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Mechanism of Action of Specific Antiserum on *Pasteurella septica*

Selective Inhibition of Net Macromolecular Synthesis and Its Reversal by Iron Compounds

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1. Circumstantial evidence suggests that the ability of the host to prevent the uptake of iron by pathogenic bacteria may constitute an important means of defence. A detailed biochemical study of the way antisera exert their antibacterial effects and the possible involvement of iron has now been initiated.

2. Work with *Pasteurella septica* antiserum has revealed a totally unsuspected way in which antiserum and complement exert their antibacterial effects. The process is very quick and specific and results in the cessation of bacterial multiplication; cells in stasis then appear to be killed by the operation of a secondary event. The inhibitory process operates by affecting the biochemistry of the bacterial cell leading first to an inhibition of net RNA synthesis and then to an inhibition of all macromolecular synthesis.

3. The presence of free iron in the serum did not prevent the initiation of the inhibition but it did allow macromolecular synthesis, cell multiplication and rapid growth to be resumed later. In contrast, the presence of haematin in the serum allowed the cells to continue multiplying in the presence of antiserum without delay. Addition of haematin to bacteria which had been inhibited by antiserum restored net RNA synthesis immediately. Net protein synthesis and multiplication were restarted within 15–20 min and DNA synthesis in 40 min.

A certain amount of confusion exists in the literature regarding the nature of the antibacterial action of normal or immune sera. Sometimes, this is due to a failure to distinguish clearly between the bactericidal and bacteriostatic effects of serum factors. For example, the action of normal guinea pig serum on *Salmonella paratyphi* C and *Salmonella typhimurium* has been described as bactericidal [1]. Closer examination of the results, however, shows that only with *S. paratyphi* is the action really bactericidal; with *S. typhimurium* the figures show typical bacteriostasis. In other cases, even when the two effects have been clearly distinguished, their relationship to each other and to the mechanisms of host resistance to bacterial infection remains obscure. Bullen and Rogers [2] have shown that while the bactericidal effects of fresh normal rabbit serum on *Escherichia coli* strain 0111 can be abolished by heating at 56 °C for 30 min, the heated serum, nevertheless, still retains the power to inhibit bacterial growth and bacteriostasis ensues. In normal horse and goat serum the presence of 15% *Pasteurella septica* antiserum has a bactericidal action on *P. septica*, but in rabbit serum its effect is bacteriostatic [3]. Heating the sera in this case abolished both the bacteriostatic and bactericidal effects. Contributing to the confusion in this field is the lack of precise biochemical knowledge of the way in which normal or immune sera actually achieve their antibacterial effects.

A substantial amount of circumstantial evidence has now accumulated from work both *in vivo* and *in vitro* which suggests that the ability of an organism to acquire iron from serum transferrin may be an essential feature of pathogenicity [2–5]. Conversely, the evidence suggests that the ability of the host to interfere with this reaction may constitute an important means of defence. Thus, the addition of sufficient iron to saturate the transferrin has been shown capable of reversing both the bacteriostatic and bactericidal effects of sera [2,3]. If it were established that the bacteriostatic power of sera was due to a derangement of bacterial iron metabolism, and that this was the primary non-cellular host defence mechanism, then it would mean that the killing or lytic effects were secondary and subsequent reactions; their reversal by iron could then be easily explained.

Clearly, the way to resolve some of these points is to determine the sequence of events in the organism culminating in bacteriostasis or death, and then to see what part iron plays in reversing the process. With this aim in mind, the effect of specific antiserum on the growth of *P. septica* in horse serum was examined; with this organism both specific antiserum and a heat-labile factor in fresh serum, possibly com-

plement, have been shown to be essential for antibacterial action [2]. Part of the work has already appeared as a preliminary communication [6].

MATERIALS AND METHODS

Organisms and Media

P. septica (strain CN3449, Wellcome Research Laboratories) was passaged repeatedly in mice until it attained a high degree of virulence, with a lethal dose of less than ten organisms. Details of the methods used for passage and storage of organisms have been described by Bullen, Wilson, Cushnie and Rogers [7].

