MEASUREMENT OF RATES OF PHAGOCYTOSIS

The Use of Cellular Monolayers

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ABSTRACT

A method has been developed for measuring the *rate* of phagocytosis rather than the quantity of particles ingested per cell when the process is virtually complete. The method, which is simpler and more rapid than those described previously, utilizes cellular monolayers, radioactive particles, and short incubation times. Under the conditions described, the rate of uptake of particles by either guinea-pig peritoneal or human blood leukocytes was proportional to both cell concentration and the time of incubation, and was independent of changes in the concentration of particles during the measurement. The particles were retained by the cells for at least 90 min. The most suitable particles so far used have been ^{32}P-labeled *Salmonella typhimurium*, and acetyl-^{14}C– or methyl-^{14}C–labeled starch particles. The oxidation of ^{14}C–labeled glucose has been studied under the same conditions that were used for the assays of phagocytosis: the greatest increase in formation of $^{14}CO_2$ from glucose-1-^{14}C occurred a few minutes after the most rapid period of phagocytosis.

INTRODUCTION

There are two main ways in which the phagocytic activity of a cell population may be studied. In the first, a measurement is made of the rate at which the added particles disappear from the extracellular medium. Typical of this class is the technique of Cohn and Morse (1). In this method, the cells being studied are presented with a known number of viable micro-organisms, and the change in this number is measured after a specified period of incubation. The results, of course, assume that the micro-organisms are killed almost immediately after they are ingested and that the number of bacteria killed is approximately equivalent to the number ingested. Under certain circumstances (e.g., short periods of incubation), this assumption may not be valid. A simpler example of this general type of methodology is that of Sastry and Hokin (2). They presented polystyrene spheres to leukocytes, centrifuged down the cells after incubation, and determined the decrease in turbidity of the extracellular fluid.

These methods, though very valuable, have a serious disadvantage, i.e. as the quantity measured is the *fall* in particle concentration outside the cells, it is necessary to incubate cells for relatively long periods in order to obtain large changes in this quantity. This generates the further problem that the particle concentration changes considerably during the course of the incubation, which may then cause a concomitant decrease in the rate of particle ingestion. Often the changes in particle concentration measured in the bacterial killing

assay are very large and are most clearly presented only when a log scale is used. Thus, only relatively large changes in phagocytic activities can be adequately measured by these methods.

The second approach is to measure the number of particles taken into the cells. This is usually done by the use of techniques based on those introduced by Hamburger (3). The cell suspension is incubated with particles, and the cells are recovered and examined microscopically. The proportion of the cells which contain ingested particles is counted. The accuracy of this method can be somewhat improved if the proportions of the cells containing specified numbers of particles are recorded (4). However, this approach is not readily applicable to the study of large numbers of samples, and recently attempts have been made in several laboratories to simplify the measurement of intracellular particle concentrations. Roberts and Quastel (5) described a method based on the extraction of ingested polymer from cells recovered after incubation with polystyrene or polyvinyltoluene spheres. The extracted material was assayed spectrophotometrically. This assay has been used most elegantly in studies by Weisman and Korn (6) on phagocytosis by *Acanthamoeba*.

Brzuchowska (7) has recently described briefly an assay of phagocytosis in which the uptake of ^{14}C-labeled *Escherichia coli* was measured. Carpenter (8) and Carpenter and Barsales (9) have published more extensive studies of the uptake of ^{131}I-labeled *E. coli* and bentonite particles by polymorphonuclear leukocytes and monocytes from spleen. The latter study approached a true rate measurement, but, since uptake was approximately proportional to particle concentration and not independent of it, the measurements did not indicate optimum rates. Proportionality between cell concentration and the rate of particle uptake was not demonstrated.

In none of these studies, except for those of Weisman and Korn (6) and Carpenter and Barsales (9), have any serious attempts been made to assay the *rate* at which particles entered the cells. In some cases (e.g. 4, 5, 9), the *time-course* of the uptake has been studied. In most cases, though, the standard assay procedure adopted involved a fairly long incubation period. Under such conditions the particle uptake usually proceeded virtually to completion, and the method assayed not the maximal phagocytic *rate* but essentially the phagocytic *capacity* of the cells under the chosen conditions.

In our experiments on the effects of various agents on the activity of phagocytic cells, we needed a simple and accurate method for assaying the *rate* of particle uptake during phagocytosis. In previous papers from this laboratory (10, 11), methods have been described for the measurement of respiration by monolayers of monocytes and for the measurement of inulin uptake by monolayers of both monocytes and polymorphonuclear leukocytes (PMN). In this paper we shall describe the way in which monolayers of polymorphonuclear leukocytes may be used for the assay of the *initial rate* of phagocytosis. As an incidental adaptation, the rate of oxidation of glucose-^{14}C may also be followed. During the development of the assay method, the whole phagocytic event has been treated as if it were a single enzymatic reaction. The results indicate that this approach yields a valid assay of particle ingestion. However, they give no indication of which portion of the over-all process constitutes the rate-limiting step.

MATERIALS AND METHODS

Isolation of Starch Particles

The isolation procedure of MacMasters et al. (12) was applied to *Amaranthus caudatus* seeds (10). These may be obtained from many seedsmen. The starch granules are not digested by leukocytes and are similar to those from *Amaranthus cruentus* (4) which were previously employed. They have an average diameter of 2.58 μ (range: 1.2–4.0 μ), and 1 mg of particles contains 8.4×10^7 particles.

Polystyrene Spherules

"Bacto-latex," a suspension of polystyrene spherules 0.81 μ in diameter, was obtained from Difco Laboratories, Detroit, Michigan. The particles were exhaustively dialyzed against distilled water before use.

Preparation of Acetyl-^{14}C–Labeled Starch

The method used was based on that of Wurzburg (13). 5 g of starch particles were suspended in 15 ml of water. The pH, measured with narrow-range pH paper, was adjusted to approximately 8.5 with NaOH. 250 mg of acetic anhydride (containing 300 μc of acetyl-1-^{14}C acetic anhydride [New England Nuclear Co.]) were slowly added to the stirred suspension. The pH was frequently readjusted to pH 8–8.5 with NaOH. 1 hr later, the pH was adjusted to about 5.0. The mixture was centrifuged, and the starch granules were recovered. They were washed six times with

water and twice with 80% ethanol. The final starch preparation contained about 15% of the added label, indicating approximately 30% conversion of the acetic anhydride.

Preparation of Methyl-^{14}C–Labeled Starch

2 g of *Amaranthus caudatus* starch particles were suspended in 20 ml of ice-cold 1:1 (v:v) 0.05 M glycine: 0.05 M NaOH. The pH of this mixture was initially 12.2. The mixture was cooled on ice and stirred. 500 μc of methyl-^{14}C-dimethyl sulfate (200 mg) was added gradually during 24 hr, and the cold mixture was stirred for a further 24 hr. The suspension was centrifuged, and the starch was washed three times each with water, 80% ethanol and methanol, and then dried. Efficient stirring was essential in all procedures in which alkaline reagents were added to starch suspensions; otherwise the starch granules became "gelatinized" or completely solubilized.

Preparation of ^{32}P-Labeled Bacteria

The bacteria used were avirulent *Salmonella typhimurium* (strain M 206) (14). They were grown, harvested, and washed by standard procedures, except that orthophosphate-^{32}P was included in the growth media. They were opsonized by incubation in 10% horse serum for 2 hr, with shaking, and the excess serum was then removed by washing the cells.

Isolation of Cells

Guinea pig PMN were elicited and harvested as described previously (10), except that in most of the experiments the eliciting agent was 15 ml of 15% (w:v) sodium caseinate in 0.9% NaCl. This solution seemed to be more consistently efficient than the 12% solution used previously and did not change the metabolic characteristics of the isolated cells. The cells were washed twice with Krebs-Ringer phosphate medium, pH 7.4 (KRP), before preparation of the monolayers.

Human blood leukocytes were prepared from heparinized venous blood after sedimentation of erythrocytes at 37° (15).

