A STUDY OF THE DISTRIBUTION OF INCOMPLETE RHESUS ANTIBODIES AMONG THE SERUM IMMUNOGLOBULIN FRACTIONS

By BARBARA E. DODD, PH.D, AND P. C. WILKINSON, M.B.B.S.

(From the Departments of Forensic Medicine and Bacteriology, The London Hospital Medical College, London, England)

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For many years now it has been realized that blood group antibodies, like many others, even though identical in specificity, display heterogeneity in physical and chemical properties and may be found associated with more than one kind of immunoglobulin.

Immunochemical techniques in fashion at particular times have been used by blood group enthusiasts to obtain more precise information about blood group antibodies. Thus, Coombs and Mourant (1) treated a human serum containing "incomplete" Rhesus (Rh) antibody with ammonium sulphate and found that the fraction containing most of the γ-globulin contained most of the incomplete antibody. There were also traces of antibody activity in the fraction containing α- and β-globulins. Mohn and Witebsky (2), in a series of papers, describe repeated dialysis of anti-Rh sera against distilled water for 18-hour periods from which they obtained three if not four separate globulin fractions with different serological properties. After a short interval, Hill, Haberman, and Guy (3) adopted the ion-exchange resin method described by Reid and Jones (4) in an effort to seek more precise separation of Rh antibodies. Later, Cann, Brown, Kirkwood, Sturgeon, and Clarke (5), by an electrophoretic method, found that Rh antibodies were distributed throughout the serum globulins and observed that "blocking" antibodies were most concentrated in gamma globulin of low mobility.

In 1955, Campbell, Sturgeon, and Vinagrad (6) explored the possibilities of separating complete and incomplete Rh antibodies by untracentrifugation and concluded that Rh saline agglutinins sedimented at a faster rate than the incomplete antibodies.

Further work on the fractionation of Rh antibodies by anion-cation exchange cellulose is described by Abelson and Rawson (7). In 1960 Chan and Deutsch (8) subjected Rh antibodies to a similar analysis with the addition of extensive studies of the effect of 2-mercaptoethanol on saline agglutinins and papain digestion on incomplete antibodies, the conclusion drawn being that the effect of both processes results in molecules which are unable to produce their usual agglutinative reactions but which are still capable of combining with the antigen.

The current methods of ion-exchange chromatography, originating from the work of Sober, Gutter, Wyckoff, and Peterson (9) and Fahey, McCoy, and Goulian (10), and density gradient ultracentrifugation (11) have not yet been extensively applied to the fractionation of blood group antibodies. The immunoglobulins isolated by the above

methods have been variously designated according to whether preference is given to nomenclature based on ultracentrifuged or immunoelectrophoretic findings. We propose to follow the example of Heremans, Vaerman, and Vaerman (12) and adhere to γ_{ss}[1]- (for 7S), γ_1A- (for β_2A) and γ_1M- (for 19S or β_2M) globulins.

It was our interest in certain Rh antibodies, which are detectable by an enzyme technique (papain) and not by antihuman globulin (13), which led us to fractionate sera which contained them by DEAE cellulose and by sucrose density gradient ultracentrifugation. The selected sera were from Rh-negative women with Rh antibodies, at various stages during pregnancy, immediately postpartum, and between pregnancies. Sera containing large amounts of complete Rh antibodies agglutinating red cells in a saline suspension were omitted since these Rh agglutinins were likely to mask the "papain-type" antibodies in which we were particularly interested.

Material and Methods

Red Cells for Titrations.—Rh-positive (CDecDE) and Rh-negative (cdecde) red cells were obtained by earprick and were used up to 48 hours from the time of bleeding. They were washed three times and then made up to convenient concentrations between 1 and 4 per cent for the tests.

Antiglobulin Sera.—Rabbit antihuman globulin sera were prepared by injection of either a 50 per cent ammonium sulphate precipitate of whole human serum or an appropriately purified immunoglobulin fraction. One mg of globulin was made up for injection in Freund's complete adjuvant. 0.5 ml of adjuvant-antigen mixture was injected, 0.25 ml into each inner ankle. Four to 6 weeks later rabbits were reinjected with 1 mg of globulin in 0.25 ml saline mixed with 0.25 ml $AlPO_4$ into several subcutaneous sites, and 0.1 ml was given intravenously. The animals were bled at 8, 10, and 12 days after the secondary injection.