The bacteria were grown for 3 h at 37 °C in papain digest broth containing 1% (w/v) of yeast extract (Difco). Cultures were harvested by centrifuging at room temperature and the organisms resuspended in a 10% (v/v) mixture of papain digest broth in 0.15 M NaCl (10% broth-saline). The total cell count per ml was obtained by the use of a colorimeter (E.E.L., filter number 622) and estimating the value from a standard graph. Suitable dilutions for inoculation were made in 10% broth-saline solutions. Viable counts were made on fresh blood agar plates in the usual way. To ensure that agglutination was not affecting the measurement of viable count, some samples were homogenized for about 7 min in an ice bath; no difference was found, however, between the homogenized and unhomogenized samples and this operation was, therefore, later omitted.

Sera

Whole normal horse serum was obtained from the Wellcome Research Laboratories (Horse serum No. 3) and kept at −70 °C. Specific *P. septica* horse antiserum (Pony PS1) was the same as that used previously by Bullen *et al.* [7]. All the sera were sterilized by membrane filtration.

Growth of Bacterial Cultures in Sera under Controlled Conditions

P. septica was grown in serum under controlled conditions in a two-necked, jacketed culture vessel similar to that described by Rogers [8], the contents of which were stirred continuously with a plastic-coated magnetic follower. Oxygen tension in the serum was maintained at a pO_2 of 80—90 mm Hg by passing a sterile mixture of an equal volume of (95% air + 5% CO_2) and (95% N_2 + 5% CO_2) through the vessel. The assembled apparatus allowed the gas mixture to enter the vessel through one side arm and to pass down a central tube to just above the surface of the serum; exhaust gas left the apparatus through another narrow side arm which also served as an inoculating and sampling point. pH was established at 7.5 by the presence of 5% CO_2 in the gas passing over the surface of the serum [9] and a temperature of 37 °C maintained by pumping water at the required temperature through the vessel's outer jacket. The serum in the vessel was equilibrated with the gas for 1—1½ h at 37 °C before inoculation. Samples were added and removed with a suitable sterile syringe fitted with a 1-in 23-gauge needle and a length of polyethylene cannula.

Incorporation of Labelled Precursors into Nucleic Acid and Protein

For studies on cellular synthesis, *P. septica* was inoculated into normal serum containing appropriate radioactive precursors 1—1½ h before specific antiserum, also containing the radioactive isotopes, was added at time zero. In control experiments, an equal volume of normal serum containing the radioactive labels replaced the antiserum. Protein synthesis was followed by the incorporation of a mixture of ^{14}C-labelled amino acids (^{14}C-labelled *Chlorella* protein hydrolysate, final activity 0.4—0.6 μCi/ml serum). RNA and DNA synthesis was measured by the incorporation of [^{32}P]phosphate ([^{32}P]orthophosphate, neutralized, final activity 1—2 μCi/ml serum). Reaction mixtures with a total volume of 15, 20 or 25 ml were used.

The assay procedures were essentially the same as those used by other workers [10—12] but modified to deal with the special difficulties arising from the fact that the bacteria were growing in serum. Addition of trichloroacetic acid to the bacteria in serum, as is usually done with cells growing in labelled media to precipitate bacterial protein and nucleic acid, was unsatisfactory since it led to the precipitation of a mass of contaminating serum proteins. To overcome this difficulty, the organisms were removed from the serum by collection on a Millipore filter and the assays carried out with the bacteria deposited on the discs.

Incorporation of ^{14}C-Labelled Amino Acids into Protein. Samples (0.5 or 1 ml) were removed from the culture vessel at intervals and mixed with 4—5 volumes of 5% (w/v) casamino acids in 0.15 M NaCl at 0 °C. This served both to dilute the ^{14}C-labelled amino acids with unlabelled amino acids and to facilitate filtration. The bacteria were collected on a 0.22 μm Millipore filter, numbered for identification, and the filter washed immediately with 2 ml ice-cold 5% (w/v) trichloroacetic acid. Filter discs were transferred to a beaker containing 150 ml of ice-cold 5% (w/v) trichloroacetic acid and washed together for 1 h; during this time the contents of the beaker were gently stirred two or three times. The contents of the beaker were then heated, at 90 °C, for 15—20 min, the discs finally being transferred to a second beaker of ice-cold 5% (w/v) trichloroacetic acid. Each filter was again washed, by suction, with 3 ml of cold 5%

(w/v) trichloroacetic acid and then dried for 30 min at 60—65 °C. Numerous radioactive samples could be processed in this way, the transfer of radioactive material from one to another being negligible; this was checked by mixing a series of blanks with the radioactive filters in the experiments. In those experiments where haematin hydrochloride was added to serum, the Millipore filters were stained dark brown. Haematin staining was removed by introducing an additional step into the assay procedure of such experiments. After washing in cold 5% (w/v) trichloroacetic acid, the filters were washed together, at room temperature, in 75% (v/v) ethanol (200 ml). The discs were then heated in 5% (w/v) trichloroacetic acid, washed and dried as before.

Incorporation of ^{32}P into RNA and DNA. Duplicate samples (0.5 ml) were removed from the culture vessels, mixed with 4—5 ml of cold Na_2HPO_4—NaH_2PO_4 buffer (0.2 M, pH 7.3), to dilute the [^{32}P]-orthophosphate, and filtered through a numbered 0.22 μm Millipore filter. After washing with 2 ml of ice-cold 5% (w/v) trichloroacetic acid by suction, the filters were transferred to a beaker containing 150 ml of ice-cold 5% (w/v) trichloroacetic acid and washed together as before. The filters were then digested for 2 h at 37 °C with 75% (v/v) ethanol (200 ml) to remove most of the phospholipid [13]; this operation also served to remove the brownish stain found on filters in those experiments where haematin hydrochloride had been added to the sera. After ethanol digestion, one half of the duplicate set of samples was put into 5% (w/v) trichloroacetic acid (150 ml) and washed again by suction with 3 ml of the same reagent. Finally, the filters were dried, as before, and the radioactivity on them determined; this represented the residue containing RNA and DNA. The other half of the duplicate set after ethanol digestion was washed with water, to remove ethanol, and the filters then digested individually in numbered tubes with 3 ml of 1 M NaOH for 15 h, at 37 °C, to hydrolyze the RNA. During alkaline digestion, the Millipore filters were partially dissolved. At the end of digestion, the tubes were cooled to 0 °C and the digest acidified with ice-cold 20% (w/v) trichloroacetic acid (7 ml). After standing in ice for 1 h, the contents of the tubes were filtered through 0.45 μm Millipore filters and the filters washed by suction with 5 ml of ice-cold 5% (w/v) trichloroacetic acid and dried as before. The radioactivity on the second set of filters was determined for the residue containing DNA. Radioactivity in RNA was obtained by subtracting that in the residue containing DNA from that in the residue containing RNA and DNA. It was shown by incorporating blanks into the experiments that the transfer of radioactive material from one filter to another during manipulations was negligible.

All filters were counted in a Beckman LS133 liquid scintillation counter using a scintillation solution containing 4 g of 2,5-diphenyloxazole in 1 l of toluene. Results are expressed as counts per min above background.

Labelling of Cells with ^{32}P or ^{14}C

In order that the labelled cells would already be adapted to grow in serum, the labelling procedure was carried out by growing them in serum containing the radioactive labels. Fresh normal horse serum (4 ml), containing [^{32}P]orthophosphate (neutralized, 8 μCi/ml serum), was inoculated with sufficient *P. septica* to give about 5×10^8 cells/ml and incubated at 37 °C for 45 min. The bacteria were collected, by centrifugation in a bench centrifuge at room temperature for 30 min, resuspended in fresh unlabelled serum (4 ml at 37 °C) and used immediately.

A similar procedure was used for obtaining ^{14}C-labelled bacteria, the [^{32}P]orthophosphate being replaced by a mixture of ^{14}C-labelled amino acids (*Chlorella* protein hydrolysate, 1—2 μCi/ml serum).

Release of ^{32}P from Labelled Bacteria

Equal portions of a suspension of labelled bacteria in serum were added to a 30% mixture of antiserum and normal serum and to normal serum alone, to act as control, and incubated under the controlled conditions described before.

Samples (1 ml) were removed from the culture vessel at intervals and mixed with 4—5 ml of cold Na_2HPO_4—NaH_2PO_4 buffer (0.2 M, pH 7.3) and the mixture filtered immediately through a 0.22 μm Millipore filter. The filter was then washed with 3 ml of cold phosphate buffer, dried in an oven at 60—65 °C for 30 min and assayed for radioactivity as described above.

In experiments where the cells were labelled with ^{14}C-labelled amino acids, the phosphate buffer wash was replaced with an aqueous solution of casamino acids in 0.15 M NaCl (5% w/v).

Uptake of Radioactive Labels by P. septica

Specific antiserum was added to bacteria which had been growing in normal serum under controlled conditions for 1.5 h, and an equal volume of normal serum added to the control. 30 min later, [^{32}P]orthophosphate (neutralized, 1—2 μCi/ml serum) was added to both vessels and its uptake by the cells measured. Samples (0.5 ml) were withdrawn at intervals, mixed with 4—5 ml of cold Na_2HPO_4—NaH_2PO_4 buffer (0.2 M, pH 7.3) and filtered immediately through a 0.22 μm Millipore filter. The filters were washed with 3 ml of cold phosphate buffer, dried and counted as described before.

