The serum opacity reaction
of *Streptococcus pyogenes*: general properties of the streptococcal factor and of the reaction in aged serum*

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Although the serum opacity factor of *Streptococcus pyogenes* was first described by Ward & Rudd (1938) as an aid to differentiating non-capsulated group A streptococci from those of other groups, it was soon evident that a large proportion of non-capsulated strains of *Str. pyogenes* failed to produce an opalescence in serum. Gooder (1961) and Köhler (1963) found an apparent inverse relationship between production of serum opacity and of the M antigen. Subsequent studies (Top & Wannamaker, 1968) have confirmed the frequency of production of serum-opacity among strains for which it has been difficult to prepare satisfactory anti-M sera, but a consistent inverse relationship was found only among type 12 strains.

Krumwiede (1954) made some preliminary investigations of the serum opacity reaction and concluded that it was due to a lipoproteinase. Rowen (1961), and Rowen & Martin (1963) have made intensive studies of the reaction in fresh human serum and concluded that the opacity was related to cholesterol esterification. He also noted considerable opacity formation in aged serum that was not due to cholesterol esterification and which is still unexplained. In this paper we will present some observations on the reaction in aged serum and on the factor responsible for it. For convenience this factor will be referred to as an enzyme, although this is perhaps premature, since the exact nature of the substrate and of the reaction is unknown.

MATERIALS AND METHODS

Strains

Strains of group A streptococci were classified by conventional methods, by T-typing (Griffith, 1934) and by M-typing (Swift, Wilson & Lancefield, 1943). Strain AN 124, a T-type 11 M-negative strain, was used in most experiments. In addition, 3497, an M-type 4 strain, AN 369, a T-pattern 3/13/B 3264 M-negative strain, together with strains of M-types 4, 11, 13, 22, 25, 48 and 49 and M-negative

strains of T-types 2, 3, 4, 5, 11, 12, 28, 8/25/imp 19 and 3/13/B 3264 were used to detect strain variations (e.g. in heat sensitivity) of the enzyme. All cultures were grown in Difco Todd–Hewitt broth at 37° C. for 18 hr. unless otherwise stated.

Measurement of serum opacity

A mixture of 0·5 ml. of enzyme sample (cell suspension or extracted enzyme solution) and 3 ml. of horse serum (Grand Island Biological Co.) was incubated at 37° C. Opacity formation was followed by measuring the increase in optical density at 475 mμ in a Beckman DU spectrophotometer. When cell suspensions were used as enzyme sample, the suspensions were diluted to give an optical density at 600 mμ of 1·00.

Cell fractionation

Suspensions of washed cells were shaken with glass beads in a Mickle disintegrator at 0° C. (Michel & Gooder, 1962) and the resultant suspension centrifuged at 10,000 g. for 30 min. at 4° C. The supernatant fluid represented the cytoplasmic material whilst the deposit contained cell wall and cell membranes, which were separated by the differential centrifugation method of Freimer (1963).

Isolation of α-lipoprotein

α-Lipoprotein was isolated by flotation (Korn, 1959). Horse serum was dialysed against running water for 3 days at 4° C. to precipitate the euglobulin fraction. The density of the supernatant (pseudoglobulin) fraction was adjusted to 1·07 by the addition of potassium bromide and the resultant solution was centrifuged at 90,000g. for 16 hr. at 4° C. in the SW 39 head of a Spinco model L ultracentrifuge. The clear colourless fraction floating on top was discarded and the density of the remainder was brought to 1·21 by the addition of potassium bromide. Centrifugation of this solution at 90,000g. for 16 hr. at 4° C. caused the α-lipoprotein to float to the top from where it could be removed in a much purified state. Potassium bromide was removed from the α-lipoprotein solution by dialysis.

Analytical methods

Glycerol was determined by the method of Korn (1959). This method is based on the conversion of glycerol to formaldehyde, the latter being estimated by colour formation with chromotropic acid. The method cannot distinguish between glycerol and glycerol-1-phosphate. Free fatty acid was determined by the method of Duncombe (1963), cholesterol and cholesterol esters by the method of Kingsley & Schaffert (1949) and protein by the modified Folin method (Lowry, Rosebrough, Farr & Randall, 1951).

