

Standardization of Allergen Extracts with Appropriate Methods

The Combined Use of Skin Prick Testing and Radio-Allergosorbent Tests

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This paper presents recommendations for standardization of allergen extract by the combined use of skin prick tests and radioallergosorbent tests. These tests provide appropriate means for quantitation of the total allergenic activity of an extract. Advantages and limitations are discussed with reference to the literature.

Key words: allergen extracts; allergy diagnosis; standardization.

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All physicians working with allergen extracts must know the quality and activity of the material they use in order to ensure safety and precision in diagnostic and immunotherapeutic procedures. Allergen extracts should satisfy the same requirements as for other biological products and drugs (3). An essential requirement is adequate standardization. Allergen extracts should be standardized with respect to biological activity (1, 5, 6, 17, 18), and methods adequate for this particular purpose (Table 1) should satisfy certain criteria (Table 2).

This paper presents recommendations for standardization of allergen extracts by the combined use of skin prick tests (SPT) and radio-allergosorbent tests (RAST).

The biological activity of the extract is established by means of SPT. A histamine equivalent (1 HEP) in prick testing is used as reference for the reactions to the allergen extract. A 1 HEP potency means that an allergen extract having this biological activity elicits a prick test reaction in most individuals who are allergic to the material, and on average produces a reaction identical

to that obtained by a similar prick test using histamine hydrochloride (HCl) 1 mg/ml (2, 4, 5, 6). The concentration of an extract with this potency is at the same time denoted as 1,000 biological units (BU)/ml.

This represents the first step already recommended by the Scandinavian Society of Allergology (5, 6) in order to end the hitherto unacceptably haphazard situation (Fig. 1a). The biological quantitation system in question is directly expressive of the biological activity as such, and is easily understood by the clinician.

The unit used (BU/ml) has the same meaning for all allergens and may be used directly as a guideline for the selection of the optimal concentration to be used to express the total amount of biologically active allergens given during challenge tests and in a course of hyposensitization (4). The system satisfies the requirements stated (Table 1) with an acceptable, although not optimal reproducibility (Fig. 1b).

RAST-based allergen quantitation is more convenient and is reproducible within a smaller range than SPT (7, 10, 13, 14, 21, 32,

Table 1

Requirements for standardization of allergen extracts.
Methods are needed to :

- measure and express in an appropriate and meaningful way the total allergenic activity.
- secure uniformity with respect to activity of different batches of the same allergen extract, and of different extracts of the same allergen.
- secure uniform activity between extracts of different allergens with identical declarations.

Table 2

Method qualities needed in allergen extract standardization:

- immunological specificity
- sensitivity
- precision and reproducibility
- available standards and reference material
- applicability for extract manufacturers
- applicability for control authorities

33). RAST makes it possible to ensure that each new production batch has the same allergenic potency as the former one (Table 1a and Fig. 1c). The RAST quantitation system does not, however, give a meaningful expression of the biological activity (Table 1b) and a given RAST unit for one allergen preparation does not necessarily indicate a similar biological activity for another preparation (Table 1c). However, a combination of the two assay systems renders a relevant and feasible approach for standardization purposes (Fig. 1d), and used together they satisfy all the requirements stated in Table 1.

Practical procedure for allergen standardization

Skin prick testing (SPT) is performed with a short-bevelled NO. 17-22 disposable needle. A drop of each solution to be tested (in duplicate) is placed on the volar aspect of the forearm or on the back of the patient with not less than 2 cm between test sites. A puncture test is performed through the

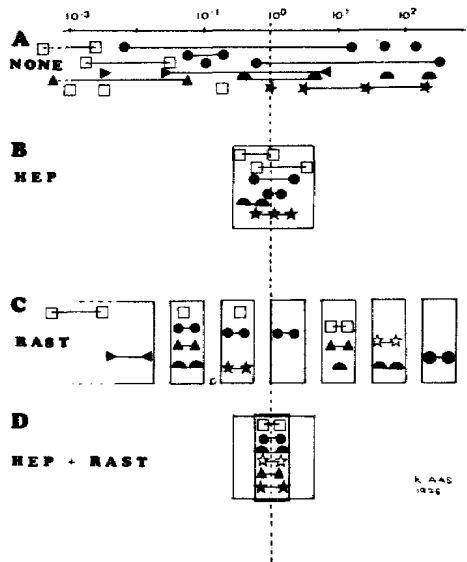


Fig. 1 Allergen extract standardization with respect to biological activity.