In studies on the uptake of amino acids by *P. septica*, the [^{32}P]orthophosphate was replaced by

^{14}C-labelled *Chlorella* protein hydrolysate (0.5 μCi/ml serum) and the phosphate buffer by a solution of casamino acids in 0.15 M NaCl (5% w/v).

Materials

Ferric ammonium citrate (Laboratory reagent, Fe 20—22%, w/v) and Haematin hydrochloride (Fe 8.7%) were from British Drug Houses Ltd. (Poole, Dorset, U.K.); the ^{14}C-labelled amino acid mixture (*Chlorella* protein hydrolysate; 52 Ci/atom carbon) and [^{32}P]orthophosphate (48.9 Ci/mg P) were from The Radiochemical Centre (Amersham, Bucks, U.K.).

RESULTS

Effect of Specific Antiserum on Growth of P. septica *in Normal Horse Serum*

To eliminate possible complications introduced by variations in the metabolic state of the organisms as they adjusted to growth in serum after their growth in papain-digest broth—1% (w/v) yeast extract, specific antiserum was added to bacteria already growing exponentially in normal serum. *P. septica* grows rapidly in fresh normal horse serum after a lag period of about 1 h [6]. In order to ensure that the cells were fully adapted to growth in serum, the time of adding specific antiserum, time zero, was always 1—1.5 h after inoculation. When specific antiserum was added to rapidly growing cells, to give a 15% mixture, the viable count continued to increase at the same rate for approximately 30 min. Cell division then stopped and a loss of viability followed. The sequence of events occurring after the addition of antiserum are seen in detail in Fig. 1.

Fig. 2 shows the effect of a fixed amount of antiserum (15%) on inocula of various sizes. In all three cases, multiplication continued at the same rate for 30 min after adding the antiserum; likewise, cell division stopped between the 30th and 40th minute. However, it can be seen that the fall in viable counts varied somewhat with the number of viable organisms present, the greater the number of cells present in relation to the antiserum, the less the killing that occurred. By increasing the amount of antiserum added to 30%, a drop in viable count similar to that seen with the small inoculum and 15% antiserum was obtained with the highest inoculum. The initial growth pattern, however, remained the same, with cell division stopping between the 30th and 40th minute. These results suggest that the serum components responsible for cellular death differ from those responsible for the inhibition of bacterial multiplication.

In the present work, enough antiserum to give a 15% mixture has been used with inocula of about 10^6 cells/ml and 30% antiserum with inocula of over 10^7 cells/ml.

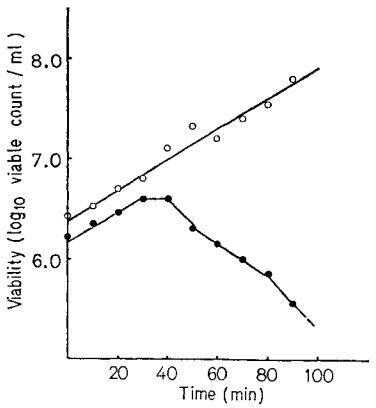

Fig. 1. *Effect of specific antiserum on the growth of* P. septica. ○, normal horse serum; ●, normal horse serum with antiserum (15%) added at zero time. An equal volume of normal serum was added to the control at the same time

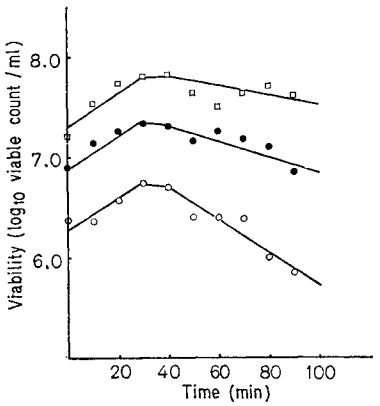

Fig. 2. *Effect of 15% specific antiserum on the growth of inocula of various sizes.* Antiserum was added at zero time. Initial viable count: ○, 2×10^6 cells/ml; ●, 8×10^6 cells/ml; □, 2×10^7 cells/ml

Changes in Cell Permeability or Complete Lysis Brought about by Specific Antiserum

It was important to know at an early stage whether changes in the growth pattern brought about by antiserum were the result of changes in the permeability or lysis of the cells, an effect which is popularly held responsible for the antibacterial action of antibody and complement [1, 14—17]. To investigate this possibility, bacteria which had been labelled with ^{32}P by growing them in serum containing [^{32}P]-orthosphosphate were added to a 30% mixture of antiserum and fresh normal horse serum and to normal horse serum alone to act as control; because of the higher cell numbers used in this experiment, the amount of antiserum used was doubled. No loss of labelled material from the cells occurred during growth in normal serum, as would be expected (Fig. 3).

