THE REQUIREMENT FOR ANTIBODY AND COMPLEMENT FOR *IN VITRO* PHAGOCYTOSIS OF STARCH GRANULES

By ROBERT A. NELSON, Jr.* and JACQUELINE LEBRUN†

Institut Pasteur, Annexe de Garches, S. et O., France, and London School of Hygiene and Tropical Medicine, London, England

(With 1 Figure in the Text)

In a preceding study on phagocytosis of bacteria as influenced by the immunological phenomenon of immune-adherence (Nelson, 1953), it became desirable to design control experiments with an antigenically inert substrate. Starch particles have been employed widely as such an inert substrate for *in vitro* measurements of phagocytosis by leucocytes. While it was known that both phagocytosis of starch and so-called chemotactic attraction of leucocytes toward starch occurred optimally in the presence of fresh serum, the action of the serum usually was ascribed to a beneficial effect on the activity of the leucocytes.

Upon analysis of certain previous publications, the concept of the immunologically inert nature of starch seemed to us to be open to question. In particular, the work of Delaunay & Lebrun (1946) suggested that complement (C′) was an active component of fresh serum involved in chemotactic activity of starch *in vitro*. Similarly, a heat-labile fraction of serum 'closely associated with both complement and clotting components of plasma' was described by Tullis (1953) as essential for the leucocyte to phagocytize starch. Despite the evidence that C′ was the active portion of the serum, both groups suggested that the favouring action of the heat-labile component was exerted solely upon the leucocyte, and Tullis termed this fraction the 'phagocytosis-promoting factor' of normal serum.‡

Against the current concept that starch is inert immunologically, Gengou (1911) demonstrated conglutination of starch granules in the presence of fresh beef serum and the fixation of C′ to starch granules in the presence of various animal sera, as measured by inhibition of subsequent conglutination of erythrocytes as an indicator system. Reasoning by analogy to adsorption of C′ on erythrocytes or bacteria sensitized with specific antibody (Ab), Gengou inferred that Ab to starch existed in normal sera. Since protein has been shown to adsorb to starch spontaneously (e.g. Przylecki & Myskowski, 1935), and since C′ is protein, it appears that the results of Gengou have been overlooked largely because data were lacking to prove the immunologically specific nature of the C′ fixation.

The present experiments were designed to reinvestigate the requirement of

* Investigator for the Howard R. Hughes Medical Institute, U.S.A.; present address, Yale University School of Medicine, New Haven, Conn., U.S.A.

† Chargée de recherches au C.N.R.S.

‡ This was reminiscent of Metchnikoff's concept (1894) of serum 'stimulins', which had largely fallen into disrepute after its refutation by Denys (1903), and by Hektoen & Ruediger (1905).

'normal' serum for phagocytosis of starch granules. Since our initial assays indicated that at least two factors, one heat-stable and the other heat-labile, were required, a detailed investigation of these factors was undertaken. This included the detection of the heat-stable factor in normal serum and the measurement of its increase in titre in animals injected with starch, and the determination of the site of action of the heat-labile component and of its qualitative relationship to the components of C′ functioning in immune haemolysis. Finally, two related studies were performed. The first concerned the development of a medium suitable for survival and motility of leucocytes but which was free from C′ activity and from Ab to starch; the second, the development of an *in vitro* assay for agglutination of starch granules by serum.

MATERIALS AND TECHNIQUES

Although various starches have been examined, primarily data on starches from rice and from *Amaranthus cruenthus* are presented, since these have proved convenient because of their small physical dimensions and low protein content. Kjeldahl nitrogen determinations on two samples of analytic grade rice starch (British Drug Houses) showed 0·03 % protein, and on one sample of *Amaranthus* starch (Northern Regional Research Lab., Peoria, Illinois) showed 0·01 % protein.