Determination of the effect of cations

Horse serum was dialysed against running water at 4° C. for 3 days. This resulted in the precipitation of euglobulins which were removed by centrifugation. The remaining pseudoglobulin fraction contained the substrate for the serum opacity reaction. Solutions of metal chlorides (analytical reagent grade) were then added

to the dialysed serum. The best mixture contained 2 ml. of dialysed horse serum, 1 ml. of the appropriate salt in the appropriate concentration and 0·5 ml. of enzyme solution (or suspension in the case of the cell-bound enzyme) in distilled water.

Heat sensitivity determination

A number of tubes containing 0·5 ml. amounts of enzyme solution (or suspension) were placed in a water bath at the required temperature. Tubes were removed at 5 min. intervals and immediately chilled in an ice-water bath. The remaining enzyme activity was determined by incubating the heated enzyme samples with 3 ml. of horse serum at 37° C. for 16 hr. and comparing the opacity produced with that produced by an unheated sample.

RESULTS

Variation of enzyme activity with age

Five ml. volumes were removed periodically from a growing culture of AN 124, merthiolate was added, and the cells were sedimented by centrifugation. The pellets were washed in distilled water, then suspended in distilled water to give an
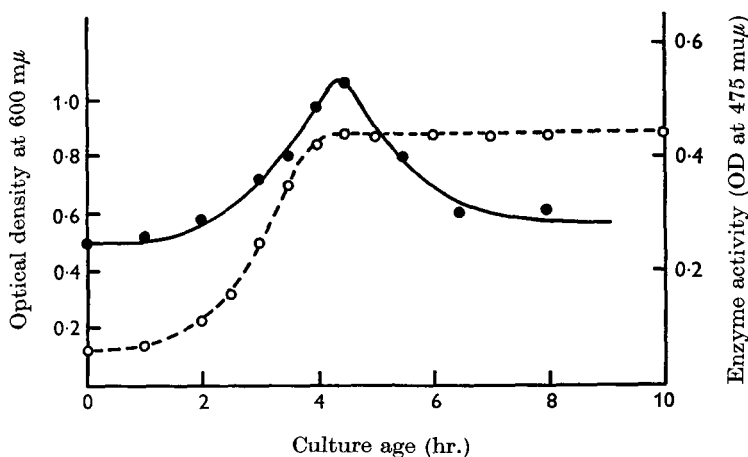

Fig. 1. Variation in the activity of the serum opacity factor of strain 3497 with age. ●—● enzyme activity; ○----○ growth curve (using the optical density at 600 mμ as a measure of the growth).

OD 600 = 1·00, and 0·5 ml. of this suspension was incubated with 3 ml. of horse serum at 37° C. for 16 hr. It was then centrifuged to remove the cellular material, and the OD of the supernatant fluid read at 475 mμ. The growth cycle was followed by measurement of the optical density of samples from the growing culture at 600 mμ.

Cells are able to produce an opacity in serum at all stages of the growth cycle (Fig. 1). The activity of the enzyme increased during the early exponential phase of growth, reaching a maximum towards the end of that phase, and decreased to a

variable extent during the stationary growth phase. This behaviour has been exhibited by all four strains tested (AN 124, AN 369, 3497, and the CDC type 22 strain).

Cellular location of the enzyme

Ward & Rudd (1938) found the enzyme in the Seitz filtrate of serum-broth cultures. Krumwiede (1954) and Rowen (1961) stated that in serum-free media the enzyme was cell bound with no extracellular activity. In initial studies with AN 124 we could find no extracellular enzyme activity, but subsequent studies have indicated that with certain strains some activity can be found in the supernatant, even when serum-free broth is used (L. W. Wannamaker, S. Skjold & F. H. Top, unpublished observations).

Table 1. *Activity of the serum opacity enzyme in cell wall and cell membrane fractions of AN* 124

	Enzyme activity	(OD 475/hr./mg. protein)
Sample	Cell-wall fraction	Cell-membrane fraction
1	0·06	0·19
2	0·02	0·14

When cells were fractionated into cytoplasmic membrane and cell wall components by mechanical breakage and differential centrifugation, no enzyme activity was detected in the cytoplasmic material but the membrane and cell-wall fraction both contained the enzyme. The enzyme activity was greatest in the membrane fraction (Table 1). Enzyme was determined in terms of increase in OD 475 per hr. per mg. protein. Protein content was estimated after extraction with 0·3 N-NaOH at 37° C. for 16 hr.