Preparation of Monolayers

Monolayers were prepared in circular (35 mm diameter) plastic tissue culture dishes (Falcon Plastics Company, Los Angeles). On arrival the dishes were 10 mm deep, but before use they were cut down on a lathe to 8 mm. This allowed the dishes to be fitted into specially cut aluminum holders for counting in the Nuclear-Chicago Model D47 automatic gas-flow counter. The cut dishes were thoroughly rinsed in distilled water and air dried. All incubations were at 37° either in a Visidome (Emil Greiner Co., New York) or on a leveled aluminum plate with a built-in heating element controlled through a Variac. The dishes were placed on the plate (or in the Visidome) and 1 ml of a suspension of either guinea pig or human leukocytes in KRP at a concentration of 0.6% (based on the packed cell volume) or less was pipetted into each. The dishes were gently agitated every few minutes, and within 15–30 min stable monolayers of cells were established on the bottoms of them. 45 min after the addition of the cell suspension, the dishes were picked up with forceps and gently rinsed through two or three beakers, each containing 150 ml of KRP at room temperature. This rinsing removed any debris and cells that had not adhered to the plastic (e.g., the erythrocytes that contaminated some preparations, especially of blood leukocytes). The monolayers were then ready for use, and, after the excess KRP had been drained off by inversion of the dishes for 30 sec on paper towels, the dishes were transferred back onto the warm plate and the appropriate subsequent solution was pipetted onto each.

In a previous study (11) monolayers of guinea-pig peritoneal PMN were used for measurements of inulin uptake. In those experiments the cell suspensions used for the preparation of the monolayers contained added serum. In the present study, this serum was omitted, and it was found that the cells adhered to the dishes and phagocytized adequately.

A monolayer prepared in this way usually contained 0.2–0.4 mg of protein. This is equivalent to approximately 1.2–2.5 × 10^6 cells per dish, or 1.3–2.6 × 10^3 cells. mm^{-2} (10).

Assay of Phagocytosis

Two slightly different methods were used which yielded similar results. (*1*) A warmed (37°) suspension of particles in 1 ml of KRP containing 7.5 mM glucose was pipetted onto each dish; *or* (*2*) 1 ml of KRP containing 7.5 mM glucose was pipetted onto each dish and 15 min later 0.05 ml of a particle suspension in KRP was added to the dishes and the contents gently mixed. In either case, the monolayers were incubated with the particle suspension for 10 min, at which time the dishes were picked up with forceps and quickly rinsed through four to six beakers, each containing 200 ml of KRP or 0.9% NaCl at room temperature, so as to wash off the unphagocytized particles. The dishes were drained by inversion on paper towels and finally by the withdrawal of the last drop of fluid into a small piece of absorbent tissue. The monolayers containing the phagocytized particles were then air dried under a warm lamp and counted as described above.

The majority of the assays reported, including all of those on human cells, were done with method *2*, with 5 mg of either acetyl-^{14}C- or methyl-^{14}C-labeled starch particles.

The standard conditions described above were derived from the results reported later in the paper, as the optimal conditions for measuring the initial rate of particle uptake by this type of cell monolayer.

Assay of Protein

After being dried and counted, the monolayers were digested overnight at room temperature in 0.5 N NaOH. Protein was assayed in a sample of the digest by the method of Lowry et al. (16). In a typical experiment, all of the monolayers prepared from a single batch of cells would contain similar numbers of cells. All of the protein assays usually fell within 10% of the mean. When phagocytosis was measured, the protein content of each monolayer was assayed.

Assay of Glucose Oxidation

Monolayers were prepared by the usual procedure and, when necessary, incubated with specified agents in 1 ml of KRP containing 2 mM glucose. The assay was started by the addition, to each layer, of 0.1 ml of 0.9% NaCl containing 1 or 2 μmoles (1 μc) of glucose-1-^{14}C. In some cases, other materials were added in suspension or solution with the glucose (e.g. 5 mg of starch granules). The dishes were gently rocked so as to mix the contents, and the lids of the dishes were then replaced by modified lids. These lids had a 2-mm hole, which was covered by a piece of parafilm, 8 mm from one edge and, suspended inside by capillarity, a 1-cm diameter piece of Whatman No. 40 filter paper carrying 0.06 ml of 1 N NaOH. Incubation was continued at 37°, usually for 20 min. The parafilm was then briefly lifted, two drops of 2 N HCl were added to the dish through the hole by the use of a fine Pasteur pipette with a greased outside surface, and the hole was covered again. The dish was gently rotated for thorough mixing and then left to stand for a further 15 min. The lid was then removed. The alkali-soaked filter-paper containing the evolved CO_2 was transferred to 10 ml of Buhler's solution (17) in a counting vial. The lid was wiped with another circle of filter paper which was transferred to the same vial, and the radioactivity was assayed with an Ansitron II automatic liquid-scintillation counter (Picker Nuclear, New Haven, Connecticut). All assays were corrected for samples carried through the same procedure in the absence of a cell monolayer. For the purposes of calculation, the protein content of the dishes was assumed to be the mean protein content of three or four unincubated monolayers prepared at the same time as those used for the assays (see above).

RESULTS

Stability of the Labeled Starch Preparations

Most of the experiments were performed with unopsonized starch, either acetyl-^{14}C– or methyl-^{14}C–labeled. Microscopic examination of both of these preparations indicated that the structure of the particles was unaffected by the preparative procedures, except that the methyl-labeled material showed a slightly increased tendency to clump. The radioactivity of both preparations was stable to washing with water, 80% ethanol, and methanol. A sample of the methyl-labeled material was dissolved in 0.5 N NaOH and dialyzed against water without any loss of radioactivity. Incubation of 15 mg of acetyl-^{14}C–labeled starch with 3 ml of a 5% (by volume) suspension of guinea pig PMN at 37° for 90 min caused the release of 14.5% of the total radioactivity in a soluble form. One-third of this released activity was recovered as respired $^{14}CO_2$. Thus, the solubilization of radioactive material when 5 mg of this starch were incubated with 1 ml of a 0.6% cell suspension (or its equivalent as a cell monolayer) for only 10 min was expected to be small (about 0.2%). In fact, neither soluble radioactivity nor $^{14}CO_2$ was detected under the latter conditions, and we were, therefore, satisfied that the labeled starch particles were stable for the duration of our assays.

In some early experiments, particle uptake was measured with starch labeled as a complex with $^{131}I_2$. However, the label was rather easily lost from these particles, and, in view of the high affinity of iodine for both lipids and proteins, its use was discontinued. Some of the observations were originally made with iodine-labeled material, but all of the data presented were obtained with other labeled preparations.

Phagocytosis by Guinea Pig Cells

APPEARANCE OF THE CELLS AFTER PHAGOCYTOSIS: Cells were examined either by phase microscopy or after staining with Wright's stain. The particles were within the cells in monolayers which had been incubated with either starch particles or polystyrene spherules. When monolayers were incubated with 10 mg of unopsonized starch for 10 min, about 77% of the cells (66–83% in four experiments) had actually ingested particles.

EFFECT OF STARCH CONCENTRATION: With methyl- (Fig. 1) or acetyl-labeled unopsonized starch, uptake was independent of starch concentration above about 3 mg per ml. Unless otherwise specified, assays were made with 5 mg per ml: under these conditions, 20% or less of the

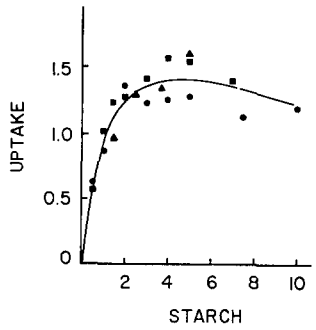

FIGURE 1 Effect of starch concentration on particle ingestion by guinea pig peritoneal polymorphonuclear leukocytes. ●, ▲, and ■ represent results obtained with three separate cell preparations. Cell concentrations were 0.2–0.35 mg of protein per dish. Incubation was carried out for 10 min at 37°. Uptake is expressed as mg of methyl-^{14}C-labeled starch granules ingested per mg of cell protein during a 10-min incubation. Particle concentration is expressed in mg of labeled starch granules added per dish, i.e. per ml.