Leucocytes were isolated from an exudate in the peritoneal cavity of guinea-pigs. The exudate was induced by injection of 6 ml. of sterile infusion broth at 6 hr., followed by 2 ml. at 2 hr. prior to the desired time for harvest of the leucocytes. The leucocytes were washed 2 or 3 times either with saline or with 0·015 M-phosphate in saline, pH 7·0.

Throughout our early experiments, both leucocytes and starch particles were mixed at a final concentration of 5×10^6 per ml., i.e. at a phagocyte-substrate ratio of 1:1, in order to simulate conditions previously employed in studies on immune-adherence and phagocytosis. As it became apparent that the rate-governing factor for phagocytosis was the degree of sensitization of the starch particles by Ab and C′, the concentration of leucocytes was increased to yield a phagocyte-substrate ratio of 4 or 6:1. The concentration of starch particles was maintained at the lower level in order to avoid either agglutination of the granules or the occurrence of 'surface' phagocytosis in a concentrated mixture, as well as to insure a sensitive end-point by having the phagocytes in excess.

For the assays of phagocytosis, either 1·0 or 2·0 ml. of the reaction mixture was placed in 15 × 100 mm. Pyrex tubes and shaken in a Warburg bath at 40 to and fro horizontal thrusts per min., or in 10 ml. Bijou bottles clamped horizontally in a circular rotator turning at 8 r.p.m.

The percentage of granules phagocytized was determined by microscopic examination of an unmeasured drop of the reaction mixture placed on a slide and covered with a cover-slip. 50–100 granules were counted at random and the percentage completely engulfed by leucocytes was recorded.

In general, glassware was treated with General Electric Dry Film. The bottles were filled with a 10 % solution in xylene, drained, heated at 160° C. for 1 hr. and washed.

RESULTS

Experiment 020955 (Table 1). Sera were collected from ten normal guinea-pigs and pooled. A sample of approximately 15 ml. of the serum was adsorbed with 2 g. of washed and packed rice starch granules for 60 min. at 0° C. The starch was removed by centrifugation and a second adsorption of the supernatant was performed with an additional 2 g. of starch.

Table 1. *Requirement for both a heat-labile and a heat-stable fraction of serum for phagocytosis of starch granules* in vitro

Serum employed	Diluent	Percentage of starch granules* phagocytized with final serum dilutions of 1/20	1/40	1/80	1/160	1/320
Fresh	Phosphate saline	100	100	58	8	0
Fresh	Heated serum	100	100	76	24	8
Heated	Phosphate saline	0	—	4	—	2
Fresh, adsorbed	Phosphate saline	6	2	2	4	2
Fresh, adsorbed *plus* heated†	Phosphate saline	98	94	50	20	2

* Percentage of starch phagocytized in duplicate control mixtures without serum: 0 and 6%.

† Final dilution of heated serum 1/30 in all mixtures.

A series of dilutions of the untreated fresh serum, mixed with starch and leucocytes, showed a capacity to induce approximately 50% phagocytosis at a final dilution of 1/80. Serum heated at 56° C. for 45 min. was inactive. Fresh serum diluted in heated serum was only slightly more active than fresh serum in buffer, indicating that the limiting factor in the assay was primarily the heat-labile component. The adsorbed serum was inactive. Complete reactivity of the adsorbed fresh serum was restored by the addition of a 1/30 dilution of heated serum.

Since adsorption in the cold with starch removed a factor replaceable by the heated serum, which by itself was inactive, and since the heat-labile component of serum was suggested by previous workers to be C′, it was inferred that the mechanism of phagocytosis of starch was a classical immunological reaction, and that the heat-stable factor was Ab.

Experiment 070654 (Table 2). Six guinea-pigs were selected for examination of the antigenicity of starch. After a preliminary bleeding, injections of starch suspended in saline were given intraperitoneally 3 times weekly for 2 weeks. A total of 40 mg. (i.e. about 12·5μg. protein impurity) was given to each animal. After the last injection the animals were rested 1 week and then re-bled.

One week later, 1 mg. of starch was injected intracutaneously into each of two abdominal sites. No reaction occurred after 1, 2, 4, 24, 48 or 72 hr.