Similarly, there was no loss of label for up to 50 min in the presence of antiserum, but at about the 60th minute the cells did begin to lose labelled material (Fig.3). It is important to note, however, that the loss of label only occurred after the cessation of multiplication; gross permeability changes, or lysis, could not, therefore, have been responsible for this event. Identical results were obtained by using cells grown in serum containing ^{14}C-labelled amino acids.

Selective Inhibition of Net Macromolecular Synthesis

Since no apparent loss of labelled material from *P. septica* was observed until about 1 h after the addition of specific antiserum, the biosynthetic capabilities of the cells under conditions leading to the cessation of multiplication and death were examined using radioactive isotopes. Bacteria were inoculated into normal serum, labelled with the appropriate radioactive isotope, 1—1.5 h before antiserum (15%) was added at zero time.

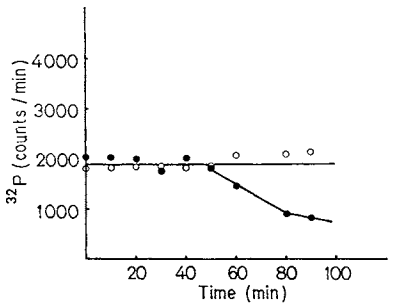

Fig.3. *Release of ^{32}P from labelled* P. septica. ○, bacteria growing in normal horse serum; ●, bacteria in 30% antiserum; antiserum was present from time zero

Fig.4A shows that protein accumulation continued normally for about 40 min after the addition of antiserum but stopped abruptly between the 45th and 55th minute. Very little loss of label occurred from the hot trichloroacetic-acid-insoluble material after this time, indicating that there was no gross destruction of the cells. Specific antiserum had a marked and early effect on net RNA synthesis (Fig.4B). This was depressed within 15 min of the addition of antiserum, well before any effect on cell multiplication was noted, and blocked completely after 40 to 50 min; a loss of label from this fraction then took place. Under similar conditions, DNA synthesis continued for approximately 90 min before stopping (Fig.4C).

Uptake of Radioactive Labels

One possible explanation for these results was that antiserum was interfering with the uptake of the radioactive labels into the cells. To see whether this was indeed the case, the uptake of [^{32}P]-orthosphosphate and the ^{14}C-labelled amino acid mixture was examined 30 min after adding antiserum; at this time the rate of RNA accumulation would have been drastically reduced and cell division virtually stopped. Fig.5 shows that there was no difference between the uptake of [^{32}P]orthophosphate by organisms in normal serum and by those in antiserum. Identical results were obtained for the uptake of the ^{14}C-labelled amino acid mixture; it is possible, however, that the uptake of one particular amino acid component of the mixture could have been affected.

The Effect of Added Iron on the Inhibitory Process

To examine the effects of free iron on the inhibitory process, enough ferric ammonium citrate was added