Extraction of the enzyme in a cell-free state

Krumwiede (1954), Rowen (1961) and Rowen & Martin (1963) obtained the enzyme in a soluble form by extraction of whole cells with urea. In their hands this gives a product of high activity and considerable stability. We have had much less success with this method and have, therefore, investigated other methods of extraction. Krumwiede also reported that the enzyme could be extracted with serum from whole cells; we have confirmed this.

Dissolution of the cell wall from wall-membrane fractions with phage-associated lysin (Maxted, 1957) releases some enzyme into solution, but the enzyme released is often of rather low activity and is unstable, 50 % of its activity being lost at −10° C. in 7 days. The addition of albumin or β-mercapto-ethanol failed to stabilize the enzyme. In addition to being unstable, the enzyme was also very impure, since it contained all of the protein material of phage-lysin itself and that released by phage lysin. Attempts at purification using ammonium sulphate precipitation were unsuccessful, since the enzyme activity was always lost. This method of extraction was highly unsatisfactory.

Extraction with 1 % sodium deoxycholate yielded a solution of enzyme of very high activity and also of great stability. Cells from 1 litre of overnight growth in Todd-Hewitt broth were Mickle disintegrated (Michel & Gooder, 1962) and the cell wall-membrane material sedimented by centrifugation at $10,000g$. for 30 min. Using this extraction method, an easily detectable opalescence (increase in OD 475 greater than 0·10 in 16 hr.) could be obtained using a 1/4000 dilution of the extract. By passing the extract through a column of G-50 Sephadex the enzyme could be freed from deoxycholate but was then less stable. Extracts freed from deoxycholate lost 50 % of their activity in 20 days at 4° C.; in the presence of deoxycholate *no activity was lost in 3 months at 4° C.*

None of the methods completely extracts the enzyme; some residual activity can always be found in the extracted cells. However, we find that the deoxycholate method of extraction is both easy to use and also very effective. All of our soluble enzyme preparations are made routinely by this method.

Enzyme substrate

Rowen & Martin (1963) have shown that, in fresh serum, extracts containing the opacity factor facilitate the transfer of fatty acids from the lecithin component of α-lipoprotein to cholesterol. A serum enzyme is responsible for the actual esterification, but the streptococcal factor makes the lecithin more available for reaction. They also showed that in aged serum there is no enhanced cholesterol esterification although there is still a serum opacity reaction. We have confirmed that in our system there is no enhanced cholesterol esterification. We have also found that pre-heating serum at 56° C. for 30 min. (which would destroy any remaining esterifying enzyme in the serum) does not reduce the amount of opalescence formed. Further, the addition of iodoacetate does not inhibit the reaction even at a concentration of 10^{-2} M. These three facts indicate that there must be some as yet unelucidated explanation for the opacity formation in our system.

In agreement with the results of Krumwiede and Rowen we found that, during the course of the reaction, the mobility of the α-lipoprotein, as determined by paper electrophoresis, is reduced. α-Lipoprotein was isolated by the flotation method and served as a substrate (or as an indicator) for the reaction in the presence of 1 % crystalline bovine serum albumin (Armour). During the course of the reaction there was a small increase in the amount of glycerol or glycerol-1-phosphate but no concomitant release of free fatty acid. We could obtain no opalescence in the absence of added albumin (in agreement with the results of Krumwiede, even though our method of lipoprotein isolation was much gentler).

It is of interest that extraction of whole serum with an equal volume of chloroform does not markedly reduce the ability to yield an opalescence when incubated with the enzyme. Obviously free chloroform-soluble lipids are not involved in the reaction.

An attempt was made to find a non-serum substrate for this streptococcal enzyme. The enzyme was unable to reduce the optical density of a suspension of streptococcal protoplast membranes (either from the homologous strain or heterologous strains). Strains which gave the serum opacity reaction were egg-yolk

negative when tested according to the method of Gillespie & Alder (1952). Further, the enzyme was unable to lyse sheep red blood cells.