One week later, three of the six animals were exsanguinated and sections for microscopic examination were prepared from the liver, spleen, pancreas, kidney and lymph nodes. A small amount of peritoneal exudate was noted in each animal, but no generalized lesions were observed. Direct microscopic examination

of the exudate showed numerous large mononuclear leucocytes containing intact starch granules.

Based upon preceding studies of Ab kinetics, the pre- and post-injection sera were titrated for Ab in the presence of starch-adsorbed C′. The titre was denoted as the reciprocal of the dilution of heated serum promoting about 50 % phagocytosis as compared with 'positive' control mixtures containing high concentrations of a serum pool.

Table 2. *Increase in phagocytosis-stimulating antibody titre in guinea-pig sera after intraperitoneal injection of* 40 *mg. of rice starch*

Guinea-pig number	50 % phagocytosis titre*	
	Pre-inoculation	Post-inoculation
4	8	64
9	<1	128
11	<4	16
12	16	16
14	6	32
15	16	128

* Titre expressed as the reciprocal of the initial dilution of heated serum promoting phagocytosis of 50 % of the starch particles after 60 min. at 37° C.

With five of the six guinea-pigs there was an increase in reactivity of the serum after injection of starch. Considerable variation occurred in the reactivity of the pre-injection sera, with titres ranging from less than 1 to 16.

Experiment 071554, *stability of Ab to heat*. Three portions of a guinea-pig serum (post-inoculation, pig 15, Table 2) were respectively heated at 60° C. for 30 min., heated at 68° C. for 30 min., and left unheated. Fourfold dilutions were prepared in buffered salt solution and the three samples titrated as described above. Approximately 50 % of the starch particles were phagocytized in the presence of C′ and of a 1/64 dilution of unheated serum, and in the presence of C′ and a 1/128 dilution of serum heated at 60° C. The serum heated at 68° C. was devoid of activity.

Experiment 011755 (Table 3). In previous assays, not shown here, it was found that starch particles pre-treated with Ab (heated serum) and washed 4 times with large volumes of saline, were quantitatively as susceptible to phagocytosis in the presence of C′ only, as were granules mixed directly with Ab and C′ and not washed. This observation, coupled with the fact (see Fig. 1) that non-Ab protein, such as gelatin or albumin, would serve as a beneficial medium for leucocytes, made it possible to design an assay to establish definitely the site of action of the heat-labile component of normal serum.

Two ml. of a counted and washed starch suspension were incubated for 60 min. at 37° C. with 1 ml. of a 1/6 dilution of fresh guinea-pig serum previously assayed and found to contain Ab and C′. A sample of 0·6 ml. was removed for assay. The remainder was washed 3 times with 15 ml. of saline. A 0·6 ml. sample of the washed granules was removed for assay. To the remainder were added 10 ml. of

'activated' papain. The mixture was incubated for 60 min. at 37° C. and then centrifuged and washed once with 15 ml. of saline. The starch was resuspended to original volume and 0·6 ml. removed for assay.

Table 3. *Site of action of C′. Reversion of opsonization by treatment with 'activated' papain**

Starch added to leucocytes in gelatin	Percentage of granules phagocytized after 45 min. at 37° C.
Starch only	4
Starch plus fresh serum	70
Starch pre-treated with fresh serum; washed 3 times	78
Starch pre-treated with serum; washed; treated with papain; washed	2

* Two g. of papain (British Drug Houses) were shaken in 50 ml. water for 1½ hr. at room temperature. The mixture was filtered (no. 1 Whatman paper) and diluted with an additional 50 ml. water. To 10 ml. of filtrate was added 1·0 ml. of 16 % cysteine; the pH was adjusted to 7·4 with 0·1 M-NaOH; and the solution incubated at 37° C. for 30 min. prior to mixing with the starch granules.