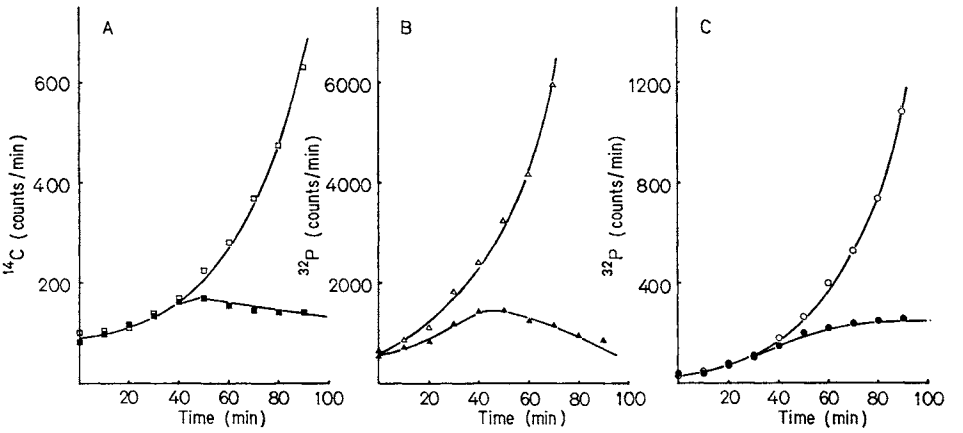

Fig.4. *Cellular synthesis following addition of specific antiserum to* P. septica. Open symbols, no antiserum; filled symbols, 15% antiserum added at time zero. (A) Net protein synthesis; (B) net RNA synthesis; (C) net DNA synthesis. RNA and DNA synthesis was measured by [^{32}P]phosphate incorporation and protein synthesis by the incorporation of a mixture of ^{14}C-labelled amino acids

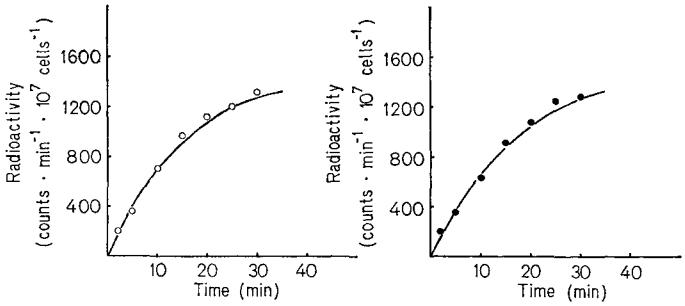

Fig. 5. *Uptake of [^{32}P]orthophosphate by* P. septica. O, bacteria in normal serum; ●, bacteria exposed to antiserum (15%) for 30 min. Time zero is the time of addition of [^{32}P]orthophosphate

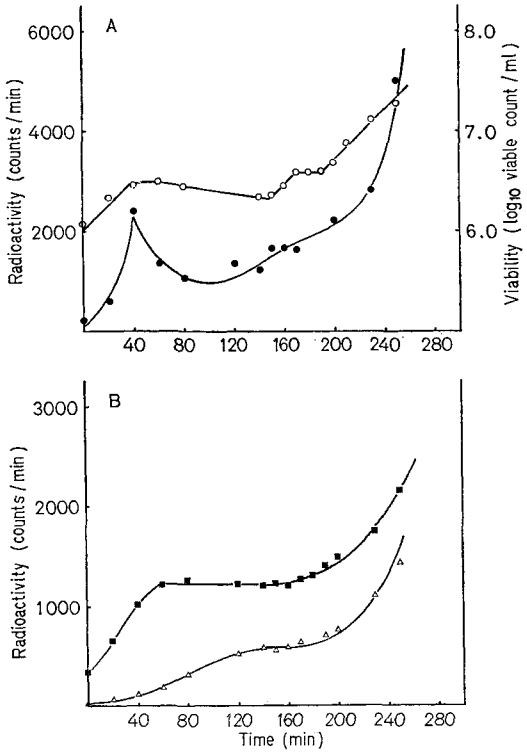

Fig. 6. *Effect of free Fe^{3+} on the inhibitory process.* Antiserum plus iron added at time zero. Sufficient ferric ammonium citrate, dissolved in 0.15 M NaCl and sterilized by autoclaving (15 lb for 15 min), was added to give a concentration of Fe^{3+} in the serum equal to twice that of the total iron-binding capacity of the transferrin (200% saturation). [^{32}P]Phosphate incorporation was used as a measure of RNA and DNA synthesis, and the incorporation of a mixture of ^{14}C-labelled amino acids as a measure of protein synthesis. O, viable count; ■, net protein synthesis; ●, net RNA synthesis; △, net DNA synthesis

to doubly saturate the serum transferrin, the iron being added at the same time as the antiserum. After addition of antiserum plus iron, cell division continued for about 40 min and then stopped (Fig. 6A). This was followed by a loss of viability thereby making the growth pattern similar to that obtained when iron was absent, although the fall in viable count was not as great. The pattern of macromolecular synthesis was also found to be virtually the same as that observed in the absence of free iron. Thus, net RNA synthesis was blocked after 40 min (Fig. 6A) and protein synthesis after about 50 min (Fig. 6B). DNA synthesis, however, continued for 130—140 min before stopping, a longer period than that found in the absence of added iron (Fig. 6B).

