Journal of Chromatography, 117 (1976) 355–363

CHROM. 8742

FLUORESCENCE OF TRYPTOPHAN-CONTAINING PEPTIDES ON PAPER OR SILICA GEL AFTER TREATMENT WITH FORMALDEHYDE, FORMALDEHYDE–OZONE OR FORMALDEHYDE–HYDROCHLORIC ACID

L.-I. LARSSON, F. SUNDLER and R. HÅKANSON
Departments of Histology and Pharmacology, University of Lund, Lund (Sweden)
(First received July 28th, 1975; revised manuscript received September 12th, 1975)

SUMMARY

Sensitive and specific procedures for the chromatographic detection of tryptophan and tryptophan-containing peptides are described. Formaldehyde gas induces strong and characteristic fluorescence from tryptophan and peptides with NH_2-terminal tryptophan residues on silica gel. On filter-paper, the detection of small amounts of these compounds requires the additional use of an oxidant, such as ozone. Treatment with formaldehyde–hydrochloric acid was used as a method for inducing fluorescence from tryptophan-containing peptides regardless of the position of the tryptophan residue in the peptide molecule. This reaction is useful for the chromatographic demonstration of small amounts of such peptides on both paper and silica gel. The spectral properties of the fluorophores of such tryptophan-containing peptides are distinctive and serve to distinguish them from all other known biogenic compounds that are capable of giving fluorescence with formaldehyde.

INTRODUCTION

Formaldehyde is a useful histochemical and chromatographic reagent for the detection of catecholamines, indoleamines and their immediate precursors[1,2]. The resulting fluorescence is characteristic of the different groups of fluorogenic compounds[1,2]. The chemical reactions that result in fluorophore formation have been investigated in detail and the concept is briefly as follows. The catecholamines and indoleamines first condense with formaldehyde in a Pictet–Spengler reaction, yielding tetrahydroisoquinolines and tetrahydro-β-carbolines, respectively. In a subsequent step, these derivatives are dehydrogenated to give intensely fluorescent dihydroisoquinolines and dihydro-β-carbolines (for details, see refs. 2–4).

From a theoretical point of view, peptides that have DOPA, 5-hydroxytryptophan or tryptophan residues should be able to react with formaldehyde to give condensation products analogous to those of the amines, provided that the fluorogenic amino acid is NH_2-terminal. This assumption has been confirmed in model experiments[5,6]. Neither DOPA nor 5-hydroxytryptophan is a common constituent of animal

peptides or proteins. Our interest was therefore focused on tryptophan-containing peptides. This paper describes the specificity and sensitivity of formaldehyde-containing reagents for the chromatographic detection of tryptophan-containing peptides. One of these modifications (treatment with formaldehyde–hydrochloric acid) permitted the detection of tryptophan regardless of its position in the peptide[7].

EXPERIMENTAL

Chemicals

L-Tyrosine and L-histidine were obtained from E. Merck (Darmstadt, G.F.R.). L-Tryptophan, indoleacetic acid, L-tryptophyl-L-phenylalanine, L-tryptophyl-L-glutamic acid, L-alanyl-L-histidine and L-glycyl-L-histidine were purchased from Sigma (St. Louis, Mo., U.S.A.). L-Tryptophyl-L-glycine, L-tryptophyl-L-alanine, L-tryptophyl-L-tyrosine and L-tryptophyl-L-tryptophan were obtained from Miles Laboratories (Elkhart, Ind., U.S.A.). L-Glycyl-L-tryptophan, L-phenylalanyl-L-tryptophan, L-prolyl-L-tryptophan, L-histidyl-L-alanine, L-histidyl-L-serine, L-histidyl-L-phenylalanine, L-histidyl-L-leucine, L-histidyl-L-lysine, L-histidyl-L-tyrosine, L-glycyl-L-histidyl-L-glycine, L-tyrosyl-L-alanine, L-tyrosyl-L-glutamic acid, L-tyrosyl-L-glycine, L-tyrosyl-L-tyrosine, L-phenylalanyl-L-tyrosine and L-valyl-L-tyrosyl-L-valine were obtained from Miles-Yeda (Kankakee, Ill., U.S.A.). L-Arginyl-L-tryptophyl-L-glycine carboxymethyl ester was a kind gift from Dr. L. Carlsson (AB Ferring, Malmö, Sweden). Synthetic tetragastrin (the COOH-terminal tetrapeptide of gastrin, L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide) was generously provided by Dr. H. J. Petersen (Leo Pharmaceutical Products, Copenhagen, Denmark). Peptavlon (N-*tert.*-butoxycarbonyl-β-L-alanyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine-amide) was purchased from ICI (Alderley Park, Great Britain) and EAE ("experimental allergic encephalitogenic peptide", L-phenylalanyl-L-seryl-L-tryptophyl-L-glycyl-L-alanyl-L-glutamyl-L-arginine) from Beckman (Palo Alto, Calif., U.S.A.). Porcine glucagon was kindly provided by Dr. H. Ege (Insulin Laboratory, Novo, Copenhagen, Denmark). Thyreoglobulin was obtained from Sigma.