Anti-γ_{ss} was prepared by injection of rabbits with purified DEAE, fraction I γ_{ss}-globulin from pooled human sera. Antisera revealing a single γ_{ss}-line only, on immunoelectrophoresis of normal human serum, were selected for use.

Anti-γ_1A was kindly supplied by Dr. J. F. Soothill of the Department of Experimental Pathology, University of Birmingham, and was absorbed with one part of 1 per cent γ_{ss}-globulin to twenty parts of serum before use. After this absorption it showed no detectable precipitation with human γ_{ss}- or γ_1M-globulins as tested by immunoelectrophoresis.

Anti-γ_1M was prepared by isolating the 19S fraction from a case of Waldenström's macroglobulinaemia by separation on sephadex G-200 followed by ultracentrifugation. This globulin was then injected into rabbits. Sera were chosen from those animals reacting optimally as tested by immunoelectrophoresis. They were absorbed with normal human serum from which the macroglobulin fraction had been removed by filtration through sephadex G-200.

Mercaptoethanol.—One part of 2 M 2-mercaptoethanol was added to 19 parts of antibody-containing serum or fraction to give a final concentration of 0.1 M mercaptoethanol. Treated and untreated fractions were incubated overnight at +4°C and titrated together immediately the following morning.

Bovine Albumin.—This was supplied by Armour and Co., Ltd., Eastbourne, England and was used at a concentration of 30 per cent.

AB Serum.—This was kindly supplied by the South London Regional Transfusion Centre and had been absorbed for the removal of any atypical blood cell antibodies.

[1] ss, *sensu stricto.*

Titration of Rh Antibodies.—The titrations of the Rh antibodies by saline, albumin, and antiglobulin techniques in the original sera and in prepared fractions were carried out by commonly used methods; therefore, technical details are omitted here. However, the following points are worth mentioning.

(*a*) The diluent for both saline and antiglobulin techniques was 0.15 M NaCl while that for the papain technique was human AB serum, which was antibody-free.

(*b*) The papain technique was a layering one and serial dilutions of whole serum or fractions were made in AB serum, then an equal volume of papain prepared according to Löw (1955) (14) was added to each tube *without* subsequent mixing. Finally, one unit volume of 1 per cent red cells of appropriate Rh type was added and, again without mixing, the tests were incubated at 37°C for 1 hour. Thus, the red cells start at the top of the fluid in each tube and travel gradually to the bottom, meeting first the papain layer and, afterwards, the test fluid. It should also be emphasized that, after the addition of the papain to the tests, they were not allowed to stand for longer than 10 minutes before being completed and placed in the incubator.

DEAE Cellulose Chromatography.—Three ml of serum was chromatographed on a column of 4.5 guanine DEAE cellulose (Whatman powder grade D.E. 50) using a cone and sphere gradient (10) of phosphate buffer from 0.01 M pH 7.5 to 0.3 M Na H_2PO_4. Fractions were pooled around the peaks, concentrated with polyethylene glycol (carbowax 20 M, G. T. Gurr, Ltd.) dialysed against 0.15 M buffered NaCl pH 7.2, and stored at −20°C before use. All protein estimations were performed at 280 mμ in a unicam spectrophotometer.

Ultracentrifuge.—0.5 ml volumes of serum were centrifuged in a Spinco model L ultracentrifuge using a sucrose density gradient from 10 to 50 per cent sucrose (11). Sera were run at 105,000 *g* for 18 hours. Fractions were collected by piercing the bottom of the tube. Sucrose was dialysed out against 0.15 M buffered NaCl pH 7.2 before use. The position of the 19S peak was checked by running a purified Waldenström's macroglobulin in a separate tube and the 7S peak by incorporation of a small amount of fluorescein-labeled 7S antibody.

RESULTS

The results of fractionation of sera containing incomplete anti-D antibodies by DEAE cellulose are shown in Fig. 1. In Fig. 1 *a* three peaks of antibody may be seen. The first peak represents antibody initially eluted at a molarity of 0.01. The second peak was eluted at just under 0.1 M and the third at about 0.15 M. It will be seen that all three peaks are detected by papain, but only the first two are detected by the antiglobulin technique. This finding was consistently repeated throughout many experiments using different sera. In experiments where an albumin technique was used to detect incomplete antibody, the pattern was similar to that using antihuman globulin; *i.e.*, the first two peaks only were detected.