In the presence of ferric ammonium citrate, regrowth of the bacteria eventually occurred, resumption of cell division taking place between the 150th and 160th minute and apparently accompanied by a wave of synchronous division (Fig. 6A). The order of resumption of macromolecular synthesis was the same as that of its inhibition. RNA was the first macromolecule to have its net synthesis restarted, this event taking place 20—40 min before any bacterial multiplication occurred (Fig. 6A). Protein synthesis started next and, lastly, DNA synthesis was re-initiated (Fig. 6B). Biosynthesis of the latter was resumed after the apparent synchronous wave of division had taken place.

Reversal of Inhibition by Haematin

In addition to ferric iron, organic iron compounds, such as haematin hydrochloride, have also been shown to work in abolishing the effect of antiserum [3, 18]. The haem compounds, however, behaved differently from ferric ammonium citrate in that bacterial growth occurred without delay [3]. In the present system, the addition of haematin hydrochloride (10 mM) to *P. septica* growing in serum, at the same time as the antiserum, allowed the cells to continue multiplying without any delay. Since haematin appeared to have its effect immediately, it was of interest to see whether it was capable of reversing the inhibitory process once net RNA, protein and DNA synthesis had stopped completely in the absence of added iron. It was decided to add haematin to *P. septica* which had been in antiserum for 120 min, since from previous work

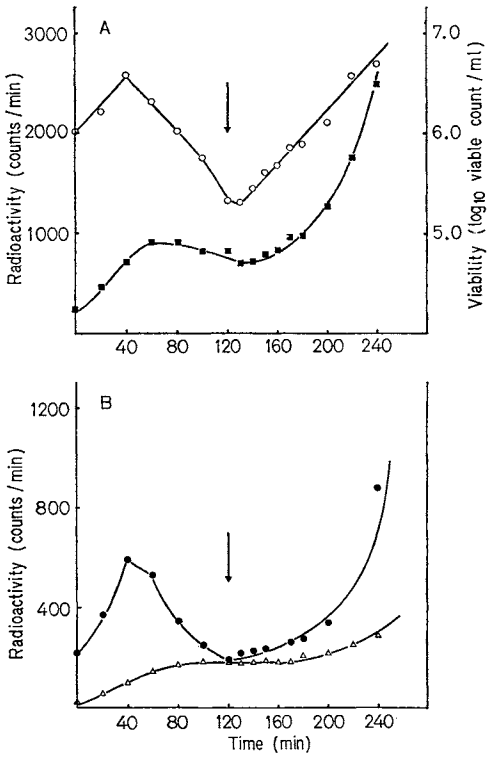

Fig. 7. *Reversal by haematin of the inhibitory effect of* P. septica *antiserum.* Antiserum (15%) was added at time zero and haematin (final concentration, 10 mM) at time 120 min, as indicated by the arrows. Haematin hydrochloride was added dissolved in 1 N NaOH (0.1 ml of the solution/20 ml serum); its addition had no significant effect on the pH. Protein synthesis was followed by the incorporation of a mixture of ^{14}C-labelled amino acids, and RNA and DNA synthesis by the incorporation of [^{32}P]phosphate. ○, viable count; ■, net protein synthesis; ●, net RNA synthesis; △, net DNA synthesis

all net macromolecular synthesis, including DNA synthesis, would have halted by that time.

Antiserum was added to *P. septica* growing in normal serum at time zero. As in previous experiments, the cells continued to multiply for 30—40 min and then stopped; a loss of viability followed (Fig. 7A). At the 120th minute, haematin hydrochloride was added to give a 10 mM solution; 10—20 min later cell division re-started again and the cells then grew at a rate similar to that in normal serum (Fig. 7A). This result differed from the resumption of growth in the presence of ferric iron, in that no wave of synchrony was seen. However, the nature of the curve indicates that the cells which do recover their ability to divide are the ones still in stasis, the ones which have not yet undergone irreversible cellular death.

In the presence of antiserum the inhibition of net macromolecular synthesis took place as described before. Protein accumulation stopped between the 45th and 55th minute (Fig. 7A); net RNA synthesis was blocked after 40 min (Fig. 7B) and a loss of label then took place from this fraction. DNA synthesis continued for 90—100 min and then stopped (Fig. 7B). On adding haematin at the 120th minute it was seen that net RNA synthesis was resumed immediately (Fig. 7B). There was a short delay of about 15 min before net protein synthesis re-started (Fig. 7A) and a longer delay before DNA synthesis was resumed (Fig. 7B). DNA biosynthesis was re-initiated about 40 min after the addition of haematin, after cell multiplication had started.