Aqueous solutions of the compounds in various concentrations were spotted on to filter-papers or silica gel thin layers in volumes of 0.5–2.0 μl.

Materials

Whatman No. 1 filter-papers and silica gel thin layers were used. The filter-papers were washed three times with 0.1 *N* hydrochloric acid, ethanol and redistilled water, in that order, and dried at 40° before use. The thin layers were prepared by coating glass cover-slips (24 × 32 mm, for routine histology) with *ca.* 100 μm of Kieselgel H (Merck). The layer was applied as a slurry consisting of 20 g of silica gel suspended in 50 ml of glass-distilled water. The plates were dried at room temperature before use.

Detection reagents

The filter-papers and thin layers were treated by one of four different procedures.

(a) Formaldehyde gas. Formaldehyde condensation of the various compounds was induced by exposure to formaldehyde gas at 65–75° for 1 h (ref. 2) in a closed jar

(1000 ml) containing about 5 g of paraformaldehyde that had previously been equilibrated in air at about 50% relative humidity.

(b) Formaldehyde gas + ozone. Ozone was introduced into the reaction vessel by discharge from a Tesla coil for 10–15 min (ref. 5), and then formaldehyde condensation was carried out as in procedure *a*.

(c) Formaldehyde gas + hydrochloric acid. Treatment with formaldehyde was carried out as in procedure *a* except that concentrated hydrochloric acid (usually 0.05 ml) was present in the reaction vessel. Controls were run without formaldehyde.

(d) Procházka method[8]. The filter-papers and silica gel thin layers were sprayed with a mixture of formalin, 6 *N* hydrochloric acid and ethanol (1:2:1) and dried in an oven at 65–75° for 1 h (ref. 9).

The sensitivities of the detection reagents were evaluated by establishing the smallest amount of the compound that was visible upon examination under UV light (HBO 200 mercury lamp with a 3-mm UG 1 filter).

In one series of experiments, treatment with formaldehyde gas as in procedure *a* was followed by exposure to the fumes of 0.5 ml of concentrated hydrochloric acid in a 1000-ml jar for about 30 min at 60–70°.

Microspectrofluorometric analysis

The spectral properties of the fluorophores and their relative intensities were determined with a modified Leitz microspectrograph[2]. The exciting light was passed through an optical system that consisted entirely of quartz components. The thin layers were placed upside-down with the silica gel facing the quartz bright-field dry condenser. The filter paper or thin layer outside the fluorescent spots was used to obtain blank spectra. All spectra were corrected for blanks and instrument errors as described previously[2]. All values given for excitation and emission maxima are the means of at least four separate recordings. For measurement of fluorescence intensities, the monochromators were set at excitation 365 nm (silica gel) or 405 nm (filter-paper) and at the maximum emission value for each fluorophore. The intensities were measured with a precision galvanometer. Each value given is the mean of at least three separate recordings.

