrically even at room temperature into nitrogen gas, formic acid, sodium cyanide, methanol, and ammonia[9]. While in acid it was rather stable, and degraded, when

heated, into equimolar nitrogen gas, formaldehyde, and methanol[9].

In the reduction of cycasin with borohydride, pH of the medium elevated gradually to near 12 along with the progress of reaction. Compared with reaction in a buffered solution at pH 12 without the reducing agent, the reduction with borohydride was different, in its production of methylamine, from the simple degradation in an alkaline medium.

The saccharide detected in the reduction of cycasin was glucose or sorbitol, but a compound of glucose bound one carbon atom, such as methyl-glucoside, was not observed. A possible existence of free aglycone was disapproved from the UV spectrophotometry. It was deduced from these results that the reducing agent attacked first the azoxy group, degraded the aglycone into smaller fragments, and consequently liberated the sugar-moiety. The freed glucose was further reduced into sorbitol in the case of borohydride. The low recovery of glucose in the catalytic reduction was attributed to some losses due to adsorption on the catalyst-carrier, carbon.

The reduction products were, as in the reduction with stannous chloride, methylamine and ammonia, as mains, and several unidentified minors. The yield of formaldehyde was low in the final stage of reaction, and was assumed to be due to further reduction into methanol or to reactions with active amines produced during the reduction. A stoichiometric relationship of these products was not observed throughout the reduction in this paper. The reaction was assumed to proceed in a complex aspect accompanied with several minor unstable intermediates, for which some more sensitive and rapid assay-procedures would be necessary. Physiological effects of the reduction products of cycasin upon experimental animals are to be studied separately.

Summary

Chemical reduction of cycasin, the toxic glycoside of cycad, *Cycas revoluta* Thunb., was studied. The sugar component of cycasin was liberated as glucose in the hydrogenation with palladium carbon as a catalyst, and it was further reduced into sorbitol in the reduction with sodium borohydride.

Catalytic reduction of cycasin progressed far more rapidly in acidic medium than in neutral. The ninhydrin-positive products were derived into dinitrophenyl compounds to be identified.

Throughout the reduction by these three procedures, a reduced form of the aglycone of cycasin was not obtained, but was degraded into smaller fragments. As the major products, methylamine and ammonia were proved together with minor unidentified amines. Production of formaldehyde was also proved, though its yield was low and suggested a possibility of some side reactions.

Acknowledgment

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References

1) Grab, D. J. and Zedeck, M. S.: Organ-specific effects of the carcinogen methylazoxymethanol

related to metabolism by nicotinamide adenine dinucleotide-dependent dehydrogenases. *Cancer Res.*, **37**, 4182–4189 (1977)
2) Horwitz, W. ed.: Formaldehyde in solutions, cyanide method. *Official Methods of Analysis of A.O.A.C.* 12th ed., p. 119, Association of Official Analytical Chemists, Washington (1975)
3) Kobayashi, A. and Matsumoto, H.: Studies on methylazoxymethanol, the aglycone of cycasin. Isolation, biological, and chemical properties. *Arch. Biochem. Biophys.*, **110**, 373–380 (1965)
4) Kobayashi, A., Yamauchi, H. and Murozono, T.: Analysis of the residual cycasin in food and other materials using cycad. *Bull. Fac. Agr. Kagoshima Univ.*, **24**, 165–170 (1974)
5) Laqueur, G. L., Mickelsen, O., Whiting, M. G. and Kurland, L. T.: Carcinogenic properties of nuts from *Cycas circinalis* L. indigenous in Guam, *J. Natl. Cancer Inst.*, **31**, 919 (1963)
6) Nash, T.: Colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem. J.*, **55**, 416–421 (1953)
7) Niederwieser, A.: Thin-layer chromatography of amino acids and derivatives. in Hirs, C. H. W. and Timasheff, S. N. eds. *Methods in Enzymology* **XXV**, p. 80, 82. Acad. Press, New York and London (1972)
8) Nippon Kagaku-kai (Japan Chem. Soc.): *Jikken Kagaku Koza* (*Handbooks of Experimental Chemistry*) **17(2)**, p. 380. Maruzen, Tokyo (1956)
9) Nishida, K., Kobayashi, A. and Nagahama, T.: Studies on cycasin, a toxic glycoside, of *Cycas revoluta* Thunb. I. Isolation and the structure of cycasin. *Bull. Agr. Chem. Soc. Japan*, **19**, 77–84 (1955)
10) Nishida, K., Kobayashi, A. and Nagahama, T.: Studies on cycasin, a toxic glycoside, of *Cycas revoluta* Thunb. VI. Polarography of cycasin. *Bull. Agr. Chem. Soc. Japan*, **20**, 122–126 (1956)
11) Satake, K. and Okuyama, F.: Colorimetry in biochemistry, amino acids. *Kagaku no Ryoiki* (*J. Japan Chemistry*), extra number **34**, p. 63. Nankodo, Tokyo (1958)
12) Yang, M. G., Kobayashi, A. and Mickelsen, O.: Bibliography of cycad research. *Fed. Proc.*, **31**, 1543–1546 (1972)