Heat sensitivity of the enzyme

The sensitivity of cell-bound enzyme to heat at 60, 80 and 100° C. revealed considerable strain variation in this respect. The rate of loss of activity of a typical heat-sensitive strain (AN 369) is shown in Fig. 2. It is seen that 50 % of the activity
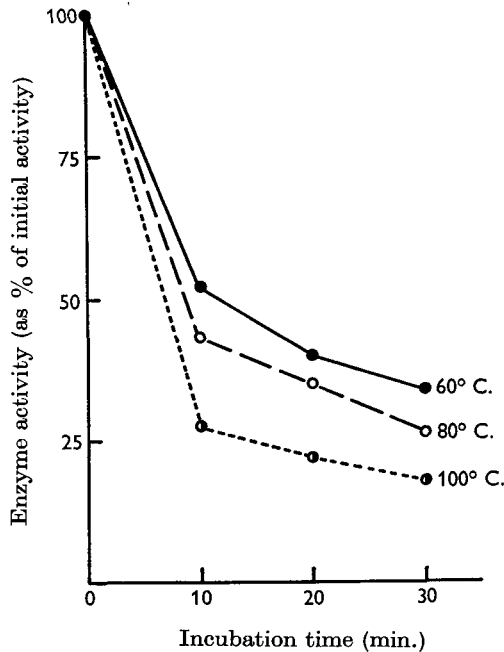

Fig. 2. The effect of heat at various temperatures on the activity of the serum opacity factor of strain AN 369.

is lost after only 10 min. of heating at 60° C., whilst 75 % is lost in 10 min. at 100° C. Approximately half of the strains tested behave in this way. The cell-bound enzyme in many strains, however, was resistant to heat at 100° C. for 60 min. and showed no loss of activity after such treatment. The heat denaturation curve for such a strain, 3497, is shown in Fig. 3. Extraction of the enzyme with deoxycholate reduces the heat resistance and removal of the deoxycholate renders the enzyme still more heat sensitive (Fig. 3). Even the deoxycholate-free enzyme of strain 3497 has a half-life of 40 min. at 100° C. The variation in heat sensitivity of the enzyme at various stages in the growth cycle was examined for strain 3497 (a heat-resistant strain) and strain AN 369 (a heat-sensitive strain). No variation in sensitivity was observed for either strain.

The pH of the suspension fluid used for incubating the enzyme is less critical than might be expected. For strain 3497 there was no reduction in activity after heating cell-bound enzyme at 100° C. for 60 min. at pH values between 4·0 and 8·6. Outside this range of pH the enzyme was much more heat sensitive (Table 2).
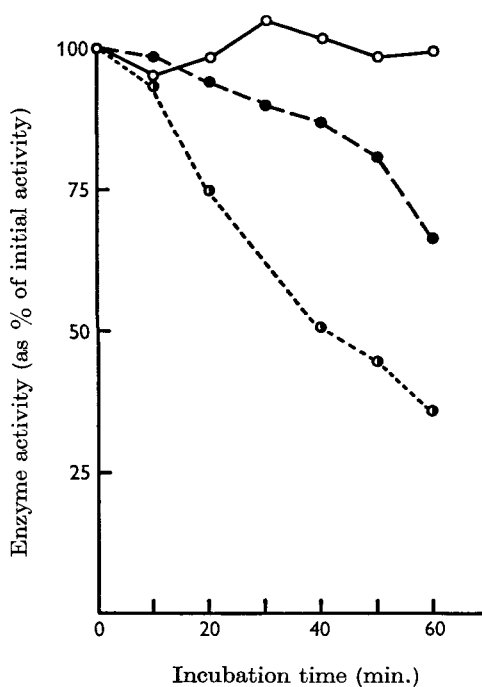
Fig. 3. The effect of heat at 100° C. on the activity of various samples of enzyme of strain 3497. ○—○ cell–bound enzyme; ●– – –● deoxycholate extract of enzyme; ◖·····◖ enzyme extract after removal of the deoxycholate.