Each of the three samples was mixed with 0·8 ml. of 5 % gelatin, 0·4 ml. of washed leucocytes in buffer, and 0·2 ml. of buffered diluent. A control mixture contained starch which had not been treated with the guinea-pig serum.

After 60 min. at 37° C. about 70 % of the starch granules which were mixed with serum were phagocytized, while only 4 % of the untreated granules were phagocytized. Since, in the mixture containing starch which had been pre-treated but washed 3 times, 78 % of the granules were ingested, it is obvious that both Ab and C′ were fixed firmly to the granules. However, the treatment of the starch–Ab–C′ complex with papain reversed the susceptibility of the granules to phagocytosis.

While it is possible that the papain, or impurities therein, may have adsorbed to the surface of the sensitized granules and thus rendered them insusceptible to phagocytosis, it seemed more reasonable to assume that the papain digested enough of the Ab or C′, or both, to reverse their sensitizing activity in a fashion analogous to the action of papain on certain antigenic complexes sensitized with Ab alone (Kalmanson & Bronfenbrenner, 1943).

This type of assay was performed also with *A. cruenthus* granules sensitized with Ab and C′. After treatment with 'activated' papain, these granules were non-reactive in immune-adherence with human erythrocytes. Sensitized granules treated with 'non-activated' papain only and with cysteine only retained complete reactivity with the erythrocytes.

It thus appears that Ab and C′ do not cause an irreversible alteration in the structure of the surface of the starch which results in increased susceptibility to phagocytosis or to immune-adherence with erythrocytes.

Experiment 031155. In an assay designed as in Table 1, the three fresh serum samples, unadsorbed, once adsorbed with starch, and twice adsorbed with starch,

were titrated for phagocytosis-inducing capacity and also for haemolytic C′ reactivity. Utilizing the 50 % end-point for lysis as measured on the spectrophotometer (Kabat & Mayer, 1948), the unadsorbed serum was found to contain 171 C′ H_{50} units per ml., the once adsorbed 103 units, and the twice adsorbed 77 units. It was calculated from the latter figure that 0·96 C′ H_{50} units in a reaction mixture of 1·0 ml. containing Ab yielded complete phagocytic reactivity, since 0·2 ml. of a 1/16 dilution of the twice adsorbed C′ promoted phagocytosis of 86 % of the starch granules in the presence of a 1/160 dilution of heated serum (Ab). Thus it is clear that a favourable correlation exists between the reactivity of guinea-pig C′ as measured in the present manner and as measured in the precise methods employed in the sheep erythrocyte haemolytic method. However, it should be noted that while two adsorptions of the C′ pool decreased the haemolytic titre by about 94 C′ H_{50} units, the phagocytosis assays were not sufficiently accurate or precise to detect a significant corresponding decline.

In order to circumvent the deficiency in precision of kinetic analyses of phagocytosis and to gain insight into the relationship of the components of C′ which function in opsonization and in haemolysis, it was felt advisable to investigate further the nature of the heat-labile component of normal serum. The recent studies of Mayer and collaborators (1954) on the components of C′ functioning in the lysis of sensitized sheep erythrocytes may be summarized in part as follows: (1) C'_1 and C'_4 only are fixed to an antigen-antibody complex at 0° C. provided Ca^{2+} is present, but Mg^{2+} is excluded; (2) fixation of C'_2 only may be induced by addition of Mg^{2+} at 0° C.; (3) fixation of C'_3 will follow but only at elevated temperatures, e.g. 37° C., and no divalent cation is required.

A suspension containing 12·5 mg. of washed rice starch in 10 ml. of saline was sensitized for 60 min. at room temperature with 1 ml. of guinea-pig normal serum globulin containing 2 mg. protein per ml. This amount of globulin was estimated to represent a moderate excess of Ab, since in previous quantitative assays, 80 μg. of this preparation induced agglutination of 0·5 ml. of starch. The sensitized granules were washed 3 times with veronal buffer deficient in Mg^{2+} and Ca^{2+}. In order to remove the divalent cations, Mg^{2+} and Ca^{2+}, the buffer and the guinea-pig serum were each passed 3 times through a column, 10 cm. long, 3 cm. diameter, packed with Amberlite resin IRC-50 (Rohm & Haas Co., Philadelphia) at a rate of about 25 ml. per min. The resin was pre-treated with 4 % sodium hydroxide and washed as outlined in the brochure supplied by the manufacturer.