RESULTS

Sensitivity and specificity of formaldehyde, formaldehyde–ozone and formaldehyde–hydrochloric acid reagents

Histidine and tyrosine and all of the peptides that lacked tryptophan failed to give detectable fluorescence with any of the treatments. As previously reported[10], treatment with formaldehyde alone (procedure *a*) gave fairly strong fluorescence on silica gel with tryptophan and peptides with NH_2-terminal tryptophan. On filter-paper, the formaldehyde-induced fluorescence of these compounds was poor (Table I). Peptides with tryptophan in the COOH-terminal or intermediate position gave no formaldehyde-induced fluorescence on either silica gel or filter-paper. On filter-paper, treatment with formaldehyde–ozone (procedure *b*) induced strong fluorescence with tryptophan and peptides with NH_2-terminal tryptophan. Peptides with tryptophan in intermediate or COOH-terminal positions remained non-fluorescent. With these compounds on silica gel, treatment with formaldehyde–ozone gave no further en-

TABLE I

MINIMUM DETECTABLE AMOUNTS (μg) OF TRYPTOPHAN, INDOLEACETIC ACID AND TRYPTOPHAN-CONTAINING PEPTIDES

Volumes of 1 μl were applied. Results indicated by dashes (—) represent amounts greater than 1 μg.

Compound	*Filter-paper*			*Silica gel*		
	Formaldehyde	*Formaldehyde–ozone*	*Formaldehyde–HCl*	*Formaldehyde*	*Formaldehyde–ozone*	*Formaldehyde–HCl*
L-Tryptophan	0.3–1.0	0.03	0.03	0.03	0.1	0.03
Indoleacetic acid	—	—	0.03	—	—	0.03
L-Tryptophyl-L-alanine	0.3–1.0	0.03	0.03	0.03	0.1	0.03
L-Tryptophyl-L-glutamic acid	0.3–1.0	0.03	0.03	0.1	0.1	0.1
L-Tryptophyl-L-glycine	0.3–1.0	0.03	0.03	0.1	0.1	0.1
L-Tryptophyl-L-phenylalanine	0.3–1.0	0.03	0.03	0.1	0.1	0.1
L-Tryptophyl-L-tyrosine	0.3–1.0	0.03	0.03	0.1	0.1	0.1
L-Glycyl-L-tryptophan	—	—	0.03	—	—	0.1
L-Phenylalanyl-L-tryptophan	—	—	0.03	—	—	0.1
L-Prolyl-L-tryptophan	—	—	0.03	—	—	0.1
L-Arginyl-L-tryptophyl-L-glycine	—	—	0.1	—	—	0.3
Tetragastrin	—	0.1	0.3	0.1–0.3	0.3	0.3
Peptavlon	—	—	0.1–0.3	—	—	0.3
EAE	—	—	0.3–1.0	—	—	0.3
Glucagon	—	—	—*	—	—	—
Thyreoglobulin	—	—	—	—	—	—

* With 10 μg of glucagon, weak fluorescence was observed.

hancement of the fluorescence induced by formaldehyde alone. Treatment with formaldehyde–hydrochloric acid (procedure *c* or *d*) gave very intense fluorescence with tryptophan and indoleacetic acid (see also ref. 8 and 11) as well as with all tryptophan-containing peptides (except glucagon), regardless of the position of tryptophan in the molecule, on both filter-paper and silica gel. On a weight basis, the tryptophan-containing polypeptide glucagon gave very weak fluorescence and thyroglobulin, which is rich in tryptophan, gave none. Procedures *c* and *d* had similar sensitivities and the results are therefore presented under the same heading in Table I. Treatment with hydrochloric acid alone induced fluorescence (albeit weak) from tryptophan, indoleacetic acid and tryptophan-containing peptides. The intensity was about 30% of that produced by combined formaldehyde–hydrochloric acid. The spectral properties were indistinguishable from those of the formaldehyde–hydrochloric acid-induced fluorescence.

Effect of hydrochloric acid concentration and time of reaction

It was to be expected that the fluorescence yield in the formaldehyde condensation would be affected by the concentration of hydrochloric acid. The effect of varying the amount of hydrochloric acid was tested using tryptophylglycine, arginyltryptophylglycine, tetragastrin, Peptavlon and glycyltryptophan on filter-paper. The results are illustrated in Fig. 1a, which shows that as little as 0.05 ml of hydrochloric acid in the reaction vessel is sufficient. Under these circumstances, the reaction was completed in 45–60 min (Fig. 1b).