Not all sera tested showed all three peaks. Fig 1 *b* shows a serum in which the second peak is absent. In Fig. 1 *c* there is only a small third peak. Fig. 1 *d* shows one of several sera in which antibody was detected in the first peak only.

The first antibody peak eluted from DEAE was made up entirely of γ_{ss}-globulin. Immunoelectrophoresis of this fraction against rabbit antihuman globulin confirmed the presence of a single γ_{ss}-line in this fraction. The second peak was eluted in a position which would correspond with the described chromatographic

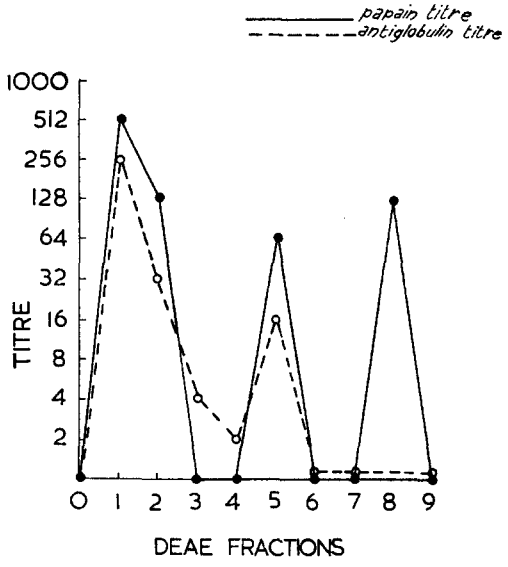

FIG. 1*a*

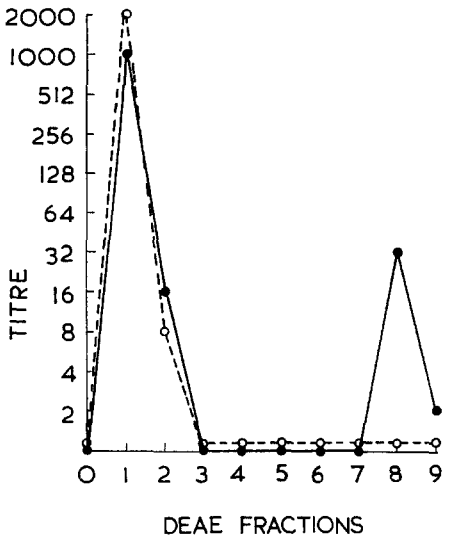

FIG. 1*b*

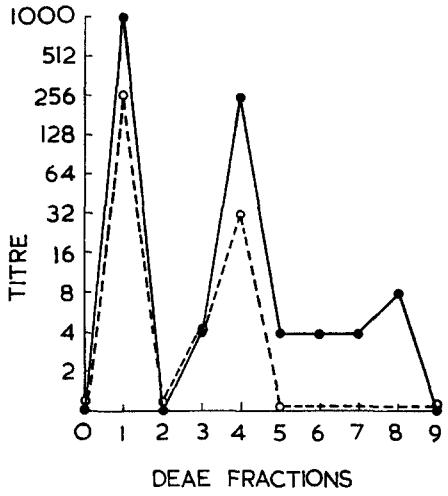

FIG. 1*c*

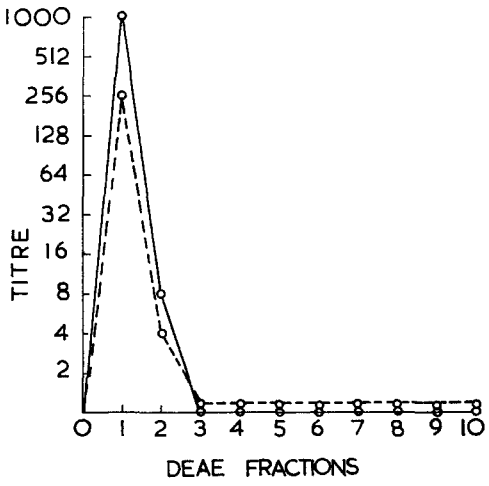

FIG. 1*d*

FIGS. 1 *a* to 1 *d*. DEAE cellulose chromatography. Distribution of incomplete Rh antibodies in four sera as tested by antiglobulin and papain techniques.