Six 1 ml. portions of the washed granules were incubated for 20 min. with six portions of 0·6 ml. of 'treated' guinea-pig C′, diluted about 1/4, under conditions outlined in Table 4. After 20 min., 10 ml. of chilled 'treated' buffer was added to each of five of the preparations, and was followed by three washings with 10 ml. of chilled 'treated' buffer. One preparation containing C′, Ca^{2+} and Mg^{2+} was left unwashed as a reactive control. The washed centrifugates were suspended to 1·6 ml. with phosphate buffer. Duplicate tubes containing 0·5 ml. of each starch sample and 2·0 ml. of freshly isolated washed leucocytes suspended in phosphate buffer containing 1 % gelatin were rotated for 45 min. at 37° C. Samples were examined microscopically to determine the percentage of starch granules phagocytized.

The result (Table 4) shows clearly that the C′ components which are required for opsonization are fixed to sensitized starch only at 37° C. and in the presence of both Ca^{2+} and Mg^{2+}. Once fixed, the C′ resists dissociation during washing with 30 volumes of buffer. Since Mayer and his associates have established that specific fractions of C′ are fixed to sensitized erythrocytes at 0° C. under the conditions used in our experiments, it would seem by analogy that all four fractions of C′ are

Table 4. *The fractions of C′ required for phagocytosis of sensitized granules of rice starch*

Starch–Ab complex treated with C′* 20 min.			Treatment of complex	Theoretical complex	Percentage of particles phagocytized after 45 min.
Ca^{2+}	Mg^{2+}	Temp.			
0	0	0°	Washed	(S–Ab)	0
0·001 M	0	0°	Washed	(S–Ab)–C'_1–C'_4	0
0	0·003 M	0°	Washed	(S–Ab)	0
0·001 M	0·003 M	0°	Washed	(S–Ab)–C'_1–C'_4–C'_2	0
0·001 M	0·003 M	37°	Washed	(S–Ab)–C'_1–C'_4–C'_2–C'_3	78
0·001 M	0·003 M	37°	Not washed	(S–Ab)–C'_1–C'_4–C'_2–C'_3	84
0·001 M	0·003 M	37°	Not washed	(S–Ab)	0
(Control without C′)					

* This C′ was titred beforehand by assays for immune adherence (*I–A*) with rice starch and human erythrocytes. From this it could be estimated that 0·6 ml. of C′ contained about three 100 % *I–A* units, i.e. a 3-fold dilution of C′ was adequate to induce *I–A* of all the presensitized starch particles mixed with erythrocytes for 60 min. at 37° C.

required for opsonization, as they are for immune haemolysis of sheep erythrocytes. Further evidence will be necessary to prove that the theoretical complexes listed are actually formed at 0° C. and with varying cations. It is of interest to note that in separate experiments with *A. cruenthus* starch, not shown here, the fixation of all four components of human C′ has been found necessary for the sensitized starch granules to undergo immune-adherence with human erythrocytes.

Experiment 090255. The decline in titre from 171 to 103 units per ml., i.e. a 39 % loss of reactivity, which resulted from one adsorption of fresh serum with starch, was remarkably high for an adsorption performed in the cold for only 60 min. In view of the question of the immunologic specificity of C′ fixation mentioned above with reference to the results of Gengou, it became important to differentiate between adsorption of C′ to the starch–Ab complex on the one hand, and an adsorption or destruction of C′ which might occur with starch independent of Ab. This provided a considerable obstacle, since all sera selected for C′ were found to contain Ab to starch.