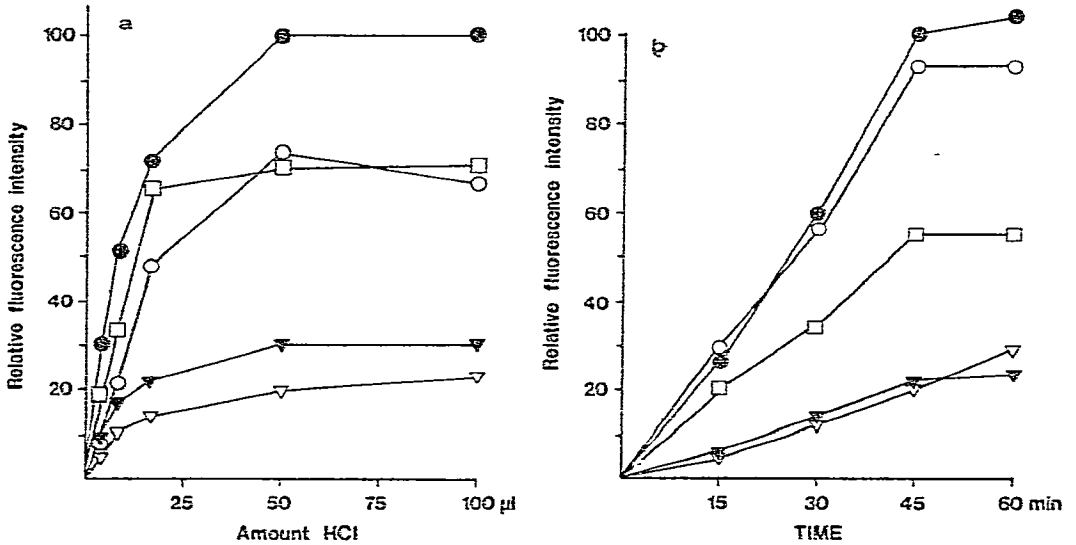

Fig. 1. Formaldehyde–hydrochloric acid-induced fluorescence of tryptophan-containing peptides on filter-paper. (a) Effect of varying the amount of hydrochloric acid on the fluorescence yield (expressed as relative fluorescence intensity); time of reaction 45 min. (b) Effect of varying the reaction time; 50 µl of hydrochloric acid in the reaction vessel. ○, Tryptophylglycine (0.3 µg); ●, arginyltryptophylglycine (1 µg); □, glycyltryptophan (0.3 µg); ▽, tetragastrin (1 µg); ▼, peptavlon (1 µg). The fluorescence intensity of the arginyltryptophylglycine fluorophore after a reaction time of 45 min is given as 100. Each value is the mean of four determinations.

TABLE II

SPECTRAL PROPERTIES (EXCITATION/EMISSION MAXIMA, nm) OF FORMALDEHYDE-INDUCED FLUORESCENCE OF TRYPTOPHAN, INDOLEACETIC ACID AND TRYPTOPHAN-CONTAINING PEPTIDES

If not otherwise stated, the concentration was 1 μg of each compound per spot. Parentheses denote minor peaks.

Compound	*Filter-paper*			*Silica gel*		
	Formaldehyde	*Formaldehyde–ozone*	*Formaldehyde–HCl**	*Formaldehyde*	*Formaldehyde–ozone*	*Formaldehyde–HCl**
L-Tryptophan	–	370/480–500	(320) 370, 450**/500–520	375/440–460	375/450	***/450–560
Indoleacetic acid	—	—	(320) 370, 450**/520–530	—	—	***/500–520
L-Tryptophyl-L-glutamic acid	—	370/500–520	(320) 370, 450**/500–520	385/490–510	385/495	***/460–550
L-Tryptophyl-L-phenylalanine	–	370/500	(320) 370, 450**/500–520	385/490–510	385/490	***/460–550
L-Tryptophyl-L-tyrosine	–	370/500–530	(320) 370, 450**/500–520	385/490–510	385/480	***/460–550
L-Glycyl-L-tryptophan	—	—	(320) 370, 450**/500–530	–	–	***/520–540
L-Phenylalanyl-L-tryptophan	—	–	(320) 370, 450**/500–530	—	–	***/520–540
L-Propyl-L-tryptophan	—	—	(320) 370, 450**/500–530	—	—	***/520–540
L-Arginyl-L-tryptophyl-L-glycine	—	—	(320) 370, 450**/500–530	—	–	***/520–540
Peptavlon	—	—	(320) 370, 450**/500–530	—	–	***/520–540

* The value of maximum emission varied with the concentration. The following amounts, applied in 1 μl, were tested: 0.03, 0.1, 0.3 and 1.0 μg.

