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Measurements of precipitin reactions by difference turbidimetry: a new method

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Summary. A new method for the measurement of precipitin and flocculation reactions between antibodies and antigens has been developed. The technique, called difference turbidimetry, involves the use of tandem cuvettes providing the opportunity of using separated and unmixed antigen and antibody as blank solutions for spectrophotometric readings in the ultraviolet wavelength range. By use of this technique genuine difference turbidity spectra have been recorded for the reaction between human serum albumin and rabbit-anti-human serum albumin IgG. It was found that difference turbidimetry at low wavelengths (e.g. 280 nm) allows the construction of precipitin curves with a very clearly expressed zoning phenomenon at a sensitivity which in terms of antigen and antibody concentrations is more than twice the sensitivity of conventional procedures. It is of special interest that the zone of equivalence differs when the same reaction between an antigen and its antibody is measured by difference turbidimetry, by absorbance of washed and redissolved precipitate, and by amount of precipitated antigen.

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INTRODUCTION

Precipitin reactions are frequently quantified by measurements of the development of turbidity at 350 nm, because this wavelength is above the normal absorption range of immunoglobulins and of protein antigens (Vincent, Harris & Yaverbaum, 1970; Davies, 1971; Ansari & Salahuddin, 1973; Nozaki, 1973). The elimination of interference of protein self-absorption in turbidimetric measurements of precipitin reactions is of special importance as the making of a precipitin curve implies either increasing antigen concentrations (α-procedure) or increasing antibody concentrations (β-procedure).

The present communication deals with a study of the possibility of eliminating self-absorption in the ultraviolet wavelength range by use of tandem cuvettes, employing separated and unmixed antigen and antibody as blank solution for spectrophotometric readings. This technique, called difference turbidimetry, allows recording of true turbidity spectra throughout the ultraviolet range. Difference turbidimetric precipitin curves have been made at several different wavelengths. The zoning phenomenon has been compared to the zoning phenomenon obtained by a conventional precipitin curve technique and by antigen binding precipitin curves.

It is demonstrated that the new technique, difference turbidimetry, provides a highly sensitive method for quantification of antigen–antibody interactions, and for recording the time course of the second slow stage of precipitin reactions.

MATERIALS AND METHODS

Monomeric and alkylated ^{131}I-labelled human serum albumin were prepared as described previously (Hill & Steensgaard, 1971). Corresponding cold iodinated human serum albumin was prepared as described by Jacobsen (1976). Rabbit anti-human serum albumin was obtained from DAKOPATTS, A/S, Copenhagen (code no 10–001, lot no 086). Quantitative precipitin curves were produced as described previously (Steensgaard, Johansen & Møller, 1975).

All spectrophotometric measurements were performed on an Aminco DW–2 spectrophotometer using split beam mode and matched tandem cuvettes (Hellma, Suprasil Quarts cuvettes, type 238). These cuvettes consist of two separated compartments each having a light path of 0·437 cm. The compartments are separated by a thin quartz plate with a mixing gap at the top allowing mixing of the content of the two compartments in the sample cuvette. Each compartment contained 1·0 ml of the appropriate solution. The antibody solution having the highest self-absorption in the ultraviolet range was consistently placed nearest the photomultiplier. Mixing of antigen and antibody was done by turning the sample cuvette upside down for 5 sec. In all experiments the reference cuvette contained the same amounts of antigen and antibody separated and unmixed as in the sample cuvette.

Difference turbidity spectra were recorded after adjusting the baseline before mixing the content of the sample cuvette by use of 15 trim potentiometers throughout the ultraviolet range 240–380 nm.

It proved important to rinse the cuvettes very carefully with a HCl/KCl buffer pH 2·2 between all measurements. All spectrophotometric operations were performed at 25°.

The concentration of antigen was varied as given in the text to figures. The final concentration of antibody in the sample cuvettes was in all experiments

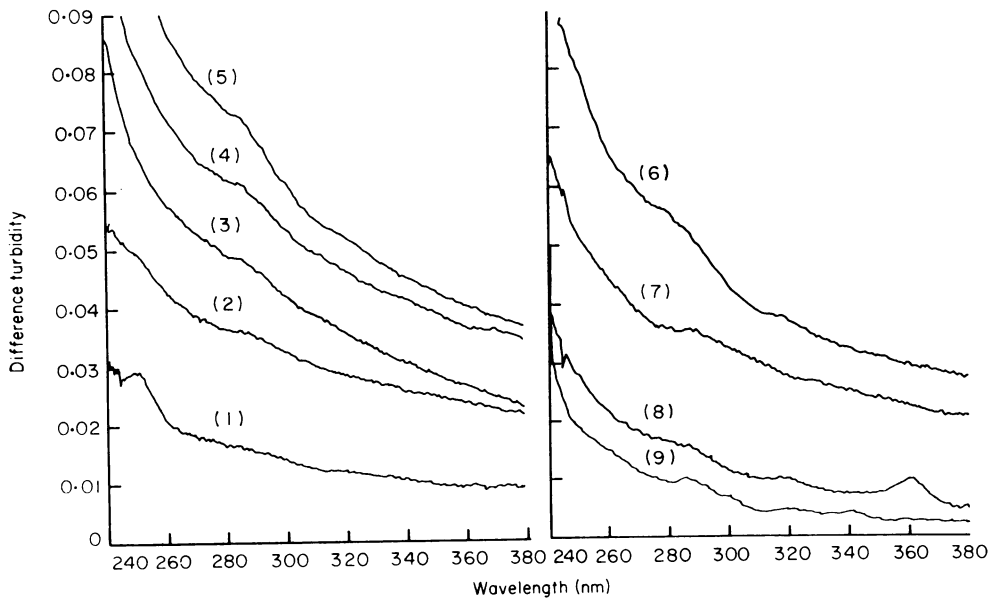

Figure 1. Difference turbidity spectra of antigen-antibody solutions obtained by using matched tandem cuvettes. The antibody (rabbit anti-human serum albumin IgG) concentration was 0·2 mg/ml in the final mixture. The antigen (iodinated human serum albumin) concentrations were 0·0012, 0·0025, 0·0037, 0·005, 0·01, 0·015, 0·020, 0·025 and 0·03 mg/ml in the final mixture. The curves are in succession denoted (1) to (9). The spectra were recorded after turbidity was developed for 600 s at 25° in the sample cuvette. The reference cuvette was loaded with corresponding concentrations of unmixed solutions of antigen and antibody.

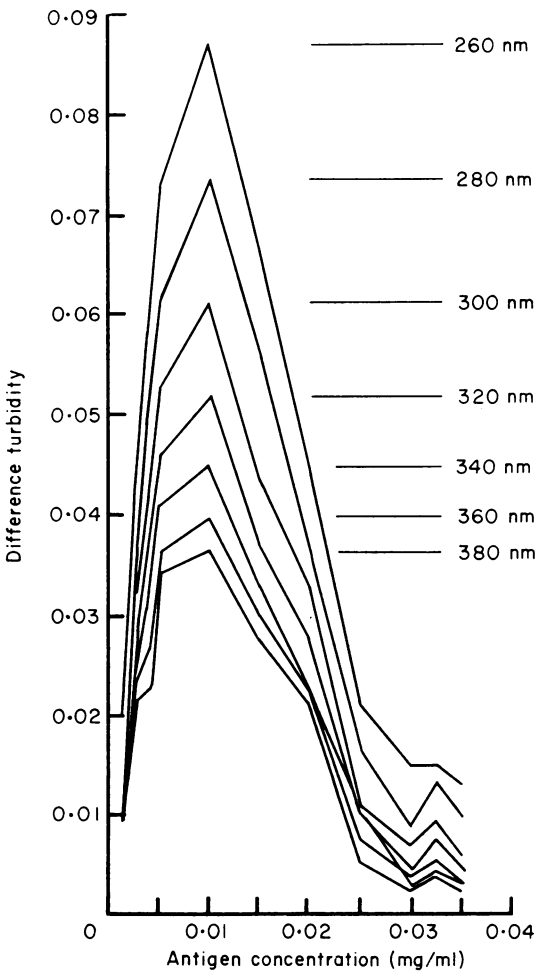

Figure 2. Precipitin curves based on difference turbidity measurements at various wavelengths. The experimental data are taken from Fig. 1.

0·2 mg/ml, calculated from an extinction coefficient of 15·0 for a 1% solution of IgG (Steensgaard *et al.*, 1975).

RESULTS AND DISCUSSION

Recording of difference turbidity spectra

Tandem cuvettes providing the opportunity of using unmixed antigen and antibody as blank solution for spectrophotometric measurements allow recording of true turbidity spectra. A series of difference turbidity spectra from 240–380 nm using the same concentration of antibody and increasing antigen concentrations has been recorded. The difference turbidity spectra after 600 sec of reaction are shown in Fig. 1. It appears from this figure that all individual spectra have common features. They all show highest turbidity at the shortest wavelength within the recorded range, and the turbidity decreases nearly unimodally with increasing wavelengths with a slight but consistent tendency of a minor peak at 280 nm.

The increase in turbidity of curves number 1 to 5 followed by a corresponding decrease in turbidity using still higher antigen concentrations, curves number 6 to 9, indicate that the difference turbidity spectra reflect a zoning phenomenon as known from conventional flocculation measurements. To investigate this further the difference turbidity at 7 selected wavelengths was plotted against antigen concentration following an α-procedure. The results are shown in Fig. 2. It appears directly that difference turbidimetric measurements clearly reflect the zoning phenomenon being characteristic of interactions between bivalent antibodies and multivalent antigens. Moreover, Fig. 2 also shows that the use of low wavelengths increases the sensitivity substantially. Thus, difference turbidity measurements at 260 nm provide more than a twofold improvement of the sensitivity as compared to direct turbidity measurements in the range of 340 to 360 nm.

Time course of the development of difference turbidity

The development with time of difference turbidity at 280 nm, chosen as a representative wavelength for difference turbidimetric measurements, is shown in Fig. 3 using varying antigen concentrations and constant antibody concentration. In this figure the recordings have been interrupted after 600 sec. It appears from the figure that two different types of development of turbidity can be distinguished. In heavy antibody excess (curves 1 and 2) the difference turbidity curve is s-shaped. The s-shaped curves probably reflect a two stage reaction indicating that the immunespecific reaction (i.e. formation of complexes *per se*) does not change the spectra of the antigen and/or the antibody themselves, but in a slower stage of the reaction which leads to formation of a precipitate turbidity is developed. With the equipment used a mixing time of 5 to 6 sec was required, and a possible s-shape of the other curves

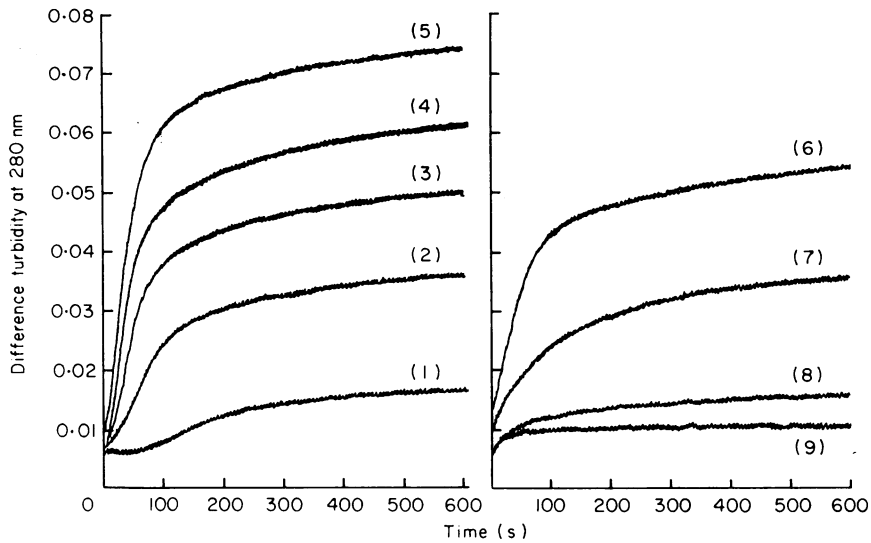

Figure 3. The time dependent development of turbidity at 280 nm. The final antibody concentration was 0·2 mg/ml and the antigen concentrations varied from 0·0012 mg/ml to 0·03 mg/ml denoted (1) to (9) as in Fig. 1.

appearing in the mixing period could not be detected. As observed by Davies (1971) by light scattering measurements the initial optical changes appear very rapidly.

It appears from Fig. 3 that a stationary state of turbidity is reached after 600 sec of reaction time. Longer reaction times have been tested, but proved impractical as the difference turbidity in the equivalence zone showed a tendency to decrease again, probably due to formation of floccules of a size that are able to sediment due to their own large size. We therefore found it most practical to use 600 sec as a standard reaction time in all experiments.

Comparison with other methods of recording precipitin curves

In Fig. 4 the difference turbidity measurements of precipitin reactions have been compared to two other ways of making precipitin curves. The first is the classical way of redissolving three times washed precipitate at pH 2·2 and measuring the absorbance at 280 nm. This was chosen because the absorbance at 280 nm mainly reflects the antibody content of the precipitate due to the large difference in extinction coeffecient of IgG and HSA. The second is measurements of radioactivity of ^{131}I-labelled antigen in the precipitate. It appears from Fig. 4 that all three methods of measuring precipitin reactions give rise to clearly expressed zoning phenomena and that the curves reach the baseline in extreme antigen excess. However, it is interesting that the point of maximum of the three curves is located at different antigen concentrations. Difference turbidity measurements give maximum at the lowest antigen concentration. Absorbance measurements of the dissolved precipitate at 280 nm show a somewhat higher point of maximum precipitation, and measuring the amount of precipitated radioactive antigen gives maximum at the highest antigen concentration.

The different appearance of the zoning phenomenon in the three techniques probably reflects differences in the underlying molecular mechanisms. In extreme antibody excess it is thermodynamically likely that antibody rich complexes of the type Ag_1Ab_n are formed (Steensgaard *et al.*, 1975; Steensgaard, Liu, Cline & Moller, 1977; Steensgaard & Frich, 1979). These complexes are accordingly characterized by having only one of their two antigen binding sites occupied. It is therefore interesting that reaction of only one site in antibody molecules is sufficient to evoke a hydrophobic change in the complexes eventually giving rise to a precipitate. The absorbance curve at 280 nm of redissolved precipitate is due mainly to the amount of antibody in the precipitate, partly because the

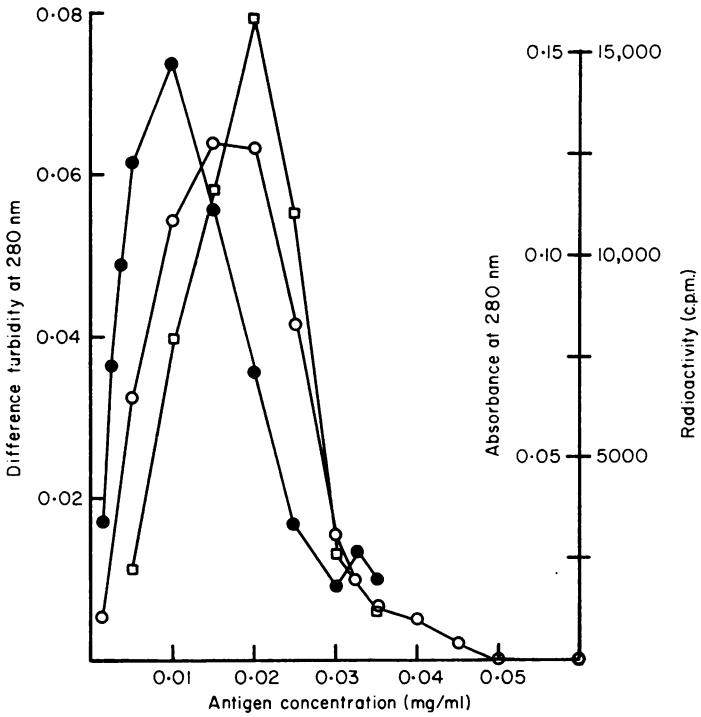

Figure 4. Comparison of precipitin curves obtained by three different techniques. In all experiments the antibody concentration was 0·2 mg/ml in the final mixtures. Difference turbidimetric measurements at 280 nm are denoted by (●). Measurements of absorbance at 280 nm of redissolved precipitates by (○), and radioactivity of iodinated antigen in the precipitates by (□)

extinction coefficient of IgG is 2·5 times the extinction coefficient of human serum albumin, and partly because precipitating complexes have an Ab/Ag ratio above 1. In case of the antigen binding precipitin curve it is interesting to note that maximum precipitation of antigen occurs in the antigen excess region of the equivalence zone of the redissolvation curve. In this part of a precipitin curve more complicated but not necessarily very large complexes are likely (Goldberg, 1952; Palmiter & Aladjem, 1968; and the previously mentioned references to our own work). In complexes formed under these conditions several antibody molecules will have both antigen binding sites occupied, and this may indicate that the occupation of both antibody sites may be an inherent advantage in the immobilization of soluble antigens.

Finally, we would like to emphasise that the experimental results presented here are performed with very low antibody concentrations (corresponding to more than a 30-fold dilution of a normal antiserum). The difference turbidity precipitin curves exhibit a very clearly expressed zoning phenomenon with low antigen concentration, and we therefore conclude that the difference turbidity principle is a very sensitive method for quantification of antigen–antibody interactions. Difference turbidity measurements have the potential to be used in future experiments to elucidate the molecular mechanism that cause antigen–antibody complexes of limited size to undergo a hydrophobic change, eventually leading to the formation of a precipitate.

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