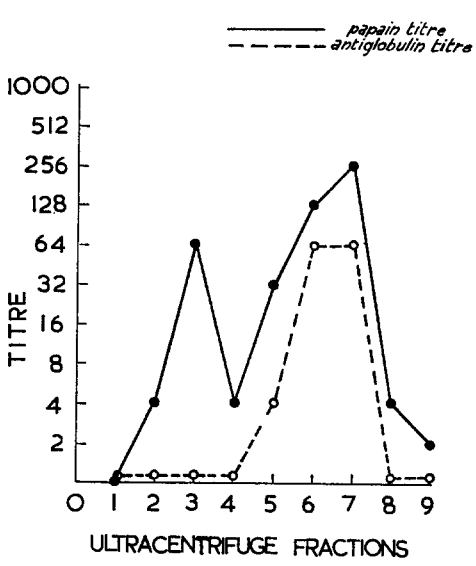

FIG. 2 *a*

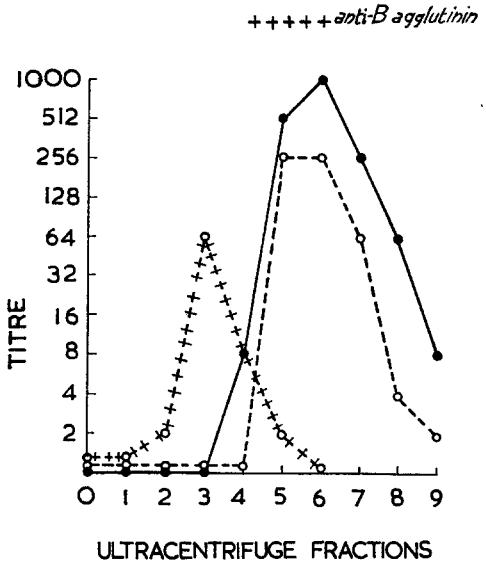

FIG. 2 *b*

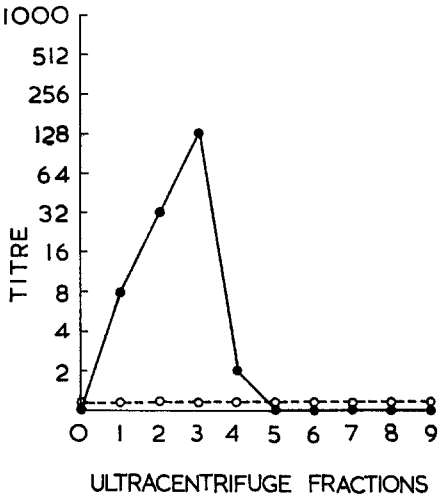

FIG. 2 *c*

FIGS. 2 *a* to 2 *c*. Ultracentrifuge patterns. Distribution of Rh antibodies. The 19S marker was maximal in tube 3 and the 7S marker maximal in tube 6 or 7 in all cases.

behaviour of γ_1A-globulin (15, 16), but this fraction would be likely also to contain electrophoretically fast moving γ_{ss}-globulin. The third peak was in the position associated with γ_1M-globulin (17, 18), but this fraction also would be contaminated with immunoglobulins trailing from earlier peaks.

Further experiments were carried out to determine more exactly how the antibodies in peaks 2 and 3 could be classified in terms of immunoglobulin. The results of sucrose density gradient ultracentrifugation experiments are shown in Fig. 2. Figure 2 *a* shows a serum containing 2 peaks of papain antibody, one in the 19S and one in the 7S position. Antibody detected by antiglobulin is present only in the 7S peak. This serum also contained saline anti-D which was confined to the 19S region. In Fig. 2 *b* a serum containing 7S incomplete anti-D

TABLE I

Fractions before and after Treatment with 2-Mercaptoethanol

Samples of anti-D	Peak I, 0.01 M PO_4		Peak II, 0.1 M PO_4 (app.)		Peak III, 0.15 M PO_4 (app.)	
	Before	After	Before	After	Before	After
Goddard	256	256	32	32	—	—
3695	16,000	16,000	256	128	8	—
4096	4000	2000	512	512	8	—
Boast 1	1000	1000	2	2	64	—
Boast 2	2000	2000	16	8	16	—
3829	512	512	64	64	8	—
Daltry	8000	8000	256	128	32	—
Boast 3	1000	1000	—	—	32	—
Anti-D	16,000	16,000	—	—	64	—

only is shown. The 19S peak is shown by the anti-B titre. Fig. 2 *c* is the ultracentrifuge pattern in a patient who showed traces only of antibody by the saline or antiglobulin techniques. The use of papain, however, revealed a good titre of incomplete anti-D. On centrifugation this papain anti-D was entirely confined to the 19S region.