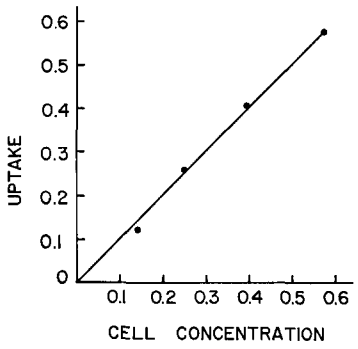

FIGURE 2 Effect of cell concentration on phagocytosis by monolayers of guinea pig peritoneal polymorphonuclear leukocytes. Incubations were carried out for 10 min at 37° with 5 mg of methyl-^{14}C-labeled starch per dish. Uptake is expressed as mg of starch ingested per monolayer during the 10-min incubation. Cell concentration is expressed as mg of protein per monolayer.

added starch was phagocytized in the 10-min assay period, and uptake was essentially independent of changes in the starch concentration.

EFFECT OF CELL CONCENTRATION: The effect of cell concentration on phagocytosis of acetyl- or methyl-labeled starch is shown in Fig. 2. Phagocytosis was proportional to the protein content of the monolayers at least up to 0.5 mg per dish.

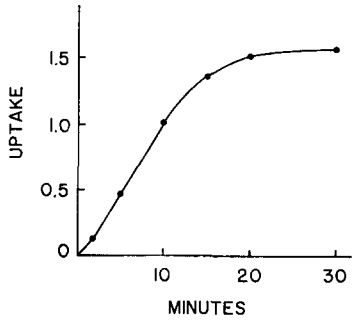

FIGURE 3 The time-course of particle uptake by monolayers of guinea pig peritoneal polymorphonuclear leukocytes. Incubations were carried out for the specified periods at 37°, with 5 mg of methyl-^{14}C-labeled starch particles per dish. The monolayers contained 0.35–0.4 mg of cell protein. Uptake is expressed as mg of starch ingested per mg of cell protein in the specified time.

TIME-COURSE OF PARTICLE UPTAKE: With 0.3 mg of protein and 5 mg of either type of ^{14}C-labeled starch, uptake was approximately proportional to the time of incubation up to at least 10 min (Fig. 3). Usually a slight lag was detected before uptake started. If the protein content was raised substantially or the starch concentration lowered, uptake sometimes declined after a shorter period.

MAGNITUDE OF UPTAKE: Fig. 4 shows the rates at which starch was phagocytized by different preparations of guinea pig and human cells (each preparation derived from a single animal or person). The data for guinea pig cells are all obtained for 10-min incubations with 5 mg of starch particles and were derived from assays with methyl-^{14}C-labeled starch (Fig. 4 *A*) and two different preparations of acetyl-^{14}C-labeled starch (Fig. 4 *B*, *C*).

ENERGY-DEPENDENCE OF THE PROCESS: It was established previously that phagocytosis by suspensions of guinea pig PMN is very sensitive to inhibitors of glycolysis and relatively insensitive to inhibitors and uncouplers of mitochondrial function (4). Table I lists the effects of these agents on phagocytosis by monolayers. The results indicate that these preparations also depend mainly on glycolytic energy for their phagocytic function, i.e. are similar to the cells previously examined in suspension (4).