In the preceding experiment the exposure of the starch–Ab complex to C′ in the absence of Mg^{2+} and Ca^{2+} at reduced temperature did not render the complex susceptible to phagocytosis or to immune-adherence. Therefore, experimental conditions were known wherein the specific fixation of C′ could be inhibited, but which probably would not influence a hypothetically non-specific adsorption or destruction of the C′.

https://doi.org/10.1017/S0022172400044259 Published online by Cambridge University Press

Fresh pooled guinea-pig serum was passed through a column of Amberlite IRC-50 three times to remove Mg^{2+} and Ca^{2+}. A 15 ml. portion of the 'treated' serum and another 15 ml. portion which was reconstituted to 0·001 M-Ca^{2+} and 0·003 M-Mg^{2+} were each mixed for 60 min. at 0° C. with 2 g. of rice starch previously washed with 'treated' saline. After centrifugation and removal of the starch these two samples of serum, and an unadsorbed sample of 'treated' serum as a control, were titrated for haemolytic C′ activity.

The unadsorbed serum contained 133 C′ H_{50} units per ml. As in the preceding assay, the serum adsorbed in the presence of Mg^{2+} and Ca^{2+} exhibited a decline of 22 % to 104 units per ml. By contrast the serum adsorbed in the absence of Mg^{2+} and Ca^{2+} declined only 7 % to 123 units per ml. The difference in loss of C′ may be interpreted to represent the specific adsorption of C′ to the starch–Ab complex. The loss of 7 % of C′ reactivity in the absence of Mg^{2+} and Ca^{2+} poses greater difficulty in interpretation. While it is possible that the responsible factor may be non-specific destruction which when encountered in other systems often goes unexplained under the term of anti-complementary action, it is equally reasonable to suspect that the loss of 10 units per ml. here encountered was due to a physical adsorption of some or all components of C′ to the starch granule at sites not occupied by Ab. Under these circumstances the adsorption of C′ would be immunologically non-specific and would not be expected to render the starch–Ab complex susceptible to phagocytosis or immune-adherence, but would at the same time effectively decrease the C′ titre of the residual serum.

Experiment 080454 (Fig. 1). As improved methods evolved during the present investigation, the results indicated that measurements could be performed with mixtures containing low concentrations of serum, e.g. with pools of C′ diluted 1/80 final, and with antiserum diluted up to 1/1280 final. As in Exps. 011755 and 031155 it became possible to perform assays with pre-treated and washed starch particles, i.e. with essentially no free protein in the reaction mixture. It therefore became critical to determine whether the resultant low concentration of protein was adequate for optimal activity and survival of leucocytes.

Series 1. 0·2 ml. samples of pre-washed starch were mixed with 0·1 ml. of a 1/20 dilution of antiserum for 5 min. at 37° C. 0·1 ml. of C′ diluted 1/8 and 0·4 ml. of leucocytes were added. 0·2 ml. of a 10 % solution of crystalline bovine albumin (Poviet Co., Holland) in phosphate was added to duplicate mixtures, and 0·2 ml. of phosphate alone was added to duplicate control mixtures. Phagocytosis was measured after 15, 30 and 60 min.

Series 2. A series of mixtures similar to the above was prepared except that the starch and antiserum were pre-incubated for 60 min. at 37° C., and then washed with phosphate to remove uncombined protein prior to adding C′ and leucocytes.

In both series a marked increase in the rate of phagocytosis occurred in the reaction mixtures containing albumin. The percentage of particles phagocytized at each time interval was slightly higher in the mixtures containing starch which had been presensitized with Ab and washed. These observations have been repeated 5 times, and similar enhancement of the rate of phagocytosis has occurred in mixtures containing either bovine albumin, guinea-pig serum albumin, which

on electrophoretic analysis was found free of alpha globulin, or gelatin. Since in no instance has phagocytosis occurred in mixtures with C′ plus either albumin or gelatin, i.e. without specific antiserum, it is likely that the enhancement is related to a favourable action of the non–Ab protein on the activity and/or survival of leucocytes.