** For peptides with NH_2-terminal tryptophan the peak at 370 nm is often dominating (especially at low concentrations), for the others the peak at 450 nm.

*** Excitation not measured because of high background fluorescence excited at >400 nm on the silica gel.

Strong fluorescence was also obtained from these peptides if the acidification was performed after exposure to formaldehyde, even when several days had passed between the formaldehyde treatment and the acidification. Fluorescence was induced from formaldehyde-treated tryptophan-containing peptides also at room temperature by exposure to the fumes of a few millilitres of concentrated hydrochloric acid for 5–10 min. The intensity of the formaldehyde–hydrochloric acid-induced fluorescence was not affected by subsequent exposure to the vapour of a few millilitres of concentrated ammonia solution for 5–15 min at room temperature.

Spectral characteristics of the fluorophores

The maxima of the excitation and emission spectra of the fluorophores formed with formaldehyde, formaldehyde–ozone or formaldehyde–hydrochloric acid are given in Table II and Fig. 2. The results obtained with the Procházka reagent (procedure *d*) were the same as those obtained with formaldehyde–hydrochloric acid (procedure *c*) and the results are therefore presented under the same heading in Table II. With formaldehyde–ozone, maximum excitation occurred at 370 nm for tryptophan and NH_2-terminal tryptophyl peptides. Characteristically, the predominant peak in the excitation spectrum for the formaldehyde–hydrochloric acid-induced fluorescence of all of the indoles tested was at 450 nm (Fig. 2, Table II). At low concentrations, however, tryptophan and peptides with NH_2-terminal tryptophan had a predominant peak at 370 nm with a shoulder at 450 nm. The value of the maximum emission was markedly concentration-dependent (Table II). Subsequent exposure to ammonia vapour (see above) did not affect the excitation maximum but induced a blue shift in the emission maximum of about 20–30 nm. As illustrated in Fig. 3, the concentration of the hydrochloric acid affected not only the intensity but also the spectral properties of the fluorescence.

DISCUSSION

Treatment with formaldehyde or formaldehyde–ozone gave fluorescence with

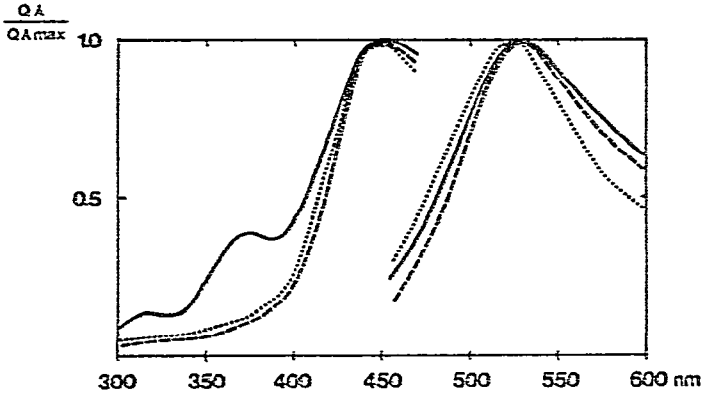

Fig. 2. Excitation (left) and emission (right) of the formaldehyde–hydrochloric acid-induced (procedure *c*) fluorescence of tryptophylglycine (———), glycyltryptophan (· · · · · ·) and arginyltryptophylglycine (— — —) on filter-paper; 1 μg of each compound per spot.