Vertical stippled line indicates activity in biological tests (SPT) corresponding to 1,000 BU/ml which means that the extract elicits SPT reactions equivalent to that of histamine 1 mg/ml (1 HEP potency) (1).

●, □, ▲, ●, ☆, ★, Different allergens
●—●, ●—●, Different batches of same allergen

droplet without causing bleeding. Histamine HCl 1 mg/ml (as histamine) is used as positive reference (5).

Efforts should first be made to establish a prick test technique which gives in about 10 patients a mean skin test reaction to the histamine reference solution $D + d \geq 8$ or $(D + d)/2 \geq 4$ mm with s.d. $\leq 20\%$. The reproducibility of the skin testing as performed by the individual physician or nurse should be optimized to the point where 10 SPTs with the histamine reference solution performed on one test individual give reactions which do not vary more than $\pm 20\%$ from the mean. Results are recorded after 15-20 min.

When the SPT technique is satisfactory, the allergen extract and histamine reference solution as well as the negative control are used in duplicate SPTs of as many allergic

individuals as is feasible. Statistically, 10–20 individuals are adequate for this purpose. The patients should have a positive history, a positive SPT to routine testing and a positive RAST (RAST class 2 or more) for the allergen in question, and should give a reaction ≥ 4 mm $(D+d)/2$ to the histamine reference solution.

They should have given their informed consent to being used for this purpose, and should not have received hyposensitization treatment with the allergen in question. In addition, at least 10 individuals who though allergic, but with negative history, negative SPT to routine testing, and negative RAST with the allergen in question, should be used as negative controls to SPT with the allergen at the highest concentrations applied in the testing.

The test extract is used in at least three, preferably five, different solutions. One of the dilutions should represent an estimated 1,000 BU/ml potency, and the others should be used in corresponding dilutions, e.g. 5×1 , 1 , 0.5 or 5×1 , 2×1 , 1 , 0.5 and 0.2 of the estimated 1,000 BU/ml concentration, or tenfold dilutions serially from the stock solution. The weal of the reaction to the SPT is outlined by a soft ballpoint pen, and the markings are transferred to mm² paper by means of tape. The largest diameter (D) and the diameter vertical to this (d) are measured in mm and the reaction is expressed as $(D + d)/2$. The reaction to allergen extract is expressed in percent of that of the histamine reference. The reactions obtained are plotted on a log-log paper, and the actual concentration of the extract corresponding to 1,000 BU/ml is extrapolated from the graph obtained.

The concentration of an extract A is adjusted to correspond to 1,000 BU/ml. The adjusted extract is subsequently used in SPT in at least 10 of the allergic individuals. If necessary, a final adjustment is carried out to give a concentration of 1,000 BU/ml or more (1). More potent extracts can be declared accordingly by indicating the dilution which corresponds to 1,000 BU/ml.

RAST

Serum is drawn from the individuals used in the SPT assay. Equal volumes of sera from at least 10 individuals with high RAST scores (RAST class 2–4) are pooled. The pooled serum is stored in suitable aliquots at -20°C .

Allergosorbents are prepared with extract A at an optimal concentration, or commercially available allergosorbents of high quality may be used. Extract A with known biological activity and new extracts (B, C) are then compared in RAST inhibition with the allergosorbent and the reference serum to establish the concentration of the new extract which corresponds to the 1,000 BU/ml concentration of extract A.

New extracts (B, C, etc.) are adjusted to a concentration corresponding to 1,000 BU/ml according to results of the RAST inhibition. They may, for a final control, be compared with the former extract A and the histamine reference by means of SPT in a few selected individuals from the former patient panel.

Rationale for using SPT with a histamine reference for assay of biological activity

In clinical practice the allergenic activity of an extract is demonstrated above all by the reactions obtained in skin testing. Skin testing is one of the most important and valuable diagnostic methods, and is available to every allergist in the world.

Histamine HCl (1 mg/ml*) is used by many as a common reference for the recording of SPT results (1, 5, 6). An allergen extract standardization making use of SPT has the particular advantage that when necessary the physician can himself check whether the extract has maintained the said biological activity. Until recently skin testing has been the method for bio-assay for all commercial allergen extracts quantitated with respect to allergenic activity.