RETENTION OF PARTICLES BY THE CELLS: After uptake had proceeded under the

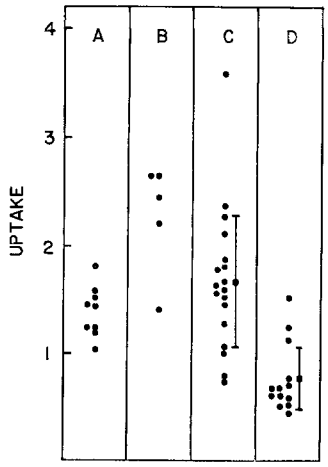

FIGURE 4 Rates of phagocytosis by a number of different cell preparations. All incubations were of monolayers containing from 0.1 to 0.5 mg of cell protein, with 5 mg of labeled starch particles, for 10 min at 37°. The four groups of data represent phagocytosis by guinea pig peritoneal polymorphonuclear leukocytes of (*A*) methyl-^{14}C-labeled starch; (*B*) acetyl-^{14}C-labeled starch (batch 1); (*C*) acetyl-^{14}C-labeled starch (batch 2); and (*D*) of phagocytosis by leukocytes from human blood of acetyl-^{14}C-labeled starch (batch 2). Uptake is expressed as mg of starch ingested per mg of cell protein in the 10-min incubation period. The bars indicate mean ± standard deviation for the measurements in columns *C* and *D*.

TABLE I

Effects of Metabolic Inhibitors

The cells were incubated for 15 min with the inhibitor in 1 ml of KRP containing 7.5 mM glucose. 5 mg of starch-^{14}C were then added in 0.05 ml of KRP, and the uptake was stopped after a further 10 min. Cell concentration: 0.3–0.4 mg of protein per dish. Results are the means of two experiments.

Inhibitor	Concentration	Inhibition
	mM	%
Sodium iodoacetate	0.3	81
Sodium fluoride	20.0	76
2,4-dinitrophenol	0.1	24
Sodium cyanide	1.0	6

usual conditions for 10 min, the dishes were rinsed for removal of excess starch, and 1 ml of KRP containing 7.5 mM glucose was pipetted onto each. The monolayers were then incubated for further periods of up to 90 min, washed again, and the number of particles still attached to the dishes was assayed. The phagocytized particles were quantitatively retained by the monolayers for the whole duration of this additional incubation period (Table II).

PHAGOCYTOSIS OF OTHER PARTICLE TYPES: Opsonized starch was phagocytized at a more rapid initial rate than unopsonized starch, and uptake stopped in less than 10 min. Opsonized

TABLE II

Retention of Phagocytized Starch by the Cells

Normal incubations for measuring phagocytosis were made with unopsonized starch-^{14}C. The rinsed monolayers were subsequently incubated for the specified periods in fresh KRP-glucose, and the amount of radioactivity remaining in the layer was determined. The results are the means of two experiments.

Period of second incubation	Retention*
min	%
0	100
10	98
20	101
30	102
60	110
90	102

* % retention is expressed as:

$$\frac{\text{mg starch per mg protein after reincubation}}{\text{mg starch per mg protein at time 0}} \times 100.$$

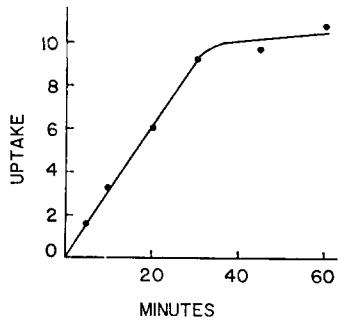

FIGURE 5 The time-course of phagocytosis of ^{32}P-labeled *Salmonella typhimurium* by guinea-pig peritoneal polymorphonuclear leukocytes. Incubations with labeled bacteria with 0.5 mg cell protein were carried out for the specified times at 37°. Uptake is expressed as cpm ^{32}P × 10^3 of opsonized bacteria ingested per mg cell protein.

