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Kinetics of allergen release from house dust mite *Dermatophagoides pteronyssinus*

W. Geissler, Ph.D., H. J. Maasch, Ph.D., G. Winter, Chem. Eng., and R. Wahl, Ph.D. Reinbek, Federal Republic of Germany

Purified bodies of Dermatophagoides pteronyssinus were extracted over different extraction times. The release kinetics of proteins, carbohydrates, and allergens were assessed by different methods. Additional attempts have been made to elucidate the extraction process also on a molecular basis. For this purpose the extracts were analyzed by isoelectric focusing, SDS gel electrophoresis, crossed radioimmunoelectrophoresis, and an immunoprint technique. Carbohydrates, proteins, and allergens were released immediately from the mite bodies but demonstrated different release kinetics. The release of carbohydrates and proteins was linear up to 48 hours but demonstrated different slopes. The major allergen P₁ demonstrated steady release up to 48 hours, whereas the overall allergenic activity as determined by RAST inhibition demonstrated typical saturation kinetics reaching a plateau after 4 to 5 hours of extraction. It was demonstrated by parallel-line assay of the RAST-inhibition curves that the allergenic determinants remained unchanged up to 48 hours. Different electrophoretic methods demonstrated that all relevant proteins and allergens were released immediately from the mite bodies. The electrophoretic patterns changed only slightly up to 4 hours. Prolonged extraction times up to 48 hours did not affect the patterns. This makes a loss of proteins or allergens by degradation unlikely. (J ALLERGY CLIN IMMUNOL 77:24-31, 1986.)

The importance of the mite species *Dermatophagoides pteronyssinus* as a potent source of allergens in house dust is well established since the fundamental work of Voorhorst et al.¹ Great advances in the development of immunoelectrophoretic techniques for characterizing allergens² made it possible to elucidate physicochemical and allergenic characteristics of mite antigens. In addition, the application of modern pro-

Abbreviations used

C ₅₀ :	50% RAST inhibition
CIE:	Crossed immunoelectrophoresis
CRIE:	Crossed radioimmunoelectrophoresis
IEF:	Isoelectric focusing
IP:	Isoelectric point
kD:	Kilo Dalton
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
RI:	RAST inhibition

From Allergopharma Joachim Ganzer KG, Reinbek, Federal Republic of Germany.

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Reprint requests: H. J. Maasch, Ph.D., Allergopharma Joachim Ganzer KG, P.O. Box 1109, D-2057 Reinbek, Federal Republic of Germany.

tein separation techniques has led to an increased knowledge about the nature of major allergenic components in mite extracts.³⁻⁵

There appear to be great differences in the protein composition of mite allergen extracts depending on the raw material used in extraction.⁶⁻⁸ Furthermore, there is some dissent about extraction conditions,^{3, 9} especially with respect to extraction times. Tovey et al.¹⁰ reported the rapid release of the major allergen P₁ from whole mite cultures. After 3 minutes about 80% of the total P₁ content was released into the supernatant. In preliminary studies with purified mite bodies, we found different release kinetics.¹¹ Our data obtained by RAST inhibition suggested a continuous release of allergens from mite bodies up to 48 hours. In this study we attempted to elucidate the effect of different extraction times on the composition of *D. pteronyssinus* extracts in more detail.

By applying several techniques available for the analysis of allergens, we studied the effects of extraction times on allergen release, allergen patterns of the extracts, and their stability.

MATERIAL AND METHODS

Source of *D. pteronyssinus*

The raw material was purchased from Commonwealth Serum Laboratories (Melbourne, Australia). Optical control demonstrated 97% of the material to be whole mite bodies, fragments, and larvae.

Preparation of extracts

Three extraction series were run independently. One percent weight per volume extracts were prepared by stirring the raw material at 4° C in Coca's solution without phenol. The extraction times were 5, 10, and 20 minutes, 1, 5, 8, 16, 24, and 48 hours. Immediately after the extraction the samples were passed through cellulose acetate filters (pore size 0.2 µm) (Sartorius, Göttingen, Federal Republic of Germany), freeze-dried, and stored at -20° C.

A further extraction series was run with slightly modified extraction times (i.e., 5 and 20 minutes, 1, 4, 13, and 48 hours). These extracts were stored at -20° pending electrophoretic analysis.

In order to minimize possible distortions of the protein patterns by salt precipitation, the extracts were dialyzed before IEF and SDS-PAGE.

Determination of protein and carbohydrates

Protein concentrations were determined by a modified Lowry procedure.¹² A calibrated solution of human serum albumin (Behringwerke; Marburg, Federal Republic of Germany) served as standard. Carbohydrates were determined by the Anthrone method¹³ with glucose as standard.

IEF

IEF was performed on PAG plates (LKB, Munich, Federal Republic of Germany) according to the manufacturer's manual. Dialyzed samples of 20 µl containing 107 µg of protein were applied to the gels.

SDS-PAGE

A polyacrylamide gradient of 3% to 22.5% containing 0.1% of SDS was used. The gel was calibrated with marker proteins from Pharmacia (Freiburg, Federal Republic of Germany). From each extract 25 µg of protein was applied to the gel. Finally, the gel was stained with Coomassie brilliant blue R 250.

CIE/CRIE

CIE was performed as described by Løwenstein² with 10 µg of protein of each sample, and 60 µl of a rabbit anti-*D. pteronyssinus* serum (ALK, Copenhagen, Denmark) per milliliter of separation gel was used. Separation in the first dimension was for 35 minutes at 8 V/cm and 15° C, and in the second dimension for 17 hours at 2 V/cm. CIE plates were stained with Coomassie brilliant blue R 250.

The method of Weeke and Løwenstein¹⁴ was used for CRIE with the following modifications: each CIE plate was incubated with 12 ml of a serum pool (1:24) collected from patients allergic to *D. pteronyssinus*. As a tracer for bound IgE, we used 500,000 cpm of ¹²⁵I-labeled anti-IgE (Pharmacia) in 12 ml of buffer. Each incubation was run overnight with gentle horizontal shaking. From each CRIE plate three autoradiographs were produced by exposing Kodak X-Omat AR films (Eastman Kodak Co., Rochester, N.Y.) at -20° C for 4, 7, and 14 days.

IEF immunoprint

A modification of the capillary blot method described by Peltre et al.^{15, 16} was used.

From each sample 40 µg of protein was focused at 10° C for 3 hours on a 0.8% agarose gel (agarose IEF, Pharmacia) containing 10% D-Sorbitol and 2% Servalyt 3-10 (Serva, Heidelberg, Federal Republic of Germany).

The power supply was set to 4 W/1000 V. Finally, nitrocellulose paper (Trans-blot, Bio-Rad, Munich, Federal Republic of Germany) was applied onto the surface of the gel, and the focused proteins were transferred by pressing for 30 minutes by means of overlaying paper towels. Surplus binding sites on the print were blocked by incubation with a 3% solution of bovine serum albumin for 10 minutes. The print was incubated overnight with 5 ml of a serum pool (1:5) of mite-allergic patients in a rotating measuring flask.

After three washing steps with 10 ml of 0.1 mol/L NaCl containing 1% Tween 20, the print was incubated in 5 ml of diluted ¹²⁵I-anti-IgE containing 500,000 cpm. Finally, the print was washed and dried. Autoradiographs were produced by exposing Kodak X-Omat AR films at -20° C for 3 and 14 days.

RI

Allergen paper disks were prepared as described by Ceska et al.¹⁷ from an internal reference preparation. RI was performed as described by Wahl et al.¹⁸ A pool of sera from 16 mite-allergic patients with RAST class 4 served as specific IgE source; ¹²⁵I-anti-IgE was from Pharmacia. The C₅₀ values and parallel-line assays were computed according to

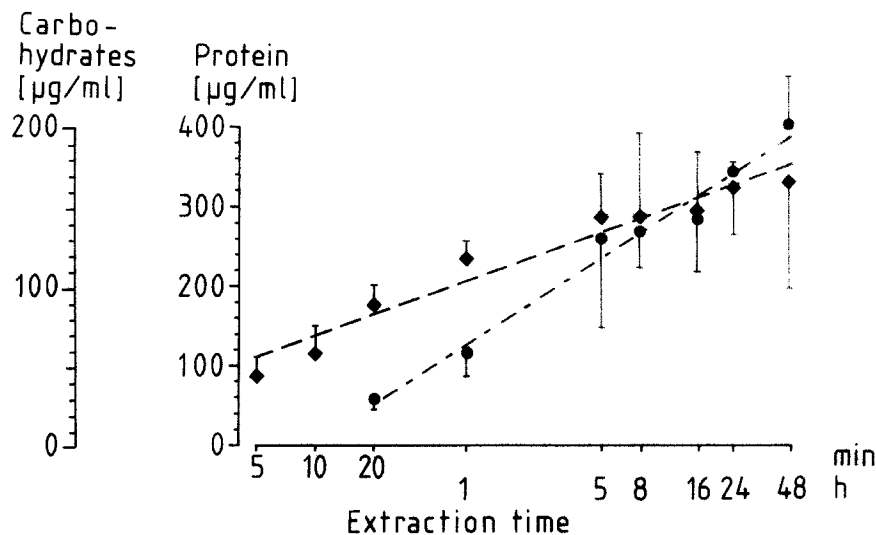


FIG. 1. Time-dependent release of proteins and carbohydrates from purified mite bodies. For details see Material and Methods. The values are means of four independent extraction series. Protein concentrations of the 5- and 10-minute extracts were 25 and 32 µg/ml. The correlation coefficient for the linear regression was calculated to 0.991 for protein (●—●) and 0.971 for carbohydrates (◆—◆).

a program suggested in a technical bulletin of the U.S. Food and Drug Administration, Bureau of Biologics.¹⁹

Determination of the major mite allergen P₁

By applying equal amounts (30 µl) of freshly prepared extracts, rocket immunoelectrophoresis was performed in an agarose gel containing 0.57 µl/cm² of an anti-P₁ serum. Areas covered by the rockets were determined by microplanimetry and were expressed in percent of the maximum peak area obtained.

RESULTS

Physicochemical analysis of the extracts

The release of proteins and carbohydrates started immediately after the contact of mite bodies with extraction fluid (Fig. 1). The semilog plot of concentrations versus extraction times yielded a linear correlation for both materials (carbohydrates, $r = 0.97$; proteins, $r = 0.99$). About 65% of the soluble proteins and 73% of the carbohydrates were extracted within 5 hours. The pH of the extracts remained constant up to 5 hours (pH 8.5 to 8.6) and rose afterwards to a final value of pH 9.3.

Analysis by electrophoretic methods

After 5 minutes the extracts demonstrated typical patterns in IEF (Fig. 2). Ongoing extraction yielded only few changes in the patterns concerning the number of bands. Some minor changes occurred up to 4 hours in the staining intensities of single bands. This

indicated that the relative amounts of single proteins were shifting.

In contrast to IEF, the changes of the patterns were more pronounced in SDS-PAGE (Fig. 3). The prominent bands in the region of 10 to 100 kD were detectable already in the 5-minute extract. During ongoing extraction, a remarkable change of the pattern appeared in the high-molecular-weight region (>100 kD). Here some proteins became visible only after 1 hour of extraction, and the changes continued up to 48 hours. Three bands with molecular weights of 105, 155, and 174 kD became visible.

Most of the antigens in CIE could be detected after 5 minutes of extraction (Fig. 4). The ongoing extraction yielded a shift of the relative amounts of the antigens only; however, the total number of antigens remained stable throughout the observed extraction period. In contrast to SDS-PAGE, no late extracting antigens were found.

Fig. 5 demonstrates the 14-day autoradiographs of the CRIE plates. Except in staining intensities, there were no differences to the 3- and 7-day autoradiographs. The 5-minute extract demonstrated an almost complete allergen pattern with regard to the number of allergens. In total, nine allergens were detected. As in CIE, the CRIE patterns demonstrated changes in the relative amounts of the different allergens up to 4 hours. After this time the typical pattern was established, and it did not change for up to 48 hours.

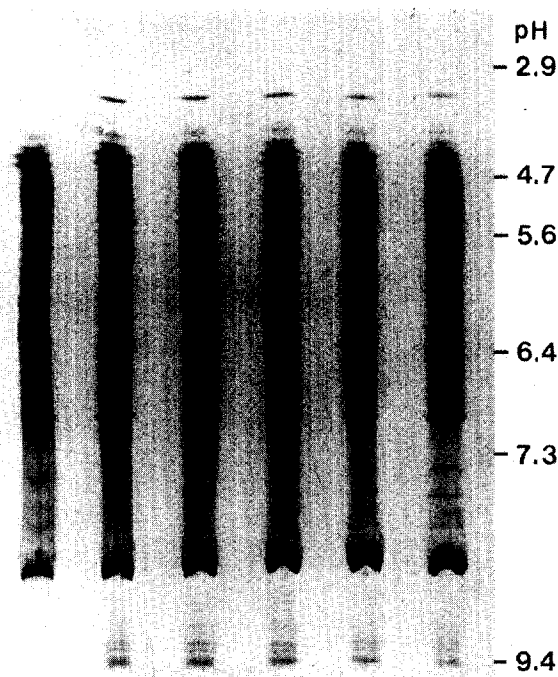


FIG. 2. IEF in polyacrylamide gel with extracts prepared from purified mite bodies with increasing extraction times. Calibration of the pH gradient was established by marker proteins (not shown). From each extract 107 μ g of protein was applied 2 cm near the cathode. Anode is on the top. Extraction times for the samples from left to right are 5 minutes, 20 minutes, 1, 4, 13, and 48 hours.

About 11 allergenic protein bands were detected by the IEF immunoprint technique (Fig. 6). They appeared in the range of pH 4.0 to pH 7.0. The bulk of the allergenic bands demonstrated IPs of pH 4.6 to 6.4. The two most prominent allergenic bands demonstrated IPs of 4.9 and 5.4, respectively.

Quantitative analysis of allergenic activity

The total allergenic activity of the extracts was determined by RI by comparing the extract volumes that elicited C_{50} (Fig. 7). The C_{50} values decreased up to 5 hours and remained constant afterwards up to 48 hours. By statistical analysis as suggested in the bulletin of the Food and Drug Administration, the RI curves within each independent extraction series were parallel.

The major allergen P_1 clearly demonstrated time-dependent extraction kinetics (Fig. 8). We found a continuous increase of P_1 concentration up to 48 hours of extraction. Furthermore, the P_1 concentrations of the different extracts correlated significantly ($r = 0.989$) with the protein concentrations of the respective samples (Fig. 9).

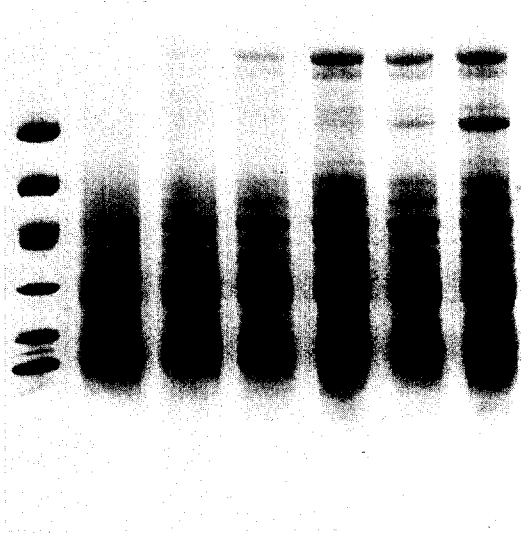


FIG. 3. Detection of *D. pteronyssinus* proteins by SDS gel electrophoresis; 25 μ g of protein was applied from each sample. Samples from left to right are molecular weight marker proteins, extracts prepared with 5 and 20 minutes, and 1, 4, 13, and 48 hours extraction times. Molecular weights of the marker proteins (from bottom to top) are 14.4, 20.1, 30, 43, 67, and 94 kD.

DISCUSSION

The crucial point in allergen extraction is to find a procedure that yields optimal allergenic activity. Furthermore, this procedure should elicit an extract that is representative for the raw material with respect to its allergen composition. The eventually chosen extraction times will be a compromise between a possible proteolytic degradation and maximum yield of allergenic activity.

It was the aim of our study to establish these conditions for allergen extracts from purified bodies of *D. pteronyssinus*. Furthermore, we wanted to study the possible sequential release of different allergens from whole mite bodies that could influence the quality of the extracts. By combining several of the available in vitro methods for the analysis of allergen extracts, we monitored the extracts prepared by different extraction times.

By straightforward methods like carbohydrate and protein measurements, we could demonstrate on a semilog plot a linear correlation of concentrations versus extraction times; however, there appears to be a difference in the release kinetics of both substances, e.g., the carbohydrate extraction is linear throughout the tested period, whereas there is a lag phase for protein in the beginning. A possible explanation might be that mite bodies need a certain time for maceration before proteins can be released. Nevertheless, sub-

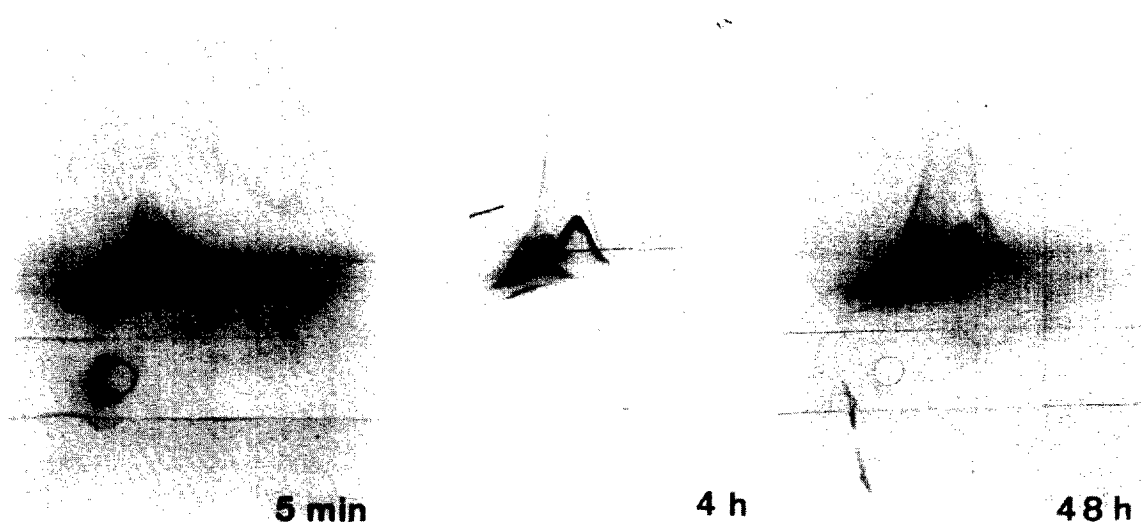


FIG. 4. CIE patterns of extracts from purified mite bodies obtained after varying extraction times. For details see Material and Methods. Before staining with Coomassie brilliant blue, CIE plates were used for performing CRIE. Only three typical extracts are demonstrated.

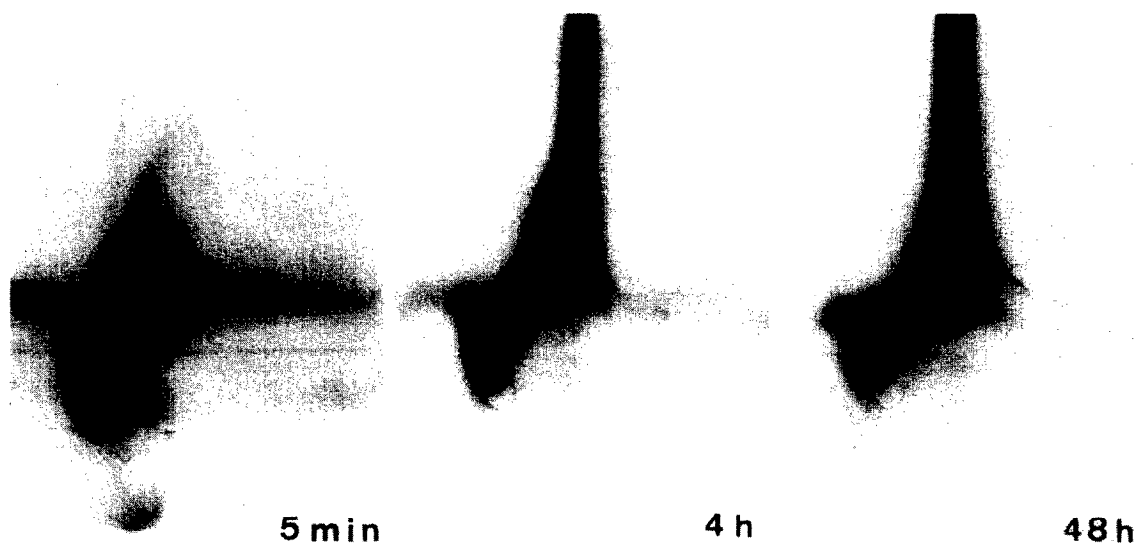


FIG. 5. Analysis of allergen composition by CRIE of extracts prepared from purified mite bodies with increasing extraction times. For the corresponding CIEs see Fig. 4. Autoradiography was performed at -20°C for 14 days on Kodak X-OMAT-AR film. Only typical examples are demonstrated.

stantial protein concentrations of 25 and 32 $\mu\text{g}/\text{ml}$ could already be measured in the 5- and 10-minute extracts (results not shown). This accounts for 6% and 8% of the extractable proteins.

The time-dependent release of proteins was further analyzed by IEF, SDS-PAGE, and CIE. All three methods separate proteins by different mechanisms. Qualitative changes in the protein composition of the extracts caused by a sequential release of proteins should therefore be easily detected. For this purpose

equal amounts of proteins of the different samples were used in different electrophoretic experiments.

In the entire extract series the IEF pattern was already completely established in the 5-minute extract. The changes occurring during the ongoing extraction affected the staining intensities of some bands only. This indicates shifts in the relative amounts of proteins in the extracts.

The results of CIE were in good agreement with the IEF patterns. The maximum number of antigens

was already present in the 5-minute extracts. However, there is a time-dependent shift in the relative amounts of the antigens. The final pattern was established after 4 to 5 hours of extraction. The results of SDS-PAGE were slightly different. The molecular weight region 10 to 100 kD, which is supposed to contain the major allergenic proteins,⁸ demonstrated typical patterns already in the 5-minute extract. However, the patterns of the high-molecular-weight region demonstrated time-dependent release kinetics for several protein bands up to 48 hours. As neither IEF nor CIE demonstrated such sequential releases of proteins, one explanation of this effect might be the occurrence of isoproteins or isoantigens, i.e., release of proteins with the same Ips and antigenic determinants but of different molecular weights.

Although IEF, SDS-PAGE, and CIE only describe physicochemical parameters of the extracts, they are in good accordance with the allergen-specific assays like CRIE, immunoprint, RI, and determination of P_1 . These methods indicate an immediate release of allergens from the source material. The IEF immunoprint clearly demonstrated that allergenically important proteins were readily released. About 10 to 11 different bands in the region of pH 4.0 to 7.0 carried allergenic determinants. Similarly, broad ranges for the IPs of allergens were also detected by others.⁸ The major mite allergen P_1 is reported to exist in at least two different forms with IPs of a relatively broad range.²⁰ Therefore, we cannot ascribe a certain immunoprint band to the P_1 activity.

A further resolution of the allergenic activity on molecular basis was done by CRIE. Also by this technique allergens could be detected in the 5-minute extract. However, the shifting of the patterns up to 4 hours indicated that the relative amounts of the individual allergens were changing.

There appears to be a shift in the ratios of allergen fractions to total protein in the extracts, which takes place during the first hour. This is well in agreement with RI results. The decreases of the C_{50} values also indicate a continuous allergen release up to 4 to 5 hours. Furthermore, the stability of the CRIE pattern after the first hour with regard to the total number of detectable allergens is reflected in the parallelism of all inhibition curves within each extraction series. In our opinion the high degree of correlation of protein and the major allergen P_1 throughout the extraction procedure demonstrates that the ratio of allergen to total protein is constant. The obvious saturation kinetics for allergen release as detected by RI probably reflects our previous findings that besides P_1 there are other very important allergens in whole mite bodies⁷

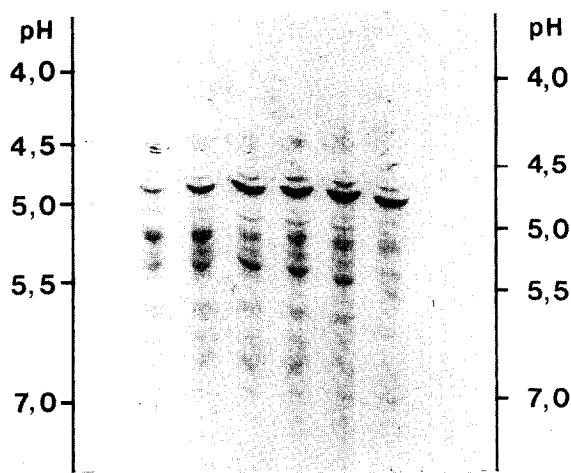


FIG. 6. Allergen patterns detected by IEF immunoprint in extracts prepared from purified mite bodies with increasing extraction times; 40 μ g of protein was applied at each lane. Extraction times were (from left to right) 5 and 20 minutes; 1, 4, 13, and 48 hours. The pH gradient was determined by cutting off 1 cm strips on each side of the agarose gel before protein transfer onto nitrocellulose paper. Strips were cut in 5 mm pieces, extracted with boiled water, and finally, the pH was determined in these extracts.

that have been underestimated when studies were done with whole mite cultures. Recently, Krilis et al.⁸ also reported on important allergens in mite bodies besides P_1 .

The different release kinetics for P_1 described by Tovey et al.¹⁰ could probably be associated with the totally different raw material used. The major source for antigen P_1 is fecal particles. Therefore, P_1 is readily released from whole mite cultures. In contrast to this, the mite bodies need a certain time for partial decomposition to release the allergens from the fecal particles in the gut.

Our findings suggest that long-time extracts of *Der-matophagoides pteronyssinus* demonstrate allergen compositions comparable to short-time extracts. With respect to reproducibility of the extraction process itself, medium and long-time extracts are superior to short-time extracts. The standard deviations for the means of the C_{50} of independent experiments were nearly one order of magnitude smaller for long-time extracts than for short-time extracts. This probably reflects some difficulties in the exact timing of the extraction procedure. The times necessary to handle the equipment before and after the extraction is nearly of the same order as for the extraction process itself when short-time extracts are done. On a quantitative basis long-time extracts yield about 10 times more allergenic activity than short-time extracts. In contrast

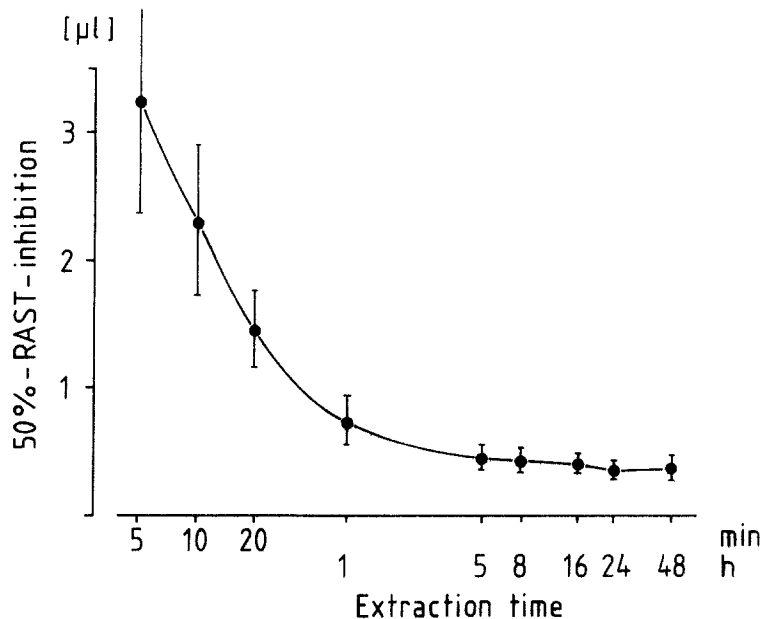


FIG. 7. Estimation of allergenic potency by RAST inhibition for extracts from purified mite bodies prepared with increasing extraction time. Allergenic activity is expressed by the extract volume necessary to achieve C_{50} . Each point is the mean of four extraction experiments.

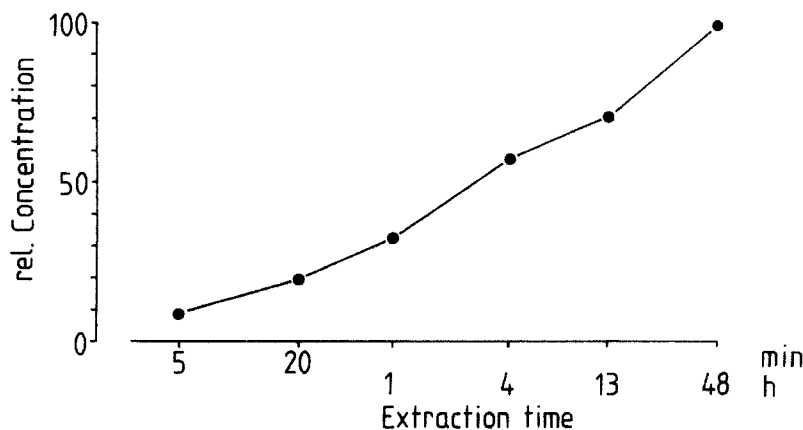


FIG. 8. Release of the major mite allergen P_1 from purified mite bodies. P_1 content of the extracts was determined by rocket immunoelectrophoresis versus a P_1 antisera. The area of the highest peak obtained was set arbitrarily as 100%.

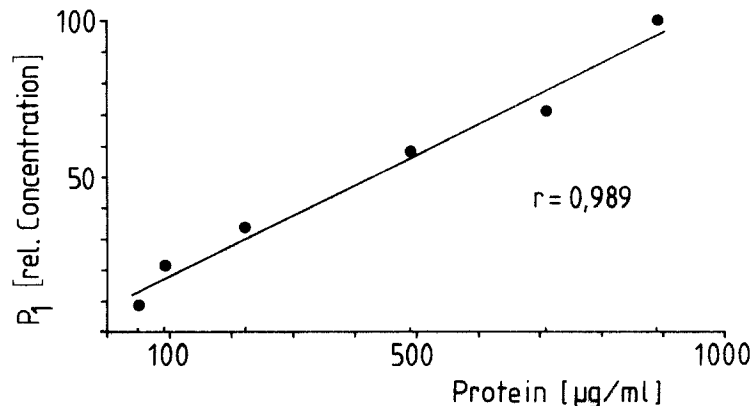


FIG. 9. Correlation of P_1 concentration expressed in arbitrary units and protein concentration of the allergen extracts.

to this, data published for short ragweed pollen extracts demonstrated different allergen compositions for short-time and long-time extracts.²¹ This is probably caused by the totally different nature of the investigated raw materials. Furthermore, this proves that data obtained for one kind of raw material should not be generalized.

Degradation of allergens in aqueous media could become a problem if inappropriate extraction conditions are used. This could result in changes in the patterns of the different electrophoretic separations or in shifting of certain bands by changes of the IP or the molecular weight. Differences in slopes of RI curves would also indicate degradation of allergens. Such changes could be detected by none of the methods used. This clearly proves that allergens from *D. pteronyssinus* are stable under the investigated conditions.

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