Quantitation by means of skin testing in a

* The concentration most commonly used is 1 mg/ml or 10 mg/ml for reference, but we recommend 1 mg/ml for standardization purposes.

limited number of allergic individuals shows a good correlation between weal size and allergen concentration (for a limited range of concentration within a certain range of weal diameter), (8, 9, 10, 16, 23, 30). However, the deviations found in non-specific skin reactivity from patient to patient are so considerable that it is not reasonable to use a precise size expressed in mm as a standard measurement of the reaction (16). Histamine appears to be the most important mediator for the allergic weal response to SPT, and is thus a suitable reference as it takes into account the individual biochemical reactivity of each patient (8, 16, 27). A histamine releaser as for example Compound 48/80 may be even still more adequate for this purpose, but is less convenient for general use and has not been well enough investigated in this context.

Prick testing has several advantages over the intracutaneous test. SPT ensures maximum safety, is less troublesome for the patient, and has been shown to correlate better with the clinical history, provocation tests and specific IgE (17, 20, 21, 24, 25, 26).

Intracutaneous testing can be used as an alternative to SPT, particularly when allergen extracts are too weak to give sufficiently strong reactions in SPT, and a precise assay of allergenic activity (concentration) can be obtained by means of intracutaneous titration techniques. However, all commercial extracts should be available in concentrations sufficient for SPT. Extra weak extracts could be classified simply as containing "less than 1,000 BU/ml". The BU/ml terminology ought to be sufficient. Confusion could arise if terms for extract potency were related to more than one type of skin test method.

Specificity, sensitivity, precision and reproducibility of skin testing

A high degree of precision and reproducibility can be achieved by perfecting intracutaneous and prick testing techniques (8, 9, 12, 17, 23, 29, 30). Squire (23) reported

a close correlation between weal diameters and histamine concentration used in SPT, and this has been confirmed in subsequent studies. Voorhorst (29, 30) reported that the accuracy of intracutaneous skin test quantitation gave a maximum deviation of 100% above and 50% below the exact value for allergenic activity.

The main reason for not obtaining still better precision was thought to be that the inclination of the log dose response line for results of testing in the skin is rather small. Differences like these, however, are found between distinct allergens and also between different populations.

Scherr et al. (22) reported a reproducibility of intracutaneous titration testing which enabled different physicians to identify in a reproducible way allergenic activity corresponding to a 1 PNU concentration of an extract of ragweed pollen with the use of a defined histamine solution as a positive reference.

Frostad et al. (12) investigated the reproducibility of skin testing with histamine and a standardized and well-characterized, purified timothy pollen preparation available as a stable lyophilized, easily soluble material. The reproducibility of 20 prick tests with one concentration in two different individuals was found to deviate less than 10% from the mean (Table 2).

Björkstén et al. (11) performed SPT with a number of allergen extracts and a rather high histamine concentration as positive reference (10 mg/ml), and reported a 21% variation coefficient. When SPTs were made with one allergen in two tenfold concentrations, the ratio of the weal diameters was found to be constant. The numeric value of this constant (c) was 1.58. From this observation, and using Björkstén's definition for the unit, the allergen concentration could be calculated ($x = \text{anti-log} (\log d - \log h) / \log c$ where c is the constant, x is the allergen concentration, and h is the diameter of the weal obtained with the histamine solution and d the diameter of the weal obtained with the unknown allergen concentration. With the

use of SPT, Belin et al. (10) calculated the allergen concentration with a confidence interval of 0.5–2.0 using 15 individuals allergic to the material in question.

Frostad, Grimmer and coworkers (12, 15) also studied the usefulness of SPT and intracutaneous testing in assessing the allergen concentration. Skin tests were performed with a purified timothy pollen allergen and the 1 mg/ml histamine reference in individuals with clinical allergy and positive RAST, as well as skin reactivity to timothy pollen. Three tenfold concentrations were used in SPT and four tenfold concentrations in intracutaneous testing. The authors reported that with SPT the 1,000 BU/ml concentration could be found with a confidence interval well between 500 BU/ml and 2,000 BU/ml, often between 700 and 1,400 BU/ml. From available data it does appear that the 1,000 BU/ml concentration can be established against a reference preparation well within the –50% to +100% range with prick testing in 15–20 unselected individuals allergic to the material.

With intracutaneous titration a 10 BU/ml quality can be established well within a similar range, as postulated by Voorhorst (30).

Rationale for using RAST

Bio-assay for allergen standardization by means of SPT is informative, but has some disadvantages. The need for a relatively large patient panel makes standardization cumbersome. The effort is worthwhile for the initial investigation and licensing of an allergen extract, but is too resource-demanding for batch-to-batch controls. However this becomes easier as modern knowledge and technology allow the production of larger batches, enabling activity testing to be carried out less frequently than before. Another disadvantage is the extreme care that is necessary to secure reproducibility and precision in standardization with SPT, and even then the confidence

interval will not be better than 0.5 to 2.0. A standardization which allows so much variation is not ideal although an important step forward. The bio-assay should be combined with *in vitro* methods which are more precise and feasible than SPT. Manufacturers need relevant *in vitro* methods as guidance in extract production, especially for batch-to-batch controls.

In vitro methods which are relevant for quantitation of allergenic activity are scarce. Major allergens that are well characterized may be quantitated by means of different modifications of immunodiffusion and for a very restricted number of allergens at the present time. Quantitation can also be achieved by means of histamine release from sensitized human leucocytes, but this technique is more cumbersome and is affected by more variable factors than RAST.

For *in vitro* methods to be acceptable for allergen extract standardization, their correlation with the biological reactions in man must be established. A number of studies report a good correlation between the results of skin tests and RAST (5, 7, 10, 13, 14, 19, 21, 32, 33). It is well established that RAST is relevant for standardization purposes and that it is specific, precise and reproducible (Table 1). For allergen activity assays, correlation coefficients of 0.998 with b 1.072 for comparison between RAST and RAST-inhibition as described by Yman (10, 19, 32), indicate that both methods are equally useful for standardization purposes.

Gleich et al. (13, 14), Yunginger et al. (33), and Noster et al. (19) compared intradermal skin test end-point titration and RAST-based techniques. The rank order correlation coefficients in the first three studies were highly significant (*Ambrosia eliator*, *Poa pratense* and *Alternaria* allergens), whilst in the Noster study a correlation coefficient of 0.91 was obtained. Using SPT and RAST-inhibition with *Betula verrucosa* allergens, Belin et al. (10) obtained a correlation coefficient of 0.98, and Grimmer et al. (12, 15) found a similarly close correlation between results of SPT and RAST inhibition for timothy pollen.

The above conclusions are valid, however, only when the allergosorbents are made from reliable material, which means allergen extracts of high quality. Conditions to be satisfied for a high degree of reproducibility and specificity in the RAST-system, are:

- The production and use of uniform discs.
- The use of adequate sera and serum dilutions.
- Precision in laboratory work
- The use of allergen extracts of the highest quality available.

It is essential to select patients and serum donors critically. Individual patients with anomalous reactions may cause confusion. The use of too few sera or a pooled serum dominated by antibodies to minor allergens may result in too much attention being paid to minor allergenic components. Furthermore, one should avoid sera containing non-reacting allergen binding or "blocking" antibodies.

RAST has considerable advantages over SPT: one is relatively independent of patient panels, the availability of standardized reagents, its convenience and capacity.

This kind of standardization is feasible for all common allergens most frequently used in biological testing and hyposensitization.

Manufacturers who cannot have their extracts quantitated by means of SPT can compare their extracts in RAST-inhibition with allergen extracts which have also been fully quantitated by SPT and RAST in combination. This should be made available for reference by an agreement between manufacturers or international reference systems. Such extracts could then be used as reference in RAST-inhibition as outlined here, provided that an adequate patient serum pool is available.

It should be realized that skin testing and RAST in their present form measure the sum of allergenic activity and not individual allergenic components. This means that with the use of patients and patient-serum reacting for example with both housedust mite and dog epithelium allergens, these

tests cannot discriminate between two housedust extract batches, the one being dominated by mite allergens, and the other by dog epithelium allergens, respectively. Qualitative or quantitative differences as to presence and distribution of different allergenic components within a total activity cannot be expressed unless more sophisticated techniques and combined methods are used. Such methods are available for internal control purposes in some laboratories (28, 31). The quantitative immunoelectrophoretic methods and crossed radioimmuno-electrophoresis seem to give the most discriminating information about the individual components in the extracts. These methods are being investigated in a Danish Allergen Standardization study, where the results of these methods are compared with those of SPT and RAST inhibition.

However, at present they can only serve as internal controls for the manufacturers, since the demonstration by these methods of a few extra components, or lack of such, in an extract can have no practical results before the purified components in question can be evaluated per se, and subtracted or added specifically. The same applies to the use of isoelectrofocusing with or without RAST of isolated fractions; this may be used similarly to crossed immunoelectrophoresis and crossed radioimmuno-electrophoresis, although inferior to the latter. RAST-inhibition can also be used if the major allergens are available for this purpose. It appears that these methods are suitable to control that all major allergens are represented in an allergen extract, unless declared otherwise.

At present and for some time to come, a combination assay of the total allergenic activity, by combining skin testing and RAST, would seem to be the first feasible approach towards relevant allergen extract standardization, and should suffice for clinical purposes. If this combined method was introduced and used, it would mean a remarkable improvement for allergy practice.

One alternative for allergen extract standardization is the production of large quantities of extracts from each allergen source and the use of selected extracts as reference on an international scale. However, this would also depend on assessment and measurement of the extract's total allergenic activity as recommended above.

Furthermore, reference extracts must be of exceptionally high quality. With the allergen reference system described, using SPT and RAST-inhibition, it would be no problem to correlate other extract productions with a given reference extract. The argument on ethical grounds against bio-assay for allergen standardization neglects the fact that many of the extracts now produced and distributed may be completely valueless or even dangerous if the biological activity of the extract is not taken into consideration. The lack of relevant standardization of allergen extracts endangers the patient, causes false diagnoses in allergy and is probably responsible for many therapeutic failures. It appears less acceptable and more unethical to inject a patient with a number of extracts of unknown quality rather than to add a small series of prick tests for allergen assay purposes to the routine SPT usually performed for diagnostic purposes. In our departments we encounter no difficulties or problems in this respect when this is done with the patient's informed consent.

REFERENCES

1. Aas, K.: Clinical and experimental aspects of standardization and purification of allergen. *Int. Arch. Allergy* 49, 44-54, 1975.
2. Aas, K.: Standardization of allergen extracts. *Dev. Biol. Stand.* 29, 341-349, 1975.
3. Aas, K.: Perspectives in allergen standardization: suggested starting requirements. *Allergy and Clinical Immunology Proceedings of IXth International Congress of Allergology. Excerpta Medica* pp. 199-202, 1977. *International Congress Series* No. 414.
4. Aas, K.: Allergenekstrakter. Nye prinsipper og tidsmessige produkter. *Tidsskr. Nor. Lægeforen.* 97, 1809-1812, 1977.
5. Aas, K. & Belin, L.: Standardization of diagnostic work in allergy. *Acta Allergol.* 27, 439-468, 1972.
6. Aas, K. & Belin, L.: Suggestions for biologic qualitative testing and standardization of allergen extracts. *Acta Allergol.* 29, 238-240, 1974.
7. Arbesman, C. E., Wypych, J. I. & Reisman, R. E.: Evaluation of RAST inhibition as a method for standardization of ragweed pollen extracts. *Int. Arch. Allergy* 53, 310-318, 1977.
8. Becker, E. L. & Rappaport, B. Z.: Quantitative studies in skin testing. II. The form of the dose-response curve utilizing a quantitative response. *J. Allergy* 19, 317-328, 1948.
9. Berlin, L.: Immunological analyses of birch pollen antigens, with special reference to the allergenic components. *Int. Arch. Allergy* 46, 300-322, 1972.
10. Belin, L., Lanner, A. & Schröder, H.: Measurement of the relative potency of *Betula verrucosa* pollen extracts by prick test and by RAST-based methods. In manuscript, 1977.
11. Bjørksten, F.: Personal communication, 1977.
12. Frostad, A. B., Bolle, R., Grimmer, Ø. & Aas, K.: A new well-characterized, purified allergen preparation from timothy pollen. II. Allergenic in vivo and in vitro properties. *Int. Arch. Allergy* 55, 35-40, 1977.
13. Gleich, G. J., Larson, J. B., Jones, R. T. & Baer, H.: Measurement of the potency of allergy extracts by their inhibitory capacities in the radioallergosorbent tests. *J. Allergy Clin. Immunol.* 53, 158-169, 1974.
14. Gleich, G. J., Leiferman, K. M., Jones, R. T., Hooton, M. L. & Baer, H.: Analysis of the potency of extracts of June grass pollen by their inhibitory capacities in the radioallergosorbent tests. *J. Allergy Clin. Immunol.* 58, 31-38, 1976.
15. Grimmer, Ø., Frostad, A. B., Sandevig, L. & Aas, K.: A practical and statistical method for the precise biological standardization of allergen extracts. In manuscript.
16. Havnen, J.: Prick test and intracutaneous test. Variability in histamine tests between different individuals and in the same individual. *Acta Allergol.* 29, 225-227, 1974.
17. Imber, E. W.: Allergic skin testing: a clinical investigation. *J. Allergy Clin. Immunol.* 60, 47-55, 1977.
18. Logan, G. B.: Toward measurement of success: from diphtheria to allergy. The Bela Schick Memorial Lecture 1976. *Ann. Allergy* 28, 1-11, 1977.
19. Noster, U., Schulz, K. H. & Schröder, H.: Assay of allergen extract potency. A comparison between skin test end point titration and RAST-based methods. (Prague 1977). In manuscript.
20. Pepys, J.: Skin tests in diagnosis. Clinical aspects of immunology, 2nd edn. p. 192. Gell and Coombs, Blackwell Scientific Publications, Oxford, 1968.
21. Pepys, J., Tangen, O., Perkins, F. T. et al.: Ex-

