THE EFFECT OF POLY-L-LYSINE ON THE
UPTAKE OF REOVIRUS DOUBLE-STRANDED RNA
IN MACROPHAGES IN VITRO

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ABSTRACT
The effect of polycations on cultured mouse peritoneal macrophages has been examined. Polycations, at concentrations greater than 5 µg/ml, are toxic for macrophages, as measured by failure of the cells to exclude vital dyes. At toxic concentrations polycations bind in large amounts to nuclei and endoplasmic reticulum, while at nontoxic levels polycations bind selectively to the cell surface. Nontoxic concentrations of polycations stimulate binding of reovirus double-stranded (ds) RNA to the macrophages by forming polycation-dsRNA complexes either in the medium or at the cell surface. These complexes enter the cell in endocytic vacuoles and are concentrated in secondary lysosomes. Despite exposure to the acid hydrolases within this cell compartment, the dsRNA and the polycation (poly-L-lysine) are conserved in a macromolecular form within the vacuolar system. The mechanism(s) by which the uptake of infectious nucleic acids and the induction of interferon by dsRNA are stimulated by polycations are discussed.

INTRODUCTION
Polycations have been reported to enhance the infectivity of single- and double-stranded viral RNA (1), of double-stranded DNA (2), and of RNA tumor viruses (3); they stimulate the cellular uptake of a variety of macromolecules (1, 2, 4–6); and they increase interferon production in cells treated with double-stranded (ds) RNA (6–8). These findings are of considerable general interest in view of the slow rate at which large molecules usually penetrate cells and the profound effects that polynucleotides exert upon cellular metabolism once they gain access to the cytoplasmic matrix.

The mechanism by which polycations stimulate the uptake of polynucleotides has been examined by several investigators (6, 7, 9–11). In general, their results are consistent with the hypothesis that polycations stimulate the binding of polynucleotides to cells by forming cationic bridges between the negative charges of the polynucleotide and the cell surface. As a result, polynucleotide-polycation-cell surface ternary complexes are presumably formed. However, the events which follow the polycation-stimulated binding of polynucleotides to cell surfaces have not been examined in detail. Comparatively little attention has been directed toward the toxic effects of polycations upon living cells and the relationship of polycation-induced changes in cell permeability to the binding of a variety of macromolecules to cells (6, 12–14). In order to clarify some of these issues, we have examined the effects of several polycations on the
viability of mouse peritoneal macrophages in vitro. We have studied the uptake, intracellular fate, and localization of poly-L-lysine in these cells and the effect of this polycation upon the binding and uptake of reovirus dsRNA. A preliminary report of these studies has been presented (33).

MATERIALS AND METHODS

[3H]Uridine and [3H]cytidine-labeled reovirus was grown and purified as described (15), dialyzed against phosphate-buffered saline (PBS) (16), and the RNA was extracted with an equal volume of water-saturated phenol at room temperature. The [3H]dsRNA-containing aqueous layer was extracted with ethyl ether, to remove any remaining phenol, and placed on a 1.5 × 7 cm methylated albumin-silicic acid column (17).

The [3H]dsRNA eluted in a single peak at 0.7 M NaCl. The peak fractions were combined, dialyzed exhaustively against PBS, passed through a 0.5 × 2 cm Sephadex G25 column which had been equilibrated with PBS, and the [3H]dsRNA-containing fractions were combined. The final solution contained 37.5 µg dsRNA/ml (1 OD at 260 nm = 50 µg/ml dsRNA (18) of sp act 1.05 × 10^5 dpm/µg.

[3H]Poly-L-lysine

[3H]Poly-L-lysine, average mol wt 50,000, sp act 1.37 mCi/µl, was prepared by New England Nuclear (Boston, Mass.) using the Wilzbach procedure. Nonradioactive poly-L-lysine was purchased from Yeda (Rehovot, Israel), poly-L-ornithine from Sigma Chemical Co. (St. Louis, Mo.). DEAE-dextran (mol wt 2 × 10^6) was purchased from Pharmacia Fine Chemicals, Inc. (Uppsala, Sweden). Poly-inosinic-cytidylic acid (poly IC) was purchased from Microbiological Associates, Inc. (Bethesda, Md.).

Macrophage Cultures

Macrophages were obtained as described previously (19) and were cultivated in medium 199 with 30% newborn calf serum (NBCS) in 35 mm plastic Petri dishes (Falcon Plastics, Oxnard, Calif.) or in Leighton tubes for 24 h before experiments. The cell concentration was approximately 10^6 cells per dish, or 5 × 10^6 cells per Leighton tube.

The experimental incubations were performed using 1.5 ml medium 199 with 1% NBCS, and dsRNA and poly-L-lysine as stated. All incubations were performed at 37°C in 95% air, 5% CO₂. To determine cell viability, cover slip cultures were incubated with 1% trypan blue or 1% nigrosine in 0.9% NaCl for 2 min before microscopic examination.

Light Microscopy

Some of the morphologic studies were performed with cells grown on flying cover slips in Leighton tubes (19). After the appropriate incubation, the cells were fixed by immersion in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 min, rinsed, mounted in water, and examined under phase-contrast optics with a Zeiss Ultraphot II microscope (Carl Zeiss, Inc., New York). Light microscopic autoradiographs were prepared with an L4 Ilford suspension (Ilford, Ltd., Ilford, England). Exposure times varied from 1 to 12 wk, after which the micrographs were developed with Microdol.

Electron Microscopy

Cells on plastic Petri dishes were fixed with glutaraldehyde for 15 min, rinsed, fixed in 1% OsO₄ in 0.1 M phosphate buffer for 30 min, and dehydrated and embedded in Epon. Semithin and thin sections were made with a Porter-Blum Microtome equipped with a diamond knife. Autoradiographs were produced according to the method of Salpeter (20) or von Gaudecker (21) as described previously (22). Exposure time varied from 4 to 10 mo. Thin sections were stained with lead according to Reynolds and were examined in a Siemens Elmiskop I electron microscope (Siemens Corp., Medical Industrial Div., Iselin, N. J.).

Binding of Labeled Materials

After incubation of cells with the isotopically labeled compound to be tested, the medium was removed and the dishes were rinsed three times with 1 ml prewarmed 0.9% NaCl solution. The cells were scraped off the dish with a rubber policeman, suspended in 1 ml of saline, centrifuged for 5 min at 1,000 rpm, and the supernatant was discarded. The resulting cell pellet was washed once in 1 ml 0.9% saline and dissolved overnight at room temperature in 1.0 ml of 1 N NaOH. A sample of 0.2 ml was counted in 15 ml Bray's solution in a Nuclear-Chicago Mark II scintillation spectrometer (Nuclear-Chicago Corp., Des Plaines, Ill.). Quenching was corrected using an external standard. The results were expressed as counts per minute per dish. Preliminary experiments showed that the cell content of dishes showed little variation (~10%) as judged by protein assay (23).

Differential centrifugation was performed with a Spinco Ultracentrifuge equipped with an SW 39 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Fractions were collected dropwise...
from the bottom of the tube and assayed for radio-
label as described above.

RESULTS

Effect of Polycations on Cell Morphology and Viability

At all concentrations over 5 µg/ml there were clear indications of cell damage (Fig. 1). After 60 min of incubation with 10 µg/ml of the nonradioactive poly-L-lysine, more than 95% of the cells were dead as judged by phase-contrast microscopy or by the uptake of trypan blue or nigrosine (Fig. 2). The toxic effects as judged by phase-contrast microscopy consisted of rounding up of the cells, cytoplasmic swelling, retraction of organelles to the perinuclear area, and abnormal refractile properties of the nuclei. The majority of the dead cells
The Uptake of Poly-L-lysine

Experiments with 12 µg/ml of [3H]poly-L-lysine (a toxic dose) showed a rapid temperature-independent binding of the material during the first hour of incubation; thereafter there was no significant additional binding of [3H]polylysine by the dead cells (Fig. 4 a). Autoradiographs demonstrated that the radioactivity in this case was located mainly in the nucleus and in remnants of the endoplasmic reticulum (Fig. 5). In contrast, when nontoxic amounts of polylysine (2 µg/ml) were added, uptake continued for 5 h (Fig. 4 a) and accounted for 1–2% of the polylysine in the medium. Uptake at this concentration was temperature dependent and inhibited by incubation at 4°C. Autoradiographs of cells incubated under these conditions showed labeling mainly over the cell surface as well as occasionally over cytoplasmic vacuoles (Fig. 6).

It should be noted that although only twice as much radiolabeled polylysine was taken up after a 1 h exposure of macrophages to a toxic concentration (12 µg/ml), as compared with a nontoxic concentration (2 µg/ml) of this compound, there was a fivefold excess of unlabeled polylysine in the former instance (Fig. 4 a). Hence, macrophages exposed to the toxic concentration bound 10-fold as much polylysine as those incubated with the compound at nontoxic concentrations.

Exposure of cells to 2 µg/ml of [3H]poly-L-lysine for 1 h with subsequent incubation in medium without the polycation for up to 6 h showed no significant loss of cell-bound radioactivity (Fig. 4 b). In contrast, washout studies with toxic concentrations (12 µg/ml) showed the loss into the medium of 50% of the labeled polycation by 6 h.

The Effect of Poly-L-lysine on the Uptake of dsRNA

In the absence of polylysine, there was insignificant binding of [3H]dsRNA to macrophages. However, addition of 2 µg/ml cold poly-L-lysine increased the uptake of dsRNA dramatically (Fig. 7). Toxic doses of polylysine also increased the uptake of [3H]dsRNA, but the extent of this effect showed marked variability in terms of time-course and total amount of [3H]dsRNA bound.

To determine whether the order in which polylysine and dsRNA were added to the cells influenced the binding of [3H]dsRNA, the experiments described in Table I were performed. Macrophages preincubated for 30 min with 2

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Figure 3  (a) Phase-contrast micrograph of macrophages after 6 h exposure to 2 µg/ml poly-l-lysine in medium 199 containing 1% NBCS. There are no signs of cell damage. X 1,500. (b) Electron micrograph from same experiment as Fig. 3a. The macrophage is well preserved. X 16,250.

µg/ml polylysine bound as much [3H]dsRNA as cells incubated for a similar time period with both [3H]dsRNA and polylysine (Table I, lines a and c). Similar results were obtained with poly-l-arginine, poly-l-ornithine, and DEAE-dextran at concentrations of 2 µg/ml. However, the polylysine-stimulated binding of [3H]dsRNA was abolished by addition of poly IC before the addi-
tion of \(^{3}\text{H}\)dsRNA (Table I, line d). When the sequence of addition of the three compounds was reversed, i.e. preincubation with poly IC, followed by polylysine and then \(^{3}\text{H}\)dsRNA, the results were similar to those obtained with polylysine alone (Table I, lines c and e). These data suggested that polylysine forms a stable complex with the cell surface and that it is the surface polylysine which complexes dsRNA and stimulates its uptake. To test this hypothesis macrophages were preincubated with unlabeled polylysine, rinsed, and incubated in the absence of polylysine alone (Table I, lines e and f). These data suggested that polylysine forms a stable complex with the cell surface and that it is the surface polylysine which complexes dsRNA and stimulates its uptake. To determine whether the decrease in the \(^{3}\text{H}\)dsRNA-binding capacity of the cells was due to elution of polylysine into the medium or to ingestion of the polylysine by the cells, macrophages were assayed for their content of \(^{3}\text{H}\)poly-L-lysine at intervals after a 4 h washout. To determine whether the decrease in the \(^{3}\text{H}\)dsRNA-binding capacity of the cells was due to elution of polylysine into the medium or to ingestion of the polylysine by the cells, macrophages were assayed for their content of \(^{3}\text{H}\)poly-L-lysine at intervals after a 30 min exposure to \(^{3}\text{H}\)poly-L-lysine. Throughout the 6 h washout period there was no loss of cell-bound radioactivity. Evidence derived from other studies indicates that there was little or no hydrolysis of poly-L-lysine after 4 h. Hence, it seems likely that the loss of \(^{3}\text{H}\)dsRNA binding capacity reflects removal of polylysine from the cell surface by endocytosis.

**The Uptake of Polycation-Polynucleotide Complexes**

Our own experimental results, together with the data reported by others (25), suggested that dsRNA is taken up by macrophages as dsRNA-polycation complexes. We therefore formed such complexes in vitro by mixing polycations and polynucleotides. Some experiments were performed with poly-L-lysine-\(^{3}\text{H}\)dsRNA complexes, but because of the relative scarcity of \(^{3}\text{H}\)dsRNA most of the experiments were done with \(^{3}\text{H}\)poly-L-lysine and poly IC. Complexes formed almost instantaneously at room temperature when solutions of poly-L-lysine and poly IC were mixed. These complexes could be sedimented by ultracentrifugation (Fig. 9). By increasing the ratio of poly IC to polylysine the formation of sedimentable complexes was enhanced (Fig. 9). When the pelletted complexes from these experiments were separated, resuspended, and added to macrophage cultures, their uptake was found to be proportional to the

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**Figure 4**: Uptake and stability of \(^{3}\text{H}\)poly-L-lysine in macrophages. (a) Macrophage cultures were incubated with 2 µg/ml (nontoxic) or 12 µg/ml (toxic) poly-L-lysine for the indicated time periods and assayed for cell-bound radiolabel. •, 2 µg/ml \(^{3}\text{H}\)poly-L-lysine; ○—○, 2 µg/ml \(^{3}\text{H}\)poly-L-lysine + 10 µg/ml poly-L-lysine. (b) Macrophage cultures were incubated with 2 or 10 µg/ml of \(^{3}\text{H}\)poly-L-lysine for 1 h at 37°C. The plates were washed and incubated for the indicated time periods in medium 199 containing 1% NBCS. Cell-bound radiolabel was assayed as described in Methods. •—•, 2 µg/ml \(^{3}\text{H}\)poly-L-lysine; •—•, 2 µg/ml \(^{3}\text{H}\)poly-L-lysine + 10 µg/ml poly-L-lysine.
FIGURE 5  (a) Light microscopic autoradiograph of macrophages incubated with 10 µg of [3H]poly-l-lysine for 1 h. Grains are seen over the cells as well as over the glass surface. × 1,500. (b) Electron microscopic autoradiograph from same experiment as Fig. 5 a. The cell is disintegrated. Grains are seen mainly over the nucleus as well as over remnants of endoplasmic reticulum. × 17,500.
FIGURE 6  Electron microscopic autoradiographs after exposure of macrophages to nontoxic doses of radioactive poly-L-lysine (2 µg/ml). Grains are found mainly over or close to the cell surface (a), and less frequently over cytoplasmic vacuoles (b and c). (a) × 24,500; (b) × 15,750; (c) × 24,500.
The uptake of $[^3H]$dsRNA (0.37 $\mu$g/ml) in the presence (---) and absence (----) of nontoxic amounts of poly-$L$-lysine (2 $\mu$g/ml).

**Fate of dsRNA**

To determine the intracellular fate of the dsRNA, macrophages were incubated with $[^3H]$dsRNA and polylysine and processed for light and electron microscopic autoradiography. Light microscopic autoradiography showed heavy labeling over the cytoplasm, especially over vacuoles in the perinuclear area (Fig. 12a). This is the locus of vacuoles derived from endocytic events. Electron microscopic autoradiography revealed that the label was located exclusively in vacuoles containing a granular material (Fig. 12b). Extraction of the cells at this time confirmed that the label was still present in the form of $[^3H]$dsRNA, as judged by its resistance to pancreatic ribonuclease digestion at high ionic strength and its susceptibility to digestion with this nuclease at low ionic strength (17, 18).

**Table I**

*Effect of Preincubation with Polycations and Polyanions on $[^3H]$dsRNA Binding*

<table>
<thead>
<tr>
<th>Order of addition*</th>
<th>Uptake $[^3H]$dsRNA (% of control, line a)</th>
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<tbody>
<tr>
<td>1st</td>
<td>2nd</td>
</tr>
<tr>
<td>(a) None</td>
<td>None</td>
</tr>
<tr>
<td>(b) None</td>
<td>None</td>
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<tr>
<td>(c) Polylysine</td>
<td>Polylysine</td>
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<tr>
<td>(d) Polylysine</td>
<td>Poly IC</td>
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<tr>
<td>(e) Poly IC</td>
<td>Polylysine</td>
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* Cells were incubated for 30 min at 37°C in medium 199 containing 1% NBCS and the compound to be tested. They were then washed in prewarmed medium and incubated for an additional 30 min with the second test compound, etc. Polylysine, 2 $\mu$g/ml; poly IC, 10 $\mu$g/ml; $[^3H]$dsRNA, 0.37 $\mu$g/ml.
FIGURE 9 Sedimentation behavior of [3H]poly-L-lysine-dsRNA complexes. 40 µg of poly IC (hatched bars), 80 µg of poly IC (open bars), or 160 µg of poly IC (solid bars) were incubated with 4 µg [3H]poly-L-lysine for 12 h at 37°C. The samples were layered over 0.25 M sucrose in 0.1 M sodium phosphate, pH 7.4, and centrifuged for 4 h at 35,000 rpm in an SW 39 rotor. Increasing amounts of sedimentable-[3H]poly-L-lysine appear as the concentration of poly IC is increased.

FIGURE 10 Uptake of [3H]poly-L-lysine-dsRNA complexes by macrophages. The uptake is proportional to the amounts of complexes available to the macrophages.

DISCUSSION
Polycations are markedly toxic for macrophages. In our experiments concentrations of poly-L-lysine (mol wt ~50,000), polyornithine, or DEAE-dextran (mol wt ~2 × 10^5) in excess of 5 µg/ml caused loss of selective permeability, as judged by the inability of the cells to exclude vital dyes, lysis of the cell membrane, and cell death. Dead cells bound about 10-fold more [3H]poly-L-lysine after a 1 h exposure than did viable ones (Fig. 4 a); in the former circumstance the radiolabeled polycation was bound to nuclei and endoplasmic reticulum (Fig. 5), while in the latter case it was localized exclusively on the plasma membrane and within endocytic vacuoles (Fig. 6). Thus the binding of polycations to cells, per se, cannot be used as a measure of biological activity and it is apparent that even a small proportion of damaged cells may give rise to spuriously increased uptake. These results cast doubt upon the biological significance of the reported entry of histones and other polycations (13, 14) into the nucleus of animal cells. Rather, it seems likely that polycations are merely reacting with nucleic acids and other anionic molecules in the nucleus and cytoplasm of damaged cells.

The absolute amount of polycation needed to alter cell viability is probably dependent upon several factors including the cell type, the degree of polymerization of the polycation, and the presence of other substances capable of reacting with the polycation, some of which may be present in serum. Cells can be protected from polycation-induced damage by poly IC or polyglutamic acid (5). This may explain why the high concentrations (25–1,000 µg/ml) of polycations used by some
FIGURE 11  (a) Phase-contrast micrograph displaying small aggregates of poly-L-lysine-dsRNA complexes taken up by macrophages after 6 h of exposure (arrows). X 1,500.  (b) Electron microscopic autoradiograph demonstrating the localization of [3H]poly-L-lysine-dsRNA complexes after 6 h of incubation. The radioactivity is seen over several large vacuoles which contain granular material. X 27,000.
FIGURE 12  (a) Light microscopic autoradiograph of macrophages incubated for 6 h with 2 µg/ml poly-L-lysine and 0.75 µg/ml [3H]dsRNA. Many grains are seen over the cell, mainly in the peri-Golgi zone. There is no labeling above background over the nucleus or the remainder of the cell cytoplasm. × 3,750. (b) Electron microscopic autoradiograph from same experiment as Fig. 12 a. Grains are seen over a perinuclear vacuole containing granular material. Similar vacuoles located more peripherally in the cell are not labeled and do not contain the granular material seen in the labeled vacuole.
investigators to stimulate nucleic acid uptake (2, 6, 11) and interferon production (8) have not destroyed the cells. Under some circumstances, toