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# Quantitative Immunoelectrophoretic Methods as Tools for a Polyvalent Approach to Standardization in the Immunochemistry of *Candida albicans*

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Quantitative immunoelectrophoresis allows a polyvalent approach to immunochemical problems, i.e., identification, quantitation, and thereby standardization of antigens and antibodies in complex mixtures, for example as in crude extracts of microorganisms and their corresponding antisera. This approach is a short cut to conventional standardization of single purified substances, since the polyvalent approach gives a precise quantitative impression as described in this investigation and therefore enables the immunochemist to precisely select the right substance(s) to be purified for further standardization and characterization. To secure the precision of the results, in complex systems it is a sine qua non to select a complex antibody standard, a complex antigen standard, and to describe the complex standard precipitate pattern which again allows a quantitative study of the reproducibility of methods and procedures. The present article describes how such a standardization was approached in this laboratory within the field of *Candida albicans* immunochemistry. By means of crossed immunoelectrophoresis, 78 water-soluble antigens were demonstrated and enumerated in an antigen standard prepared from one strain of *C. albicans A* (B 311 Hasenclever). The antibody standard consisted of purified and concentrated rabbit antibodies. The migration velocity of each antigen was indicated in relation to purified human albumin. Not all precipitates could be seen in one immunoplate; therefore a standardized procedure was worked out showing reproducibly 54 precipitates. The reproducibility of quantitation by the crossed-immunoelectrophoresis procedure was determined for each of 30 antigens by repeated measurements; the relative standard deviations ranged from 2.4 to 15.4% and were below 10% for 24 antigens. A simple standardized antigen production procedure was described in great detail, and by quantitative determinations on 30 antigens the procedure was found to be satisfactorily reproducible. By means of crossed-line immunoelectrophoresis in the modification called absorption of antibodies in situ, it was found that strain B 311 contained no specific antigens in comparison to eight other strains of *C. albicans*. Thirty antigens were quantitated in antigen preparations made by the standardized procedure from the eight strains, and each antigen concentration was expressed as a percentage of the antigen standard. For each antigen a significant inter-strain variation was observed. Of 30 antigens, fifteen were satisfactorily stable after storage at −20 C for 1½ years. The laboratory methods and procedures described in this article thus work with high precision and allow a rapid collection of quantitative data concerning many individual antigens and their corresponding antibodies without purification of antigens. A new complex antigen standard can be made with satisfactory precision from strain B 311. The production of a similar complex antibody standard is a major problem; therefore the main problem in the intra-laboratory standardization seems to be the change from one complex standard to another. In inter-laboratory standardization on the

complex level, there seems to be a minimum demand that the first laboratory distributes the antibody standard and that other laboratories use the methods and procedures of the first laboratory.

Purified and standardized microbial antigens are desirable objectives in serological tests for diagnosis in infectious diseases. It is generally a difficult job to single out individual antigens to be purified for such tests, since the antigenic complexity of many microorganisms is very high and there is a huge number of parameters in immunochemical experiments on microbial antigens and their antibodies. In the early stages of such studies it is reasonable to work with crude antigen extracts containing the widest possible range of antigens. Quantitative studies using such complex reagents can be performed by means of the quantitative immunoelectrophoretic methods recently reviewed by Axelsen and Bock (2). In clinical trials the human antibodies of diagnostic importance might be pointed out by the method of Krøll (2, 13), or as described with examples from *Candida* serology by the method of Svendsen and Axelsen (15). A great advantage of these methods is that the strength of the individual antibodies (specificities) can be measured without previous purification of individual antigens. Another aspect is that cross-reactions (total or partial immunochemical identity) between a multitude of antigens in two complex antigen samples, for example in crude extracts of two different species of microorganisms, can be revealed by these methods (6). In this way a connection can easily be established between diagnostic and taxonomic studies to indicate the degree of species specificity of a certain human antibody.

This polyvalent, quantitative approach obviously can be a shortcut in microbial serology in order to point out interesting single antigens or antibodies on the background of a precise impression of the complexity in the system studied. However, a certain degree of standardization is necessary and can be obtained at the complex level in the initial stages of such studies. The previous demonstration of 68 water-soluble antigens in *Candida albicans* (1) is further analyzed in the present study to show how such a standardization can be approached. The investigation comprises 9 steps: (i) production of a standard pool of purified rabbit antibodies against water-soluble antigens from *C. albicans*; (ii) production of a standard pool of water-soluble antigens from *C. albicans*, extracted by a standardized procedure; (iii) determination of the maximal number of precipitates that could be revealed by crossed immunoelectrophoresis by using these standards; (iv) enumeration and characterization of these antigens; (v) qualitative and quantitative investigation of the reproducibility of a standardized immunoelectrophoresis procedure; (vi) investigation of the reproducibility of a standardized antigen production procedure; (vii) qualitative investigation of the interstrain variation; (viii) quantitative investigation of the interstrain variation; and (ix) quantitative and qualitative investigation of the stability of the standard antigen. On the basis of the results obtained in this and earlier studies, the approach method of an inter-laboratory standardization on the complex level is outlined.

## MATERIALS AND METHODS

**Strains of Candida albicans.** Strain B 311 (Hasenclever) was used for the production of the standard antigen and for immunization of rabbits. This strain was also used in the previous studies (1, 2, 15) and is, at present, one of the *C. albicans* strains most often used in immunochemical studies on *C. albicans*. The following strains were kindly made available by A. Stenderup, Institute of Medical Microbiology, University of Aarhus: 1/m, 2/m, 4/m, 7/m, 772/70, 882/70, 903/70, and 963/70.

**Rabbit antibodies.** Immunization of rabbits with the *C. albicans* antigen (strain B 311) and the methods for purification and concentration of the antibodies were described earlier (1). The standard antibody preparation, coded *St. Ab. 3 to 8*, used in the present study was produced by pooling the antibodies of 11 rabbits from 6 bleedings of each rabbit (bleeding numbers 3 to 8 after the antigen for immunization was changed [1]). This standard antibody preparation had a protein concentration of 27 g per liter, and 85% were gamma globulins. To 100 ml of this standard was added 3.75 ml of rabbit gamma globulin against human albumin (batch no. 0.29, standard strength, Dakopatts A/S, Copenhagen), and this mixture was used for some of the immunoelectrophoreses and coded *St. Ab. 3 to 8 + anti alb.*

**Standardized procedure for production of antigen.** Forty-eight-hour cultures of strain B 311, on Sabouraud dextrose agar (Oxoid) 37 C), were gently scraped off the plates with glass rods and suspended in sterile saline (0.154 M NaCl); the volume of saline was approximately double that of the culture. In this way 25 batches of culture were produced, each batch weighing ½ to 3 g more than 10.0 g (vide infra). Each batch was submitted to the following standardized procedure. The culture was centrifuged three times in a preweighed 50-ml glass tube in an ordinary laboratory centrifuge. (i) The first centrifugation was at 1,400 × *g* for 10 min. The supernatant fluid was discarded, and the sedimented culture was resuspended in saline (about 1:1, vol/vol). (ii) The first step was repeated. (iii) The third centrifugation was

at 4,000 × *g* for 15 min after which the supernatant fluid was discarded.

After this washing procedure the centrifuge tube was again weighed, and removal of culture was carefully performed until 10 g of the wet culture remained in the tube. The 10.0-g culture was resuspended in 10.0 g of saline and transferred to a Braun homogenization bottle (50 ml) containing 50.0 g of glass beads (0.45–0.50 mm) and 12.0 g of saline. Homogenization was performed in a Braun cell-homogenizor (MSK) at 2,800 rpm for 120 s by using liquid $CO_2$ as a coolant. After this procedure controls assured that the bottle was ice-cold, and that the contents were not frozen.

The homogenized culture without glass beads was submitted to ultracentrifugation at 4 C for 60 min at 105,000 × *g* (Beckman ultracentrifuge L2-65 B, rotor 65). After the centrifugation there was a turbid surface layer, and above the dense sediment there was another turbid zone. These two layers were avoided in pipetting the clear supernatant fluid which was used as antigen. In this way 25 different batches of antigen were produced from the strain B 311. Fifteen batches were pooled and used as a standard antigen, coded *St. Ag. 71*, and ten batches were kept separate. Storage was at −20 C in samples of 100 µliters in small plastic tubes closed with rubber stoppers. By refractometry the colloid concentration of *St. Ag. 71* was determined to be 32 g per liter with human serum as the standard (the refractive increment at 20 C was 0.00587). Antigen was also prepared by the standardized procedure from each of the eight other strains of *C. albicans*.

**Crossed immunoelectrophoresis.** Crossed immunoelectrophoresis was carried out as described earlier (1) with 1% agarose gel (Litex, Glostrup, Denmark) in barbital buffer (pH 8.6, ionic strength 0.02) by using the DL immunoelectrophoresis equipment (Dansk Laboratorieudstyr A/S, Copenhagen, Denmark). The connection between the gels on the plates and the buffer of the vessels consisted of 1-cm-thick gel wicks; the vessels contained barbital buffer (pH 8.6, ionic strength 0.07). To prevent antigen contamination of the buffer vessels, dialysis members were interposed between the electrophoresis gel and the contact gels (Krøll, personal communication).

All first-dimensional electrophoreses were run at 12 C, applying 10 V per cm for 55 min. All second-dimensional electrophoreses were run at 12 C, applying 2 V per cm overnight. Dimensions of the plates were 10 by 10 cm, and the thickness of gels was 1 mm. The washing, drying, and staining was performed as described earlier (1).

To visualize as many antigen-antibody systems (precipitates) as possible, series of crossed immunoelectrophoresis were run with varying amounts of *St. Ag. 71* (0.1, 1, 4, 7, and 10 µliters) and *St. Ab. 3 to 8* (2.5, 5, 10, and 12.5 µliters per $cm^2$ second-dimensional gel). To study the reproducibility of the crossed immunoelectrophoresis procedure and to study the variation of antigen concentration in different antigen batches, standard crossed immunoelectrophoreses were run as follows: 50 µliters of *St. Ag. 71* were mixed with 50 µliters of a standard solution of purified human albumin (prepared from Human albumin, trocken, reinst, op. nr. 3566, Behringwerke AG, Marburg/Lahn, Deutschland) containing 0.245 g per liter and stored at −20 C in samples of 100 µliters in the same freezer as *St. Ag. 71*. Two microliters of this mixture were submitted to first-dimensional electrophoresis; the application of antigen was carried out throughout the study with the same Carlsberg double constriction pipette. The second-dimension gel contained 5 µliters of *St. Ag. 3 to 8 + anti-albumin* per square centimeter. The antigen preparations from the eight different strains and different samples of *St. Ag. 71* after storage at −20 and 8 C were also submitted to the standard crossed immunoelectrophoretic techniques.

**Quantitation of the antigens.** In the crossed immunoelectrophoresis the area enclosed by a precipitate is proportional to the antigen-antibody ratio of the system (8). As the antibody amount and the volume of antigen were kept constant in the standard immunoelectrophoresis, the size of the areas were proportional to the concentration of the individual antigens in the antigen samples. After magnification at ×8.3, the areas were integrated according to Weeke (17) by using a semiautomatic planimeter. Each area on one immunoplate was scanned twice, and the mean was expressed as percentage of the mean of the reference (albumin) area (4 scannings) on the same plate.

**Crossed-line immunoelectrophoresis.** Crossed-line immunoelectrophoresis (13) was performed in the modification described by Axelsen and Bock (absorption of antibodies in situ; reference 2). The analysis was performed as the standard crossed immunoelectrophoresis (described above) with the modification that a ditch (4 by 80 mm) was punched out anodically in the first-dimension run after the first-dimension electrophoresis of *St. Ag. 71* was mixed with human albumin. The ditch was filled with 300 µliters of an antigen preparation made from another strain than that of B 311. After diffusion of this antigen into the gel, the edges of the ditch were pushed together, *St. Ab. 3 to 8 + anti-albumin* was poured onto the rest of the plate, and the second-dimension electrophoresis was performed. If no peak precipitates (except the albumin peak) was seen in the plate, the antigen of the ditch contained all of the antigens of *St. Ag. 71* (2). (For further details of this method see Literature Cited; 2, 6).

**Statistical analysis.** The significance of the difference between the repeated determinations on the same batch and the determinations on the different batches of antigen made from strain B 311 was established by using the F-test. *P*, the probability of difference being due to chance, was obtained from tables for the test.

## RESULTS

By running a series of crossed immunoelectrophoreses with varying amounts of the standard *C. albicans* antigen (*St. Ag. 71*) and varying amounts of the standard preparation of rabbit antibodies against *C. albicans* (*St. Ab.*

*3 to 8*), it was possible to detect a total number of 78 individual precipitates in the diferent immunoplates, indicating the existence of 78 water-soluble antigens in *C. albicans*. All 78 antigens (precipitates) were enumerated beginning with no. 1 in the anodic end, and ending with antigen no. 78 in the cathodic end of the first-dimensional electrophoresis. The relative migration velocity of each antigen was determined by measurements of the position of the peaks of the precipitates and appears by using human albumin as the reference (Table 1).

A standard crossed immunoelectrophoresis was used as the basis for further studies of the antigens. Figure 1 shows such a standard immunoplate in which purified human albumin served as a reference. Figure 2 is a drawing of Fig. 1 with enumeration of the precipitates. Only 57 precipitates could be detected in the immunoplate of Fig. 1. Of these 57 precipitates, only no. 2, 6, and 45 did not appear regularly in 10 consecutive standard immunoelectrophoreses. In other words, the standard procedure repeatedly revealed 54 antigens of *C. albicians* (no. 78 a, b and c are regarded as one complex antigen). These 54 standard antigens are footnoted with a *c* in Table 1. After staining intensities were determined, the 53 precipitates were divided into four groups (Table 1).

The reproducibility (or precision) of the antigen quantitation by the standard crossed immunoelectrophoresis was studied by planimetric determinations of the areas delimited by 30 precipitates in 10 immunoplates produced by running (during 1 week) 10 samples of the standard antigen, *St. Ag. 71*, mixed with human albumin. The area of each precipitate was expressed as percentage of the albumin reference on the same plate. The antigens selected for quantitation and the arithmetic means and relative standard deviations of the 10 determinations appear in Table 1. The relative standard deviations ranged from 2 to 4% (antigen no. 7, 11, 16, 18, 28, 33, 36, 40, 44, and 51) to 12 to 16% (antigen no. 13, 15, 46, and 78). For 24 antigens, the relative standard deviation was below 10%. With one exception (antigen no. 15), the relative standard variation was below 10% for all antigens producing areas larger than 0.44 $cm^2$; in addition these precipitates were more distinct than those having areas lower than 0.44 $cm^2$.

The reproducibility of the antigen production procedure was studied quantitatively by quantitation of the same 30 antigens in 10 different batches of antigen made from the same strain (B 311). By means of the F-test the data were compared to those obtained in the 10 determinations on the same batch (vide supra). Significant differences ($P < 0.05$) were observed for 10 antigens (no. 7, 11, 16, 18, 21, 28, 40, 49, 51, and 61), and 4 of these (no. 7, 18, 28, and 51) reached $P$ values below 0.01.

The qualitative comparison of the eight *C. albicans* strains to strain B 311 (*St. Ag. 71*) was performed by means of the crossed-line immunoelectrophoresis (13) in the modification called absorption of antibodies in situ (2). The experiments showed that in relation to these other strains the B 311 strain contained no specific antigens, and no reactions of partial identity (6) were observed. In the crossed immunoelectrophoresis that were used for the quantitation of the individual antigens, vide infra, the precipitate patterns were very much like those of Fig. 1 of *St. Ag. 71* with the exception that some quantitative variations were seen, especially if the plates were superimposed. The only qualitative difference from *St. Ag. 71* was observed in antigen no. 78. The triple composition of this precipitate (Fig. 2) was not observed clearly in all strains.

The quantitative interstrain variation can be seen in Fig. 3, in which all of the antigen concentrations were expressed as percentage of the concentration of the standard (*St. Ag. 71*). The lowest concentration in relation to *St. Ag. 71* was 12% (Ag. no. 78, strain 882/70), and the highest concentration was 324% (Ag. no. 72 strain 903/70). Forty percent of the observations were higher than *St. Ag. 71* +2 SD, and 27% of the observations were lower than *St. Ag. 71* −2 SD. For none of the strains were the concentrations within the range of *St. Ag. 71* ±2 SD. The mean concentrations of each antigen for all 8 strains ranged from 67 to 176% of *St. Ag. 71*, 4 means were below that of *St. Ag. 71* −2 SD, and 11 mean values were higher than *St. Ag. 71* +2 SD. The 4 antigens which showed the smallest interstrain variation were no. 13, 33, 40, and 51.

The concentration of 30 antigens of *St. Ag. 71* after storage at −20 C up to 18½ months appears in Fig. 4. As judged from this figure, 15 antigens (no. 7, 11, 15, 16, 18, 21, 25, 26, 28, 33, 49, 51, 61, 62, and 63) seemed to be relatively stable. Also qualitative changes could be seen in the plates. After 18½ months the anodic peak of no. 28 (Fig. 1 and 2, and Table 1) increased considerably; the major peak of antigen no. 49 was now the cathodic one; no. 58 was considerably broader (the cathodic shoulder had disappeared, and a new shoulder appeared beneath no. 51); antigen no. 73 moved backwards to

TABLE 1. *Characterization of the individual antigens of Candida albicans and the precision of quantitation*[a]

| Antigen no. | RMV | Stain | Area | SD (%) | Antigen no. | RMV | Stain | Area | SD (%) |
|---|---|---|---|---|---|---|---|---|---|
| Alb. ref. | 100 | ++++ | 100.0 | | 40[b,c] | 80 | +++ | 150.8 | 3.6 |
| 1 | 150 | | | | 41[b,c] | 70 | + | | |
| 2[b] | 140 | | | | 42[b,c] | 65 | + | | |
| 3 | 140 | | | | 43 | 75 | | | |
| 4 | 135 | | | | 44[b,c] | 75 | ++++ | 219.6 | 3.9 |
| 5[b,c] | 120 | ++ | | | 45[b] | 70 | + | | |
| 6[b] | 120 | + | | | 46[b,c] | 65 | +++ | 3.8 | 15.3 |
| 7[b,c] | 120 | ++++ | 57.7 | 2.8 | 47[b,c] | 65 | ++++ | 31.7 | 4.8 |
| 8[b,c] | 110 | +++ | 2.2 | 15.4 | 48[b,c] | 65 | +++ | | |
| 9[b,c] | 115 | + | | | 49[b,c] | 65 | ++++ | 169.3 | 4.2 |
| 10[b,c] | 110 | + | | | | (50) | | | |
| 11[b,c] | 110 | +++ | 98.6 | 2.9 | 50[b,c] | 60 | ++ | | |
| 12 | 110 | | | | 51[b,c] | 60 | ++++ | 59.8 | 3.4 |
| 13[b,c] | 110 | +++ | 9.0 | 13.6 | 52 | 65 | | | |
| 14 | 110 | | | | 53 | 60 | | | |
| 15[b,c] | 105 | +++ | 78.5 | 12.9 | 54 | 60 | | | |
| | (90) | | | | 55[b,c] | 55 | ++ | | |
| 16[b,c] | 100 | ++++ | 35.2 | 2.7 | 56[b,c] | 60 | ++ | | |
| 17[b,c] | 100 | ++ | | | 57[b,c] | 60 | ++ | | |
| 18[b,c] | 100 | ++++ | 43.7 | 2.4 | 58[b,c] | 55 | +++ | 379.1 | 5.8 |
| 19[b,c] | 95 | ++ | | | 59[b,c] | 50 | ++ | | |
| 20[b,c] | 95 | + | | | 60 | 55 | | | |
| 21[b,c] | 90 | ++ | 17.8 | 6.1 | 61[b,c] | 60 | ++++ | 46.8 | 4.6 |
| 22 | 95 | | | | 62[b,c] | 50 | ++++ | 17.4 | 8.0 |
| 23 | 90 | | | | 63[b,c] | 45 | ++++ | 23.4 | 6.9 |
| 24 | 95 | | | | 64[b,c] | 40 | ++++ | 154.5 | 6.3 |
| 25[b,c] | 95 | +++ | 99.2 | 4.6 | 65[b,c] | 45 | ++++ | 105.9 | 4.4 |
| 26[b,c] | 90 | +++ | 48.9 | 5.7 | 66[b,c] | 45 | + | | |
| 27 | 90 | | | | 67 | 45 | | | |
| 28[b,c] | 90 | ++++ | 111.5 | 2.4 | 68[b,c] | 45 | + | | |
| | (100) | | | | 69 | 45 | | | |
| 29[b,c] | 85 | ++ | | | 70[b,c] | 45 | + | | |
| 30[b,c] | 85 | ++ | | | 71[b,c] | 40 | ++++ | 50.6 | 6.4 |
| 31 | 90 | | | | 72[b,c] | 40 | +++ | 28.2 | 6.8 |
| 32[b,c] | 85 | ++ | | | 73[b,c] | 35 | +++ | 36.4 | 5.3 |
| 33[b,c] | 80 | ++++ | 49.4 | 3.3 | 74 | 35 | | | |
| 34[b,c] | 90 | + | | | 75 | 25 | | | |
| 35[b,c] | 80 | +++ | | | 76[b,c] | 20 | ++++ | 24.0 | 11.6 |
| 36[b,c] | 80 | +++ | 17.3 | 3.7 | 77 | 20 | | | |
| 37 | 90 | | | | 78ab[b,c] | 0–60 | +++ | 34.0 | 13.0 |
| 38[b,c] | 80 | +++ | | | 78c[b,c] | 0–70 | ++ | | |
| 39[b,c] | 80 | +++ | | | | | | | |

[a] Antigen enumeration is according to Fig. 2. Relative migration velocity (RMV) is in relation to the albumin reference. Two figures (see antigen numbers 15, 28, and 49) indicate that one antigen has two peaks; the minor peak is in parentheses. Stain refers to visual distinctness after staining of the standard crossed immunoelectrophoresis (++++: very distinct, +: very faint). Area is in percentage of albumin precipitate (100 per cent = 1.27 $cm^2$) mean of 10 determinations on the antigen standard, *St. Ag. 71*. Standard deviation (SD) is in percentage of mean calculated on the basis of data from 10 determinations on the antigen standard (the same batch).

[b] The precipitate was present in the standard crossed immunoelectrophoresis of Fig. 1.

[c] Regularly occurring precipitates in repeated standardized experiments.

antigen no. 76; and the complex precipitate of no. 78 was less distinct. The concentrations of the same antigens were also determined in samples stored at 8 C. After 3 months at this temperature most antigens had disappeared completely. Those remaining were no. 7, 51, and 78 and a few others. The breakdown of the antigens was started after about 1½ months at this temperature.

## DISCUSSION

To secure the precision of the many quantitative data that can be collected without purifica-

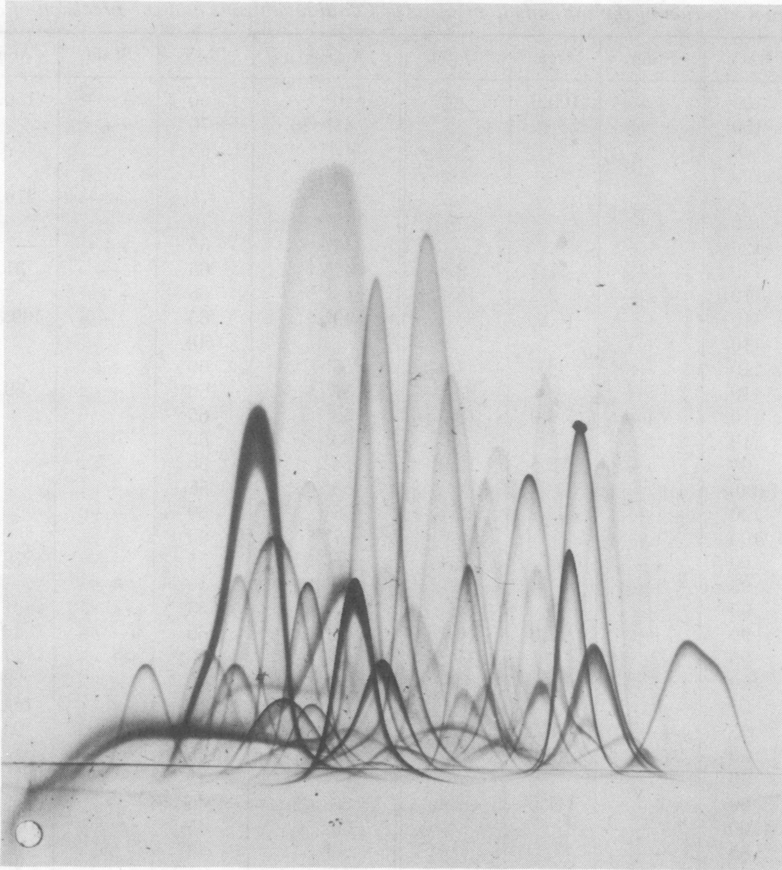

FIG-1. *Standardized crossed immunoelectrophoresis of water-soluble Candida albicans antigens (standard preparation) mixed with purified human albumin. The second-dimension gel contained purified and concentrated rabbit antibodies against C. albicans (standard preparation) mixed with monospecific rabbit antibodies against human albumin. The dot indicates the albumin precipitate, which served as a reference. (Anode to the right during the first-dimension electrophoresis and anode at the top during the second-dimension electrophoresis. Stained with Coomassie brilliant blue R.)*

tion of antigens by means of the new quantitative immunoelectrophoretic methods (2), it is necessary to select complex antigen standards and complex antibody standards, i.e., to secure a certain degree of standardization in the initial stages of the investigations. The present study comprised nine steps with the purpose to investigate the reproducibility of the methods and procedures employed for the standardization of *C. albicans* immunochemistry in this laboratory. Each step will be commented on, emphasizing technical details. Finally, the results will be briefly discussed outlining the inter-laboratory standardization method which should be followed.

(i) In studies like the present very little is known beforehand about the stability of the antigens. In contrast to this fact, the stability of precipitating rabbit antibodies is known to be high since the drop in antibody titer of preparations produced and stored as in this study has been shown to be less than 2% per year (N. M. G. Harboe, personal communication). Furthermore, the relative fall in titer can be presumed to be the same for all of the antibodies (specificities), especially when the antibodies are pooled from several rabbits. The precipitin responses of individual rabbits injected identically with the same *Candida* antigen have been found to differ very much (3), indicating difficulties in the production of similar (standardized) polyvalent antisera even within the same laboratory. Even very complex antisera can be compared quantitatively (2, 13, 15), but this can be a complicated and laborious job. Purification of individual antigens is greatly facilitated if the fractionations are followed by the method of Harboe and Svendsen (16) by

using an antiserum which reveals as many antigens as possible, i.e., an antiserum of high quality in terms of the polyvalent approach. The same pool can be used as a reference in different studies on precipitins, for example as described by Svendsen and Axelsen (15). As long as standardization is only at an experimental level the possibility for exchange of standard antisera between different investigators is very important. In consequence of all of these facts the cornerstone of the standardization in studies like the present after the initial studies (1) should be to produce a large standard pool of rabbit antibodies of high quality. Such a pool, coded *St. Ab. 3 to 8*, was prepared in this study during 9 months by pooling the antibodies of 11 rabbits from 6 consecutive bleedings of each rabbit. The immunization schedule and the procedures for purification and concentration of the antibodies were reported earlier (1). In the steady state each rabbit is injected every 6 weeks, and 8 days

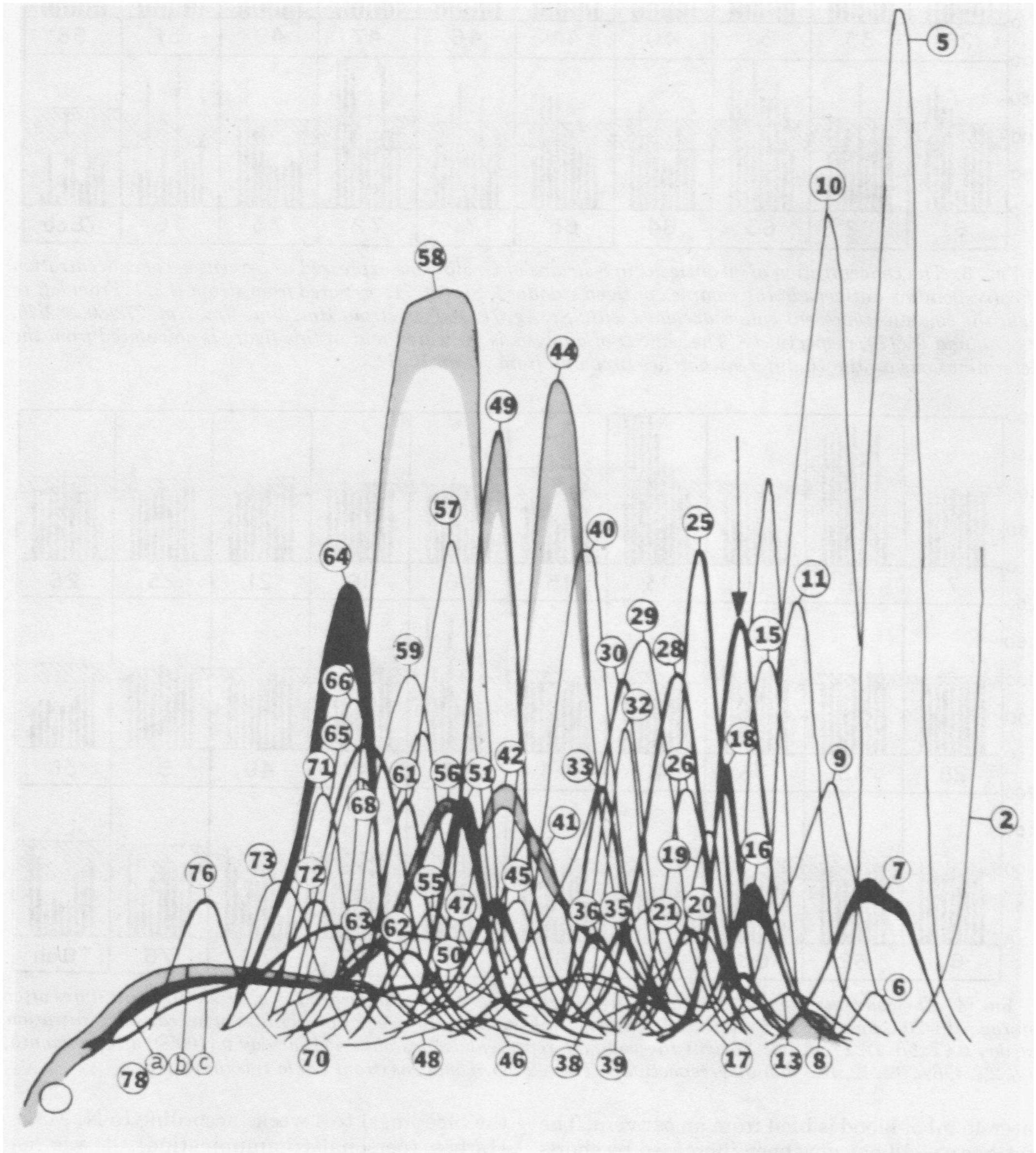


FIG. 2. *Drawing of Fig. 1 with enumeration of the precipitates. The arrow indicates the albumin reference.*

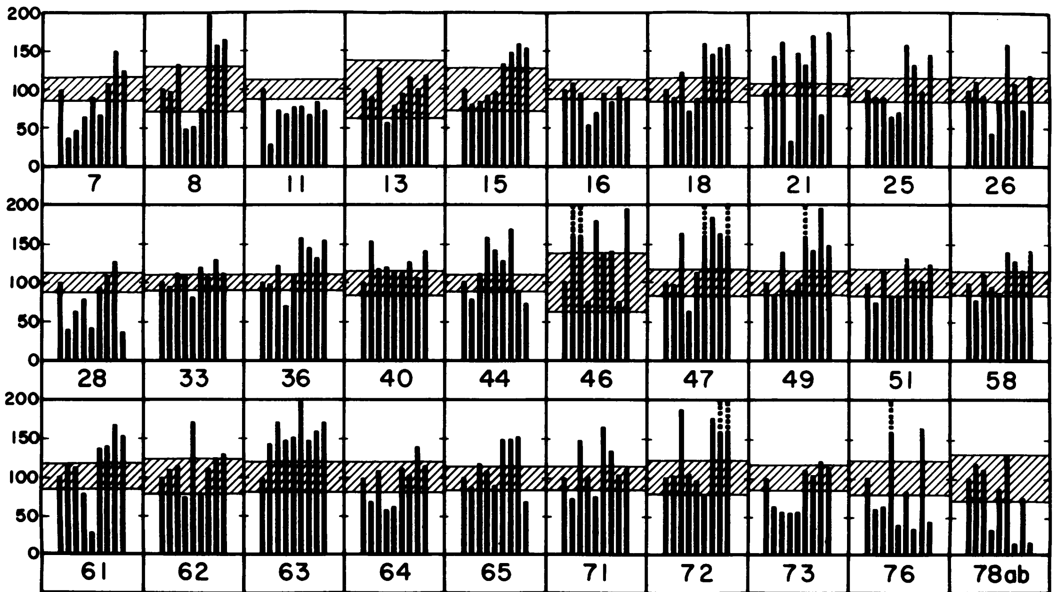


FIG. 3. *The concentration of 30 antigens in 8 strains of C. albicans expressed as percentage of concentration of corresponding antigens in the complex antigen standard, St. Ag. 71, prepared from strain B 311. From left to right the columns represent values obtained with: St. Ag. 71 (100 %), strain 1/m, 2/m, 4/m, 7/m, 772/80, 882/70, 903/70, and 963/70, respectively. The ±2 SD of analysis is indicated and in this figure is calculated from the determinations on the 10 different batches prepared from strain B 311.*

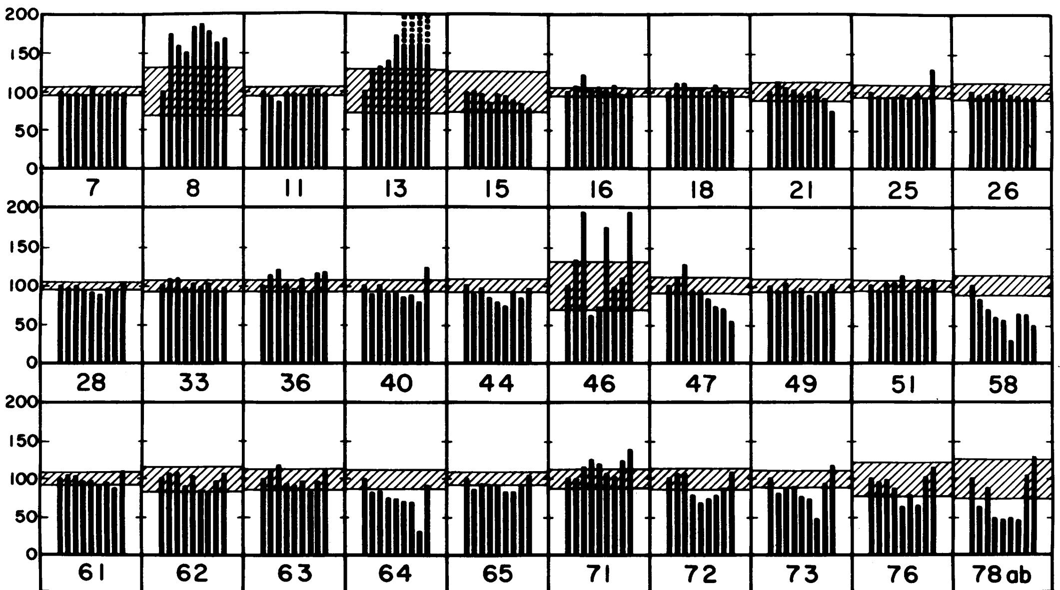


FIG. 4. *The concentration of 30 antigens in the complex antigen standard, St. Ag. 71, at different times after storage at −20 C for up to 18.5 months. The concentrations are expressed as percentage of average concentration at day 0 (Table 1). From left to right the columns represent values obtained: at day 0 (100%), after ¾ month, 1½, 2½, 4, 6¼, 3¼, 12, 18½ months, respectively. The ±2 SD of analysis (from Table 1) is indicated.*

later 45 ml of blood is bled from an ear vein. The antibody yield has now been increased by shortening the intervals between the injections (and the bleedings) to 4 weeks according to N. M. G. Harboe (personal communication). It was not mentioned in the previous report (1) that the

rabbits chosen for immunization were selected among offspring of animals which were good precipitin producers when immunized similarly with human serum proteins; this probably has some significance for the high quality of the standard antibody pool described in this article.

(ii) Production of water-soluble antigens from *C. albicans* was performed by means of a homogenization procedure, followed by an ultracentrifugation. The procedure was essentially the same as described in the earlier report (1), but in the present study each step was further standardized to perform the extraction in a reproducible way. The individual steps in the extraction procedure will be discussed; it should be kept in mind that the significance for each antigen of the suggested changes in the procedure is accessible to qualitative and quantitative investigations by means of the quantitative immunoelectrophoretic methods (2) without purification of the antigens. The antigens were extracted in 0.154 M NaCl; however, demineralized water might be used in future studies since additional membrane-bound antigens probably can be solubilized in this way (7). The principles for extraction of membrane-bound antigens have been reviewed by Kahan and Reisfeld (11). More than one initial washing of the culture may be superfluous, since no antigens were detected in the substrate (1). The reason for the repeated washings was that it was thought to be convenient to work without too many components from the substrate, for example during future fractionations on columns. An obvious disadvantage of the repeated washings is that hypothetically extracellular antigens excreted in the medium can be lost; however, if present extracellularly, they are probably also, to some extent, present intracellularly. With regard to the quantitative reproducibility, the weighing of the culture before the crushing is obviously a very important step in the procedure. A meaningful weighing is dependent on the degree of standardization of the preceding centrifugation (washing). Again, more than one washing need not be necessary. The proportion between the amount of the wet culture (10.0 g) and the amount of extraction medium (22.0 g of saline = 10.0 g + 12.0 g, the last 12.0 were necessary for keeping the glass beads suspended) was chosen to get a concentrated solution of antigens, since only small volumes are applied in the gels for immunoelectrophoresis. Furthermore, dilution of small volumes (for example in a 50-$\mu$liter scale) can be performed with great accuracy, if necessary, in contrast to concentration procedures which additionally might alter the antigens. Also dialyses were avoided since they may introduce changes which are difficult to control: they may denaturate antigens, cause losses, and finally they are time-consuming. If the purpose of study had been to obtain a great yield of antigens the extraction volume could have been increased to 50 or 100 ml by washing the glass beads after the crushing procedure. To simplify the procedure, the crushing time (120 s) probably can be shortened. It can be expected that denaturation of antigens can occur during the homogenization, especially if the cooling is not effective. Therefore, immediately after the homogenization, controls assured that the bottles were cold. If a bottle was warm, or on the contrary if the contents were frozen due to too much cooling, the sample was excluded from this study. The final ultracentrifugation was performed to dispose of insoluble cell debris, the antigens of which do not migrate in the immunoelectrophoresis. Furthermore, the cell debris disturbs the exact application of antigen in the wells of the gel. More membrane-bound antigens might have been extracted if demineralized water had been used as extraction medium. If the purpose of study had been to investigate membrane antigens, the sediment might have been submitted to further extractions by means of detergents (11). The standard pool of antigen, coded *St. Ag. 71*, was produced by pooling 15 batches of one strain (B 311) at 4 C. Until analysis the antigen was stored at −20 C in samples of 100 $\mu$liters in small plastic tubes. Once a sample was thawed at room temperature it was submitted to immunoelectrophoresis within a quarter of an hour, and the antigen not used was discarded. In this way alterations of the antigens due to repeated thawing and freezing were avoided. In future studies it would probably be an advantage to quick-freeze the antigen samples in liquid $N_2$ and subsequently store at −70 C. (At our laboratory −70 C was not available when this study was performed.) It might also be worthy to stabilize the antigen by means of protease inhibitors (E. Bock, personal communication); these could be added to the extraction medium before the homogenization of the cells. In the present study the only precautions taken against proteolytic or other enzymatic damage to the antigens were that during and after the homogenization the antigen was kept cold, stored as quickly as possible, and the immunoelectrophoreses were run just after the thawing of the antigen samples.

(iii) To reveal the maximal number of antigens in the standard antigen (= the maximal number of precipitins in the standard antibody pool), a number of crossed immunoelectropho-

reses were run with different ratios between the antigen and the antibody amount. A close examination of the different precipitate patterns showed a total number of 78 individual precipitates. This is 10 precipitates more than earlier reported (1); however, new preparations of antigen and antibody were used. It might be added here that to reveal as many precipitates as possible, it is of importance that the destaining of the immunoplates is performed with care; a faint blue background staining secures the detection of very faint precipitates. In the previous report (1) the dimension of the plates was 10 by 20 cm, and 67 precipitates could be counted in a single plate. For convenience 10- by 10-cm plates were used in the present study, and at no time could all 78 precipitates be detected in one plate.

(iv) All of the precipitates were enumerated beginning in the anodic end. Unfortunately it was not possible to compare the enumeration of the 16 *C. albicans* antigens described by Biguet et al. (4, 5) to that of the present study due to technical differences. By mixing the standard antigen with purified human albumin, and the standard antibody with antibodies against albumin, the relative migration velocity of each antigen could be determined in relation to the albumin (Table 1).

(v) At this stage of the study it was calculated that too much of the costly antibody pool would be used if all 78 antigens were to be further investigated. It was therefore decided to continue with a cheaper standardized immunoelectrophoresis procedure (Fig. 1 and 2). The reproducibility of this procedure was qualitatively very good, since 54 of 57 precipitates occurred in all of the 10 runs performed during one week. The 54 antigens are footnoted with a *c* in Table 1. After their degree of visual distinctness in the 10 plates had been determined, the 54 precipitates were divided into 4 groups (Table 1). It might be thought that some or all of these antigens could be different pieces of the same crude antigen molecule which could have split during the extraction procedure, e.g., due to proteolysis. If so, reactions of partial identity would have been expected to occur between some of the apparently individual antigens. These reactions have recently been studied in model experiments by Bock and Axelsen (6) and Axelsen and Bock (unpublished data), but in the present study no reactions of this kind were observed, except for the fuzzy precipitate no. 78 which deserves special attention, vide infra. However, microheterogeneity of the individual antigens was observed in a few cases, for example precipitate no. 15, 28, 49, 58, and 64 were not regular symmetrical peaks. If the purpose of study had been to reveal microheterogeneity of the individual antigens the original Laurell procedure (9, 14) should have been used instead of the Clarke and Freeman procedure (8). Precipitate no. 78 was not clearly defined (fuzzy) and seemed to consist of at least 3 partially identical antigens, called a, b, and c (Fig. 2); precipitate no. 78 consists of protein-polysaccharide complexes (supposedly mannan from the cell wall).

Reproducibility (or precision) of the antigen quantitation was expressed as the relative standard deviation for each antigen of ten repeated determinations. As demonstrated by Weeke (17) a reference can improve the precision of the method. In this study purified human albumin was used as reference for five reasons: (i) it is commercially available and sufficiently standardized; (ii) it is a stable protein, (iii) monospecific anti-albumin is also commercially available in a sufficiently standardized form, (iv) the albumin was found to have a convenient place among the anodic *Candida* precipitates, and (v) human albumin is a well known protein and therefore seemed to be a reasonable reference for a description of the position of the *Candida* antigens after the electrophoresis. In contrast to Weeke's use of carbamylated transferrin as a reference for the study of serum proteins, it was necessary to add anti-albumin to the anti-*Candida* standard before the immunoelectrophoreses. To avoid errors due to this procedure, one (sufficiently large) pool of *St. Ab. 3 to 8 + anti-albumin* was produced and used throughout the study. For 22 of the 30 antigens the use of the albumin reference improved the precision of the quantitation. Twenty-four of the thirty antigens had relative standard deviations below 10%, indicating a very good precision for the method since only 10 determinations were preformed for each antigen (to spare the costly anti-*Candida* antibodies). In Weeke's study (17) on human serum proteins the coefficients of variation were generally somewhat higher. However, Weeke's study was performed over 6 months and included a person-to-person variation. The observation by Weeke that the precision decreased with decreasing areas was also seen in this study, and it was evident that antigens producing less distinct precipitates were determined with less precision than distinct ones. The main error of the less distinct proteins and those with small areas therefore lies in the scanning procedure. It should be noted that the precision for an antigen which produces a small area can be increased by increasing the antigen amount applied in the

well; an interesting small precipitate can therefore be easily studied more precisely by this simple manipulation (simultaneously the precision is changed for some other antigens.). Similarly the less distinct precipitates can be made more distinct by increasing both the antigen amount and the antibody amount in the system; in this way the precision of the antigen quantitation can be increased for the antigens in question.

(vi) The precision of the antigen production procedure obviously could be improved since the variation in the data obtained from 10 different batches exceeded that of the 10 repeated determinations on the same batch for 10 antigens ($P < 0.05$). These 10 antigens were generally determined with highest precision in the repeated determinations on the same batch (Table 1). However, for the remaining 20 antigens the precision could not be improved by using the 0.05 level of significance, and at the present stage of standardization there was found no reason for trying to improve the reproducibility of this procedure.

(vii) No qualitative interstrain variation was found; i.e., strain B 311 contained no specific antigens in relation to the eight other strains. Although only eight strains were investigated (to spare costly antibodies) in this one-way reaction (i.e., only antibodies against one strain), the results indicate that there are no qualitative antigenic differences between different strains of *C. albicans*. The A-B serotype differences of Hasenclever (10) might have been overlooked by this method since pure mannans are probably not moving in the electrophoreses; immunochemical differences of the mannans might be expected to be reflected in antigen no. 78 which is presumed to consist of protein-mannan complexes. However, it was not the purpose of this study to investigate the A-B serotyping. On the basis of these results it therefore seems justified to use *St. Ag. 71* as the reagent for studies on human precipitins of *C. albicans*: the risk that antibodies should be overlooked due to interstrain variations is negligible. For the same reason *St. Ag. 71* can be used as the standard for studies of immunochemical inter-species variations.

(viii) It is clear from Fig. 3 that a significant quantitative interstrain variation was observed for all antigens. Possibly a future serotyping could be performed on the basis of quantitative differences as revealed with this method.

(ix) Storage experiments showed that the concentrations of 15 antigens were stable over a period of 1½ years when stored at −20 C in small plastic tubes with rubber stoppers. The nonstability of the remaining 15 antigens appeared as an apparent decrease or increase in concentration. As long as the assay is immunochemical, a loss of antigenic determinants can cause an increase in concentration (which is naturally only apparent, just as the concentrations of myeloma proteins are often overestimated when polyclonal immunoglobulins are used as standards). A decrease in concentration could be due to regular split of molecules; there may be many other explanations for such changes. It should be remembered that human albumin was used as the reference. Therefore, if an antigen is stable for 1½ years, for example as with antigen no. 7 (Fig. 4), this signifies that the stability of no. 7 equals that of human albumin under the experimental conditions. A stability such as that of human albumin is highly satisfying. Of the 15 antigens with satisfying stable concentrations, 2 (no. 28 and 49) showed a change in profile indicating a shift in mobility; these two antigens therefore cannot be considered stable in the same respect as the remaining 15 antigens. From a standardization point of view it is unlucky that all antigens were not as stable as no. 7. However, the quantitative capacity of the methods nevertheless offers a possibility for corrections of unknown samples if *St. Ag. 71* is used as a standard at a time where the antigen under investigation has a known decreased concentration in *St. Ag. 71*.

To secure the comparability of quantitative data over longer periods of time the intra-laboratory standardization was performed by making a large pool of antigen, a large pool of antibody, and by working out standardized methods and procedures. In this way highly reproducible results were obtained, and errors were kept to a minimum. Problems arise when new antigen or antibody standards have to be introduced. In the polyvalent approach of this study these problems are much greater than in the conventional biological standardization on single substances, since many parameters have to be expressed in terms of the old standard. Figure 3 clearly illustrates the importance of using only one strain for preparation of new antigen standards. The change of the complex antibody pool is probably the most crucial point and could be the main source of error in the intra-laboratory standardization. However, the rabbits can be kept alive for years, and the differences between the pools of the same rabbits can be kept to a minimum if the individual bleedings of each rabbit are studied before the pooling. With these precautions, the possibilities for obtaining comparable results over long periods of time should be good by using the

quantitative immunoelectrophoretic methods.

It is obvious that confusion may arise if the nomenclature of *C. albicans* antigens as described in this article has to be introduced into another laboratory. It is a minimum demand for such a project that exactly the same methods and procedures are used; therefore methods and procedures were described in great detail in this article. However, even if the same strain of *C. albicans* was used as immunogen the project probably would have few chances for success due to the very great qualitative individual differences in the antibody response of rabbits and it would take at least 1 year before this could be decided due to the long-term immunizations used in these studies. The new approach to microbial serology by means of the quantitative immunoelectrophoretic methods probably will show a similarly high antigenic complexity of many other microorganisms, the risk being an immense confusion in the literature concerning the number and names of individual antigens in microorganisms. The 78 antigens of *C. albicans* shown in this article clearly illustrate this danger. Therefore the safest and quickest, intralaboratory standardization method that can be recommended is that the first laboratory distributes the antibody standard. The antigen standard can be produced safely according to the method described in this article. Probably most of the precipitates can be recognized from Fig. 1 and 2 of this article, but it might be necessary to make a few runs with the original antigen standard. Even if only 30 or 40 precipitate numbers could be transferred from the first to other laboratories, this would mean an immense progress in regard to the possibilities for obtaining large numbers of directly comparable data on molecular level, since many data in these complex systems can be obtained in a limited number of immunoelectrophoreses.

It was stated in the introduction that purified and standardized antigens were desirable objectives in serological tests for diagnosis in infectious diseases. However, as demonstrated in this article, purification of antigens is not necessary to obtain specific quantitative knowledge about individual antigens (or antibodies). Neither need purification be necessary for standardization. Furthermore purification procedures may cause partial loss of the antigen or of antigenic determinants; therefore the purified substance may be less suited for the intended purpose. Problems of this kind can be elucidated and overcome by the polyvalent approach as presented in this article.

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