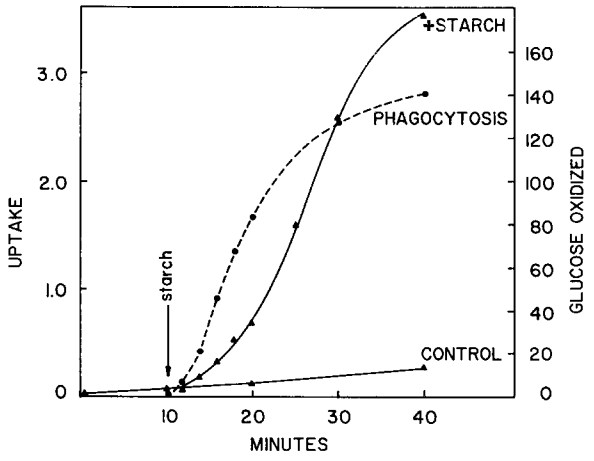

FIGURE 6 A comparison of the time-course of phagocytosis and that of the associated stimulation in oxidation of glucose-1-^{14}C in guinea pig peritoneal polymorphonuclear leukocytes. The incubation details are given in the text. Particle uptake (--●--) is expressed as mg of starch ingested per mg of cell protein. $^{14}CO_2$ evolution (—▲—) is expressed as nmoles $^{14}CO_2$ evolved (or glucose oxidized) per mg of cell protein. 5 mg of starch particles were added after 10 min of incubation (arrow).

^{32}P-labeled bacteria were phagocytized at a slower initial rate than starch, and uptake continued for a considerably longer period (Fig. 5). The layers were also observed to phagocytize polystyrene spherules extensively.

Oxidation of Glucose-1-^{14}C by Guinea Pig Cells

The labeled glucose preparations used in these experiments released approximately 0.1% of their total radioactivity as CO_2 on addition of acid, even in the absence of cells. As this quantity was of the same order of magnitude as the amount of glucose oxidized by monolayers in the absence of starch, the values of oxidation rates for control monolayers must be regarded as approximate, unless many observations are made.

KINETICS: $^{14}CO_2$ output was independent of glucose concentration from 2.5 to 10 mM, was proportional to protein content up to at least 0.5 mg per dish, and was proportional to time for at least 40 min of incubation. Unstimulated cells oxidized about 20 nmoles of glucose per mg protein per hour (e.g. Fig. 6).

EFFECTS OF PARTICLES ON GLUCOSE OXIDATION: In these measurements, a slightly modified technique was used. The measurement of glucose oxidation followed the addition of glucose-^{14}C and substitution of a lid modified for CO_2 trapping as described under Methods. However, after 10 min of incubation the parafilm covering the hole in the lid was briefly lifted, and 0.1 ml of NaCl (0.9%) or 0.1 ml of NaCl containing 5 mg of starch was added. The hole was then quickly recovered. Incubation was continued for varying periods and the reaction was stopped with HCl (see Methods). The time-course of particle uptake was followed simultaneously by the use of an identical set of monolayers. As is seen in Fig. 6, the onset of the maximum rate of phagocytosis preceded the onset of the maximal stimulation of glucose oxidation by a few minutes and did not taper off at the time when particle uptake was diminishing.

Phagocytosis by Human Leukocytes

The time-course, protein-dependence, and effects of starch concentration with human leukocytes were similar to the corresponding data for the guinea pig cells. Human cells phagocytized less starch than guinea pig cells (Fig. 4 *D*). The data with the human cells were obtained with the acetyl-^{14}C-starch (batch 2) which gave the guinea pig data of Fig 4 *C*.

GLUCOSE OXIDATION BY HUMAN LEUKOCYTES: Glucose was oxidized at rates somewhat slower than those for the guinea pig cells, and the respiratory stimulus associated with the addition of starch particles was of about the same magnitude. In two experiments, the unstimulated and stimulated rates of oxidation were: Experiment a, 15 and 108; Experiment b, 3.5 and 77 nmoles of glucose oxidized per mg protein in 20 min. These figures may be compared with the data for guinea pig cells given in Fig. 6.

DISCUSSION

In the method for assaying phagocytosis which has been presented above, particle ingestion is measured as the uptake of radioactive particles (starch-^{14}C or bacteria-^{32}P) by monolayers of cells in small tissue-culture dishes. We believe that this method

offers several advantages over other available methods:

1. This method can be used for measuring the ingestion of any particle, irrespective of its size, for which a suitable radioactive label can be devised. Alternatively, particles of any type might be used, the monolayers dissolved in NaOH as described here for protein determination, and the particle concentration measured by a suitable means. The problems associated with assays in which particles are used which are difficult to separate from cells by differential centrifugation are not encountered.

2. The timing of the incubations does not depend on arresting the ingestion process either by cooling or by the addition of metabolic inhibitors to the medium. It is, therefore, easy to make measurements over very short periods.