The results of the treatment of DEAE fractions with 2-mercaptoethanol are shown in Table I. Antibody titres in peaks 1 and 2 were unaffected by 2-mercaptoethanol in almost all the fractions tested. The titre was reduced by one tube in a few cases. In the third peak, on the other hand, the antibody titre was reduced to zero in all cases; in sera containing saline anti-D, the titre was also reduced to zero.

It seemed clear therefore from these results that the antibody in peak 3 on DEAE was γ_1M-globulin. This fraction contained the saline agglutinating anti-D and also an incomplete macroglobulin antibody detectable only by papain and not by antiglobulin sera or albumin.

The middle peak was likely to contain γ_1A-globulin or γ_{ss}-globulin. In order to test for the presence of incomplete anti-D in these immunoglobulins, rabbit antiglobulin sera prepared specifically against γ_{ss}-, γ_1A-, and γ_1M-globulins were used. Peaks 1 and 2 were tested directly by the usual antiglobulin test for Rh antibody. Peak 1 reacted only with anti-γ_{ss} ; peak 2 reacted with anti-γ_1A but also with anti-γ_{ss} . All three peaks were also tested by antiglobulin consumption tests. This was necessary because the papain-type antibody in peak 3 was not revealed by the usual antiglobulin test.

The antiglobulin consumption test was effected by mixing equal parts of the DEAE peak to be tested with the appropriate rabbit antihuman globulin serum. The mixture was left on the bench for some hours and then in the cold overnight. The precipitate was removed by centrifugation, and a titre by papain technique of the Rh antibodies was made on the supernatant fluid and compared with the original Rh titre of the fraction.

TABLE II

Antiglobulin Consumption Test

	Anti-γ_{ss}	Anti-γ_1A	Anti-γ_1M
Peak 1	+	−	−
Peak 2	+	+	weak +
Peak 3	+	−	+

+, reduction in titre of Rh antibody after mixture with the particular antiglobulin serum; −, no reduction in titre.

A complete or almost complete inhibition of the activity of the Rh antibody in any peak fraction after treatment with a specific antiserum was taken to imply that the Rh antibody belonged to the class of immunoglobulins against which the rabbit antiserum had been prepared. Results of these tests are shown in Table II. Not unexpectedly, there was some cross-reaction between the anti-γ_{ss} reagent and the Rh antibody containing globulin of all three peak fractions. In addition, there was slight cross-reaction between anti-γ_1M and the immunoglobulins of peak 2. The most important feature to note is the absolute specificity of the anti-γ_1A which is reactive solely with the immunoglobulin of peak 2. In this peak an Rh antibody titre of 32 was reduced to 0 after treatment with the anti-γ_1A.

A direct titre of the Rh antibody in this peak by the antiglobulin method gave a value of 64 using a wide spectrum antiglobulin reagent and a value of 32 using the specific anti-γ_1A.

These results led us to the conclusion that the three incomplete anti-D peaks on DEAE contain γ_{ss}-, γ_1A- + some γ_{ss}-, and γ_1M-globulin antibodies respectively.

DISCUSSION

Several workers have shown the presence of incomplete Rhesus antibodies in the γ_{ss}-fraction of serum. In contrast, saline anti-D is associated with the γ_1M-fraction (17–19). In these studies however, treatment of D-positive red cells with papain was not used, neither had the significance of γ_1A-globulin as an antibody globulin been realised until recently. In the present communication, evidence is presented that *incomplete* Rhesus antibodies occur in all three immunoglobulin fractions. The distribution of such antibodies among these fractions is variable. Some of the sera studied lacked a second and third peak on DEAE cellulose. We would agree that the major proportion of the incomplete Rhesus antibody appears most often in the γ_{ss}-fraction. On the other hand, sera are found in which all of the antibody is γ_1M and is only detected by the papain technique (see Fig. 2 *c*).