Table 2. *Variation in heat sensitivity with the pH of the suspension fluid for enzyme from strain* 3497

pH of the suspension fluid	Time at 100° C. for 50% reduction of enzyme activity (min.)
3·0	20
3·9	60
5·8	60
6·9	60
8·6	60
9·3	7

Other characteristics of the enzyme

The pH optimum of cell-bound and cell-free enzyme was determined using as substrate whole serum and α-lipoprotein. The pH optimum using the crudest enzyme substrate system (whole cells with whole serum) was 5·8 (Fig. 4); this was also the optimum pH for the purest system—cell-free enzyme with α-lipoprotein.

The enzyme was sensitive to the proteolytic enzymes trypsin and pepsin (both enzymes were crystalline products from Armour and Co. and were used at a concentration of 0·005% for 2 hr. at 37° C.), but was resistant to streptococcal

proteinase. A crystalline product (which was kindly given to us by Dr S. D. Elliott) was used at a concentration of 0·01 %; the reaction was performed at 37° C. for 2 hr. at pH 7 in the presence of 0·05 M sodium thioglycollate. This latter observation is consistent with the fact that some of the best producers of α-lipoproteinase are also good producers of proteinase. The enzyme was destroyed
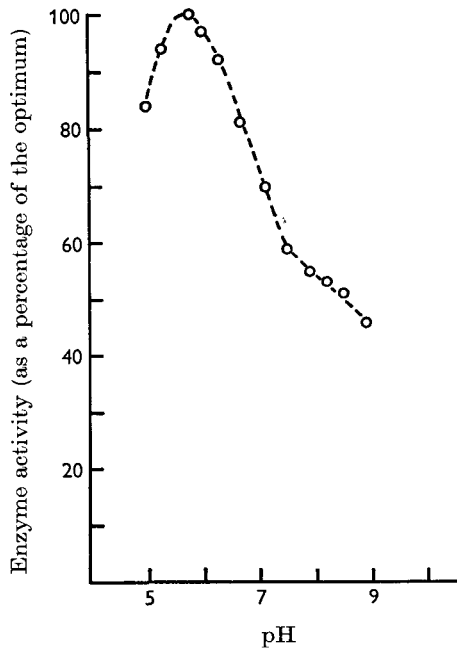

Fig. 4. Variation in the activity of the serum opacity enzyme with pH.

Table 3. *Effect of cations at various concentrations on the activity of cell-bound enzyme of strain AN 124*

OD 475 produced at various cation concentrations

Cation	3×10^{-1}M	3×10^{-2}M	1×10^{-2}M	3×10^{-3}M	1×10^{-3}M	3×10^{-4}M	1×10^{-4}M	0
K$^+$	1·05	0·70	0·57	0·47	0·49	0·49	0·48	0·50
Na$^+$	1·02	0·71	0·59	0·50	0·49	0·51	0·50	0·50
Mg^{++}	0·31	0·39	0·52	0·74	0·72	0·47	0·45	0·50
Ca^{++}	0·19	0·31	0·57	0·96	0·79	0·47	0·45	0·50

by incubation with 0·1 % formaldehyde or with 0·05 M sodium periodate for 30 min. at room temperature. The cell-bound enzyme, after such treatment followed by extensive washing with saline, was unable to produce any opacity in serum in 16 hr at 37° C. The inactivation with periodate was not reversible by treatment with reducing agents such as sodium arsenite or sodium thioglycollate.

The effects of cations on the reaction are complex. High concentrations of the divalent cations Ca^{2+} and Mg^{2+} (0·1 M or above) inhibited the reaction of both cell-bound and cell-free enzyme whilst lower concentrations had an activating effect

(Table 3). High concentrations of the univalent cations K^+, Na^+, Li^+, and NH_4^+ (10^{-2} M or above) activated cell-bound enzyme but were without effect on cell-free enzyme. The effects of the univalent cations were additive, as were the effects of the divalent cations. The action of univalent and divalent cations on cell-bound enzyme were not independent, since 0·3 M-NaCl failed to enhance the serum opacity reaction in the presence of divalent cations at a concentration of as little as 10^{-3} M. Further, the activating effect of 10^{-2} M-Ca^{2+} or Mg^{2+} was abolished by the presence of univalent cations. Univalent cations did not interfere, however, with the repression of the opacity reaction by higher concentrations of divalent cations.