This result disagrees with the report of Tullis (1953) that albumin was inhibitory to phagocytosis. Since the present experiments indicate that gelatin will substitute quantitatively for albumin in enhancing leucocyte activity, and since the

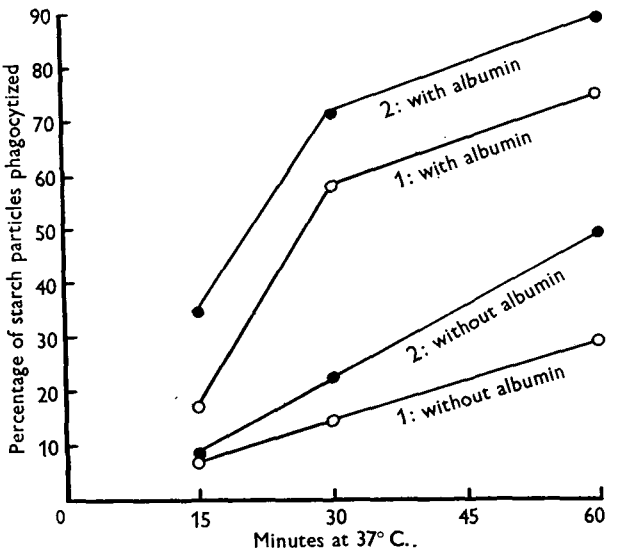

Fig. 1. Enhancement of phagocytosis of rice-starch particles, pretreated with Ab and C′, in mixtures containing 2 % albumin.

guinea-pig serum albumin was free from detectable globulin, these results further conflict with a more recent report of Tullis (1954) that a phagocytosis-enhancing effect of certain albumin preparations was due to an alpha-globulin impurity.

Experiment 022155 (Table 5). The tendency for starch granules to sediment rapidly from suspension hindered for some time the development of a convenient visual measurement of agglutination. However, a homogenous spread of the washed and sedimented granules from an unclumped suspension occurs on the surface of curved depressions on a plastic plate. It has been found that agglutinated suspensions sediment more rapidly and more unevenly and tend to fall down the curved surface *en masse* or in small cascades, thus providing an easily apparent visual end-point for agglutination measurements.

0·2 ml. samples of 5-fold dilutions of the fresh guinea-pig serum pool and of the twice adsorbed serum pool described in Table 1 were mixed with 0·5 ml. of suspensions of rice starch and of *A. cruenthus* starch. Agglutination, which was verified by direct microscopic examination of each preparation, occurred at a 1/625 dilution of fresh serum with rice starch, and at a 1/125 dilution of fresh serum with

Amaranthus starch. The residual agglutinating activity of the serum after two adsorptions with rice starch was markedly depressed, and was evident only at a 1/5 dilution with both starch preparations. Control mixtures containing starch plus saline only, and starch plus a 1 % solution of guinea-pig serum albumin showed no agglutination.

Assays with rice starch were recorded after 30 and 60 min. at room temperature. Agglutination, as judged by the pattern technique, was not apparent for about 2 hr. with *Amaranthus* starch, presumably because of a tendency to settle less rapidly due to its smaller size.

Table 5. *Agglutination of starch by dilutions of guinea-pig serum utilizing the patterns produced on plastic plates**

Type of starch	Treatment of pool of guinea-pig serum	Agglutination of starch† with serum dilutions				
		1/1	1/5	1/25	1/125	1/625
Rice	Unadsorbed	+	+	+	+	±
	Twice adsorbed‡	+	+	−	−	−
Amaranthus cruenthus	Unadsorbed	+	+	+	+	−
	Twice adsorbed‡	±	±	−	−	−

* Manufactured by Prestware Ltd., London. The depressions are $\frac{3}{10}$ in. in depth and $\frac{3}{4}$ in. in diameter, and have round bottoms.

† No agglutination occurred in control mixtures with saline or with 1 % serum albumin in saline.