As in the case of ferric iron, the order of resumption of macromolecular synthesis in the presence of haematin was the same as that of its inhibition: RNA, protein, DNA. The important difference is that addition of haematin restored net RNA synthesis immediately.

DISCUSSION

This work has brought to light a totally unsuspected way in which antiserum and complement exert their antibacterial effect. In the case of *P. septica* the process operates by affecting the biochemistry of the bacterial cell leading first to an inhibition of net RNA synthesis and subsequently to an inhibition of all macromolecular synthesis. It is very quick and specific, and results in the cessation of bacterial multiplication. Subsequently, cellular death occurs, but as yet nothing is known about this killing event. The evidence suggests that the bactericidal effect is an independent phenomenon, induced by serum, and one which only occurs after bacteriostasis has been established.

The present results, therefore, differ considerably from the currently accepted ideas about the mechanism of action of serum factors against pathogenic bacteria, ideas which have simply invoked gross breakdown of permeability barriers or complete lysis of the bacterial cell [1, 14—17]. The fact that added iron or haematin reverses the inhibition argues against the inhibitory process being due to extensive cellular damage, but it is consistent with the view that the mechanism by which serum factors stop bacterial growth involves interfering in some way with the organism iron supply. The idea, proposed by Fletcher [19], that iron, in excess of the binding capacity of transferrin, overcomes the antibacterial action of serum factors merely by allowing a protective coat of serum protein to be bound to the bacterial cell surface is unlikely to be true. It is difficult to envisage entities as different as ferric iron, haematin and haemoglobin [3] operating mechanically in the same way to affect the bacterial surface. It is also difficult to see how the presence of haematin could reverse the inhibition once it had occurred, or to explain why the presence of excess ferric iron in serum did not prevent the inhibition taking place initially but did allow resumption of growth later.

It is not yet clear whether the active agent responsible for reversing the inhibition is haem or ferric iron itself. The long delay that occurs before the resumption of growth in the presence of ferric iron could indicate a necessity for its conversion to a haem group; on the other hand, the rapid effect of haematin may simply be a reflection of a more efficient and more rapid uptake mechanism. Whichever it may be, it would seem that, because of the rapidity with which inhibition occurs, a mechanism much more specific than a simple overall iron starvation is involved. Rapid depletion of iron or haem from a specific and crucial cellular role, for example, could be envisaged.

Whilst this paper was in preparation, Melching and Vas [20] reported on the bactericidal effects of serum on *E. coli* 0111. Their results showed that serum components rapidly affected bacterial metabolism. In particular, they found an early effect on RNA accumulation. This was reduced after 15 min reaction time and blocked completely after 30 min; a loss of label from this fraction then occurred making the pattern of inhibition similar to that found in the present study. DNA and protein accumulation were subsequently affected. Their results also showed that metabolic alterations preceded the loss of permeability control in the cells, or their lysis, a result which agrees with the results of the present investigation. In view of these similarities, and of the fact that Bullen and Rogers [2] have shown the antibacterial effects of rabbit serum on *E. coli* 0111 to be reversed by iron, it is worth considering the possibility that cellular death in this organism, as in *P. septica*, is an event subsequent to the cessation of bacterial multiplication.

The exact nature of the biochemical lesion in *P. septica* has not been determined, but the fact that RNA is the first macromolecule to have its net synthesis interfered with in the presence of antiserum and again that this is the first macromolecule to have its net synthesis resumed in the presence of added iron or haematin, strongly suggests that the inhibitory process is related to RNA metabolism. The observation of similar sequences of inhibition in other systems strengthens this view. The antibiotic rifampicin, for example, interferes directly only with RNA synthesis [21—23]; however, by inhibiting the synthesis of RNA it indirectly affects the synthesis of protein and DNA [24]. Interference with RNA accumulation in *P. septica* could be the result of an altered rate of RNA synthesis, of RNA degradation or of a combination of both. The possible involvement of iron or haem in RNA synthesis or in its control is particularly intriguing. Gross interference with the energy supply of the cell is unlikely since inhibition of net protein, RNA and DNA synthesis occurred at different times. However, it is conceivable that slight changes in the availability of ribonucleotides could be involved. Recently it has been shown that RNA accumulation is extraordinarily sensitive to the pool size of purine nucleoside triphosphates [25—27]. Future work will be concentrated upon unravelling more of the biochemistry of the inhibitory process and in determining the precise role played by iron.

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