DISCUSSION

The work reported here was done with aged serum so the results differ somewhat from those obtained by Rowen using fresh serum. For example, preheating the serum or adding iodoacetate, both of which were reported by Rowen to inhibit the reaction in fresh serum by repressing or destroying the cholesterol esterifying enzyme in the serum, had no effect on the serum opacity reaction in the system used here. This was presumably because the serum enzyme was no longer present, having been lost during the aging process. This conclusion is supported by the fact that we could detect no enhancement of cholesterol esterification during the serum opacity reaction.

These studies suggest that the cell-bound enzyme responsible for the serum opacity reaction may be primarily associated with the protoplast membrane. The proportionately smaller amount of enzyme activity found in the cell wall may be due to contamination of the walls with membrane material or to the leakage of the enzyme from the membrane through the cell wall. The latter explanation is not incompatible with the failure to detect extracellular enzyme in some strains, since cell-free enzyme can be very unstable. Enzyme leakage with subsequent stabilization provides a possible explanation for the apparent extraction of the enzyme with serum, since it is hard to envisage a membrane-bound enzyme being extracted so readily from whole cells. Another cell-bound activity of group A streptococci, the cell-bound streptolysin (Ginsburg, Harris & Grossowicz, 1963) is also extractable with serum. This appears to be able to diffuse into the medium and bind to lipoprotein, albumin and other constituents.

The difference in effect of univalent cations on cell-bound and cell-free enzyme is of interest. Since they have no effect on cell-free enzyme, the activation must be indirect. Presumably they assist in exposing the enzyme to the substrate via some action on the cell membrane. Since the action of univalent cations is inhibited by the presence of divalent cations, it could be postulated that this is competition for sites at the membrane, with the divalent cations being more tightly bound. The effect of divalent cations does not depend on the enzyme being cell bound; consequently it can be postulated that they are acting directly on the enzyme-substrate system and that this is a true case of cation inhibition.

All preparations of soluble enzyme leave much to be desired with respect to purity. Preliminary investigations using sucrose gradients, DEAE-cellulose columns

and ammonium sulphate fractionation proved fruitless. However, the enzyme appears to be precipitable with alcohol and this, together with its resistance to streptococcal proteinase, may provide a means of obtaining more satisfactory preparations. The best method of obtaining a stable enzyme preparation with high activity is to extract the cell wall membrane fraction with 1 % sodium deoxycholate at 0° C. for 16 hr. Deoxycholate has detergent properties and will dissolve protoplast membranes; it has also been shown to be capable of fractionating bacterial cell walls (Hill, 1967) and may prove to be a valuable tool in the extraction of enzymes or antigens from cells.

SUMMARY

The capacity of certain strains of *Streptococcus pyogenes* to produce opacity in aged horse serum has been studied. Cells from all stages of the growth cycle are able to produce opacity. Maximal activity is reached towards the end of the exponential phase of growth.

Examination of cell fractions obtained by mechanical breakage and differential centrifugation suggested that the cell-bound activity is predominantly associated with the membrane fraction. Extraction with sodium deoxycholate yields a soluble fraction of high activity.

There is considerable strain variation in heat stability of the serum opacity factor. Cell-bound activity is often quite resistant to heat, whereas extracted activity is less stable.

Low concentrations of divalent cations have an activating effect, whereas high concentrations inhibit the serum opacity reaction. High concentrations of univalent cations are without effect on the cell-free enzyme but have an activating effect on the cell-bound enzyme.

For both the cell-bound and the cell-free enzyme the pH optimum was 5·8.

Although sensitive to trypsin and pepsin, the serum opacity factor appears to be resistant to streptococcal proteinase. Its activity is destroyed by formaldehyde and by periodate but is unaffected by a number of reducing agents.

Pre-heating of the serum or the addition of iodoacetate did not affect the serum opacity reaction. The enhanced cholesterol esterification previously described with fresh serum appears to be a secondary reaction. Even when isolated by relatively gentle methods, α-lipoprotein serves as a substrate only in the presence of crystalline serum albumin.

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