- amination of cocksfoot pollen (*Dactylis glomerata*) extracts by skin prick test in man, RAST-based allergen assay and protein content. *Dev. Biol. Stand.* 29, 284-292, 1975.
22. Scherr, M. S., Grater, W. C., Baer, H., Center, J. G., Hale, R. & Berman, B. A.: Report of the committee on standardization. I. A method of evaluating skin test response. *Ann. Allergy* 20, 30-34, 1971.
23. Squire, J. R.: The relationship between horse-dandruff and horse-serum antigens in asthma. *Clin. Sci.* 9, 127-150, 1960.
24. Stenius, B.: Skin and provocation tests with *Dermatophagoides pteronyssinus* in allergic rhinitis. Comparison of prick and intracutaneous skin test methods and correlation with specific IgE. *Acta Allergol.* 28, 81-100, 1973.
25. Stenius, B., Wide, L., Seymour, W. M., Holford-Strevens, V. & Pepys, J.: Clinical significance of specific IgE to common allergens. *Clin. Allergy* 1, 37-55, 1971.
26. Stenius, B. & Wide, L.: Prick, scratch, intracutaneous tests and specific IgE in the serum. *Acta Allergol.* 29, 227-228, 1974.
27. Swain, H. H. & Becker, E. L.: Quantitative studies in skin testing. V. The whealing reactions of histamine and ragweed pollen extract. *J. Allergy* 23, 441-451, 1952.
28. Varga, J. M. & Ceska, M.: Characterization of allergen extracts by gel isoelectrofocusing and radioimmunosorbent allergen assay. *J. Allergy Clin. Immunol.* 49, 274-284, 1972.
30. Voorhorst, R. & van Krieken, H.: Atopic skin test reevaluated. II. Variability in results of skin testing done in octuplicate. *Ann. Allergy* 31, 195-204, 1973.
29. Voorhorst, R.: Standardization of allergens by an improved skin-testing technique. *Dev. Biol. Stand.* 29, 352-361, 1975.
31. Weeke, B. & Löwenstein, H.: Allergens identified in crossed radioimmuno-electrophoresis. *Scand. J. Immunol., Suppl.* 1, 149-153, 1973.
32. Yman, L., Ponterius, G. & Brandt, R.: RAST-based allergen assay methods. *Dev. Biol. Stand.* 29, 151-165, 1975.
33. Yuninger, J. W., Jones, R. T. & Gleich, G. L.: Studies on *Alternaria* extracts by the direct RAST and by RAST inhibition. *J. Allergy Clin. Immunol.* 58, 405-413, 1976.

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