3. It is not necessary, after incubation, to expose the cells to a series of washes by differential centrifugation in order to separate away the unphagocytized particles.

4. If it is necessary to preincubate the cells with an added agent and to remove the agent before measuring particle ingestion, this is very easily achieved by a rinsing of the monolayers as described above.

Although the monolayer method has, as yet, been applied only to a small number of cell and particle types, it could be applied to any type of cell which will adhere firmly to a glass or plastic surface. For instance, Carpenter and Barsales (9) described some studies of particle uptake by monolayers of monocytes cultured from spleen. Monolayers are also easily prepared from peritoneal monocytes, alveolar macrophages (10), or, more recently, eosinophils (unpublished data). It must be stressed that appropriate incubation conditions (particle concentration, time of incubation, etc.) should be determined for each type of cell if valid measurements of *initial phagocytic rates* are wanted. For example, when the method described here was applied to the uptake of opsonized acetyl-^{14}C-starch particles by guinea pig peritoneal monocytes (10), particle uptake was proportional to the period of incubation for only slightly over 5 min.[1]

The method is readily amenable to monitoring by microscopy, since the dishes have satisfactory optical qualities. We have not noted adherence of particles to the plastic between cells and have found that negligible association of particles with the dishes ocurred in the absence of cells. As far as we can tell microscopically, the vast majority of particles in a dish with normally phagocytizing cells is within the cells, rather than merely attached to them; this is consistent with the data indicating severe depression of uptake by metabolic inhibitors. Microscopic evaluation should be included in the assessment of the method, with each type of cell to which it was applied, before acceptance of the method as a routine assay system.

The chief difference between the monolayer method described here and most of the previous assay methods lies in the ease with which uptake of particles can be measured after incubations of only a few minutes. The polymorphonuclear leukocyte ingests *E. coli*, protein-coated bentonite particles, or unopsonized starch for only about 20 min (Fig 3 and reference 8), opsonized starch for a shorter period, and polystyrene particles for less than 10 min (5). *Salmonella typhimurium* are taken in over a somewhat longer period (Fig 5). This means that, if useful measurements of the rates of particle ingestion by these cells are to be made, appropriately short incubation times are essential. This has not usually been the case in previous studies, and the published data on this type of cell thus largely reflect measurements of the total quantity of particles within the cells after completion of phagocytosis (i.e., the *capacity* of the cells for particle uptake under the specified conditions has been measured). This would apply, for example, to the results of Cohn and Morse (1), Sbarra and Karnovsky (4), Roberts and Quastel (5), and Sastry and Hokin (2). In some cases, assays have been based on experiments in which the suspending medium has been almost completely devoid of particles at the end of the incubation (e.g. reference 18). In all of these studies, the reported effects on phagocytic activity might represent variations in either *rate of* or *capacity for* uptake, or in both: the data would not differentiate between changes in these two variables. This difference may well account for apparent discrepancies between our results and those of other workers. For example, whilst we found effective inhibition of phagocytosis by guinea pig PMN at low concentrations of iodoacetate (Table I), Carpenter (8) found considerably lower levels of inhibition, even when he used much higher iodoacetate concentrations. If iodoacetate depressed only the rate of uptake of particles by these cells, and not their eventual capacity for ingestion, then this result might be simply a reflection of the longer incubation times used by those workers.

[1] C. Peeters and E. A. Glass, unpublished data.

Serum, or serum factors, have often been implicated both in the attachment of cells to surfaces and in the phagocytic process itself. We have not routinely used serum in the method described in this paper. However, for some cell-types, it might be advantageous to include serum in the medium during the cell-attachment process (e.g., Oren et al. [10]). With respect to the role of serum factors in particle ingestion, we have observed, as expected, that either pretreatment of certain types of particles with serum or inclusion of serum in the medium during phagocytosis greatly increased the rate of ingestion (unpublished data). One area of investigation in which the assay method we have described might be of real value is the study of the roles of serum factors in phagocytosis (e.g., reference 19).

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