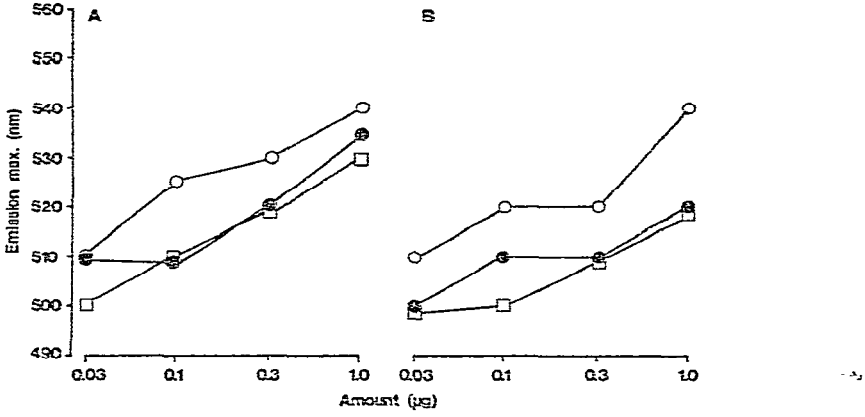

Fig. 3. Formaldehyde–hydrochloric acid-induced fluorescence of tryptophan-containing peptides on filter-paper. Concentration-dependent shift in the emission maxima of the fluorescence of tryptophylglycine (○), arginyltryptophylglycine (●) and glycyltryptophan (□). A, 0.05 ml of hydrochloric acid in the reaction vessel; B, 0.5 ml hydrochloric acid. Each value is the mean of four determinations.

tryptophan (but not with histidine or tyrosine) and with peptides with NH_2-terminal tryptophan. The results with the two model systems, filter-paper and silica gel, differed in that tryptophan and the peptides with NH_2-terminal tryptophan gave strong formaldehyde-induced fluorescence on silica gel but very weak fluorescence on filter-paper. Possibly the products are more easily oxidized on the silica gel[12]. This assumption is supported by the observation that formaldehyde induced strong fluorescence with the above compounds on filter-paper provided that the reaction took place in the presence of ozone. In neither of the two model systems did treatment with formaldehyde or formaldehyde–ozone induce more than weak fluorescence with peptides that had tryptophan in COOH-terminal or intermediary positions. Treatment with formaldehyde–hydrochloric acid (procedures *c* and *d*), however, induced very strong fluorescence with tryptophan, indoleacetic acid[11] and all peptides that contained tryptophan, regardless of its position in the peptide molecule. Tryptophan-containing proteins and polypeptides gave, on a weight basis, no (thyroglobulin) or weak (glucagon) fluorescence. Procedures *c* and *d* gave identical results.

The results clearly show that the tryptophan residue of the peptides is responsible for the fluorescence induced by formaldehyde–hydrochloric acid. Intense fluorescence also resulted from indoleacetic acid and from peptides with tryptophan in COOH-terminal or intermediary positions. Thus, a free tryptophan α-NH_2 group is not required. It is probable that the fluorophore is a derivative of the indole nucleus, conceivably a 2-substituted product[13], and that acid catalyzes its formation.

ACKNOWLEDGEMENT

This study was supported by grants from the Swedish Medical Research Council (04X-3764, 04X-4499).

REFERENCES

1 A. Björklund, B. Falck and R. Håkanson, *Anal. Biochem.*, 35 (1970) 264.
2 A. Björklund, B. Falck and Ch. Owman, in S. A. Berson (Editor), *Methods of Investigative and Diagnostic Endocrinology*, Vol. I, J. E. Rall and I. J. Kopin (Editors), *The Thyroid and Biogenic Amines*, North-Holland, Amsterdam, 1972, p. 318.
3 S. M. Hess and S. Udenfriend, *J. Pharmacol. Exp. Ther.*, 127 (1959) 175.
4 S. Udenfriend, *Fluorescence Assay in Biology and Medicine*, Academic Press, New York, 1962, p. 166.
5 R. Håkanson and F. Sundler, *J. Histochem. Cytochem.*, 19 (1971) 477.
6 R. Håkanson, A.-K. Sjöberg and F. Sundler, *Histochemie*, 28 (1971) 367.
7 L.-I. Larsson, F. Sundler and R. Håkanson, *J. Histochem. Cytochem.*, 23 (1975) in press.
8 I. M. Hais and K. Macek, *Handbuch der Papierchromatographie*, Band 1, Fischer Verlag, Jena, 1958, p. 588.
9 A. Björklund, B. Falck and R. Håkanson, *J. Chromatogr.*, 47 (1970) 530.
10 L. Edvinsson, R. Håkanson and F. Sundler, *Anal. Biochem.*, 46 (1972) 473.
11 E. Stahl and H. Kaldewey, *Z. Physiol. Chem.*, 323 (1961) 182.
12 A. Björklund, B. Falck and R. Håkanson, *J. Chromatogr.*, 40 (1969) 186.
13 A. Previero, G. Prota and M.-A. Coletti-Previero, *Biochim. Biophys. Acta*, 285 (1972) 269.