There is now good evidence for antibody activity of various specificities in the γ_1A-globulin fraction of human sera. Schultze (20) found antibodies against diphtheria, tetanus, *Salmonellae*, poliovirus, and ABO isohaemagglutinins in the γ_1A-fraction, although his preparations were not absolutely pure. Heremans *et al.* (12) described antibodies to *Brucella abortus* and to diphtheria toxoid in γ_1A-fractions of high purity. We would confirm that antibodies to *Brucella* occur with great regularity in this fraction (21). Reagins have been reported as occurring in the γ_1A-fraction (15, 22, 23). Kunkel and Rockey (24) reported ABO agglutinins detected in high titre by purified antisera against human γ_1A-globulin.

The present work shows that, in certain sera at least, part of the incomplete anti-D titre is contributed by γ_1A-globulin molecules in addition to γ_1M and γ_{ss}. We feel that other antibodies may also be mixtures of the three types of immunoglobulin (12) and that there may be a danger of oversimplification in classifications of antibodies which describe them as entirely 7S or entirely 19S as the case may be.

The method of immunisation and the time interval following stimulation are known to play an important part in determining the distribution of many antibodies among immunoglobulin fractions. The data here presented are insufficient to relate patterns of distribution of Rhesus antibodies among these fractions to the stage or type of immunisation. It may be said that antibodies detected by papain only which are γ_1M in nature, are present early in immunisation. Four sera obtained 10 days postpartum all showed a high middle peak on DEAE but peak 3 (γ_1M) was virtually absent.

Comments on the "Papain-Type" Rh Antibody.—This antibody reveals itself as an incomplete macroglobulin which, in certain selected sera, can be isolated in the γ_1M-region on both DEAE and ultracentrifuge fractionation (see Figs. 1 *a* and 1 *b* and 2 *a* and 2 *c*). It fails to give a positive antiglobulin test when an attempt is made to sensitise cells with it, this despite the fact that antiglobulin

reagents were used which contained plenty of anti-γ_1M. This failure to react is not due to a failure of the antibody to react with its corresponding globulin, as can be shown by results of antiglobulin consumption tests. Moreover, as shown in a previous paper (13), a positive antiglobulin can be obtained with the routine wide spectrum antiglobulin reagent when D-- red cells are used for sensitisation instead of the more usual cells of type CDecDE.

A recent paper (25), describes the finding of an Rh antibody belonging to the γ_1M-globulin fraction which would not agglutinate cells in saline or give a positive antiglobulin reaction but produced agglutination after incubation with bromelin. This is, no doubt, the same as, or a similar, incomplete macroglobulin to the papain-type antibody described here.

The use of the D-- cells (*i.e.* Rh-positive cells having D and lacking Cc and Ee) probably has the effect, like papain treatment, of increasing the number of antigen sites available for this type of antibody.

A point of difference between these incomplete macroglobulins and some other macroglobulins of blood group specificity, *e.g.* anti-Le^a, is that they do not appear to bind complement. The papain titre is unimpaired when inactivated sera are used, and the use of fresh human serum as a source of complement does not bring about a positive antiglobulin reaction with normal Rh-positive (CDecDE) cells.

SUMMARY

Sera containing Rh antibodies were taken from Rh-negative women at intervals during pregnancy and postpartum. A selection of these was fractionated on DEAE cellulose and by density gradient ultracentrifugation.

The distribution among the immunoglobulin fractions γ_{ss}, γ_1A, and γ_1M of Rh antibodies in the sera of Rh-negative women during pregnancy was analysed by these fractionation procedures and also by treatment with 2-mercaptoethanol and by testing with rabbit antisera prepared against purified immunoglobulin fractions.

The experiments showed that incomplete Rh antibodies occurred in the γ_{ss}-, γ_1A-, and γ_1M-globulin fractions, although the bulk of the antibody was in the γ_{ss}. In addition, evidence that the previously described (13) "papain-type" antibody is an incomplete γ_1M-globulin is presented.

We have pleasure in acknowledging the keen technical assistance of Miss D. A. Eeles who performed many of the Rh antibody titrations.

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