‡ The serum was adsorbed twice with rice starch at 0° C., as outlined in Table 1.

Differences in agglutination occurred with sera from different species. Sera from adult chickens showed most marked reactivity, guinea-pig sera produced moderate agglutination, while human sera produced only slight agglutination. Pooled serum from 2-day-old chicks and from 13-day-old chick embryos were not reactive. It is suggested that these results reflect the content of Ab produced during normal conditions in response to as yet unknown antigenic stimuli.

SUMMARY

Against the concept that starch is inert immunologically and, therefore, an ideal substrate for *in vitro* studies on leucocytes, the results here recorded demonstrate that there are at least two components of serum which are specifically required for phagocytosis of starch. The available evidence suggests that the heat-labile component is C′, and the heat-stable component is specific Ab. The latter, which is a serum globulin, conforms to the definition of Ab from three standpoints: (1) it is adsorbed to the starch granule at low temperature and cannot be eluted by washing with saline at pH 7·0; (2) although it is found in 'normal' serum, it appears in increased quantities in the serum of animals injected with starch; and (3) once combined with the starch granule, it exerts three *in vitro* immunological effects, i.e. opsonization, induction of immune-adherence, and agglutination. As evidence that the latter reactions are due to specific Ab, the enhancement of phagocytosis and the induction of immune-adherence are manifested *only* in the presence of C′.

The measurement of Ab or C′ has been complicated by the finding that fresh normal serum to be employed as C′ has invariably contained Ab to starch. This situation is similar to that which exists with a variety of micro-organisms, e.g. *Micrococcus aureus*, against which the majority of normal animals and human beings possess circulating Ab. To obtain C′ free of Ab, fresh guinea-pig serum was adsorbed in the cold with starch. Despite the low temperature which would be expected to reduce the C′ fixation, about 30–40 % of C′ activity was lost during adsorption for 60 min. at 0° C. When the adsorption was performed in the absence of Mg^{2+} and Ca^{2+}, the loss of C′ was lessened considerably. This is interpreted to mean that at least two factors contributed to the loss of C′ cited above: (1) the specific fixation of some or all components of C′ to the starch–Ab complex, which requires the presence of Mg^{2+} and Ca^{2+}, and which occurs even in the cold; and (2) the non-specific action of starch in adsorbing C′, or perhaps destroying C′, which does not require Mg^{2+} or Ca^{2+}.

Since rice starch contains small amounts of protein (0·03 %), as well as undetermined amounts of lipid, and since 40 mg. of starch were injected into the animals, the possibility exists that the antibody described here is directed against either of these two impurities. On the other hand, a tentative hypothesis is offered that the amylose or amylopectin components represent the antigen. This concept appears plausible in view of the recent descriptions by Heidelberger, Aisenberg & Hassid (1954) of the antigenicity of glycogen, by Kabat & Berg (1953) of the antigenicity of dextran, and by Grabar (1953) of the antigenicity of gelatin. A brief survey has indicated that Ab to starch is found in sera from several different species, including man. As yet, no comprehensive study of the role of this Ab in pathologic processes has been undertaken.

While the concept proposed by Tullis of a specific 'phagocytosis-promoting factor' of normal serum acting on leucocytes would appear to be invalidated by the present findings, the possibility of course remains that heat-labile elements in serum other than C′ may contribute to the activity and survival of leucocytes. Nonetheless it would seem unnecessary to perpetuate the phrase 'phagocytosis-promoting factor'. The suggested specificity in this term is not justified since any substance which promotes survival of leucocytes, and hence their ability to phagocytize, might properly be called a phagocytosis-promoting factor.

Gratitude is expressed to Dr George Brecker, National Institutes of Health, U.S.A., who supplied *Amaranthus cruenthus* starch, and to Dr M. E. Mackay, Lister Institute, London, who provided purified protein fractions of guinea-pig serum.

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(*MS. received for publication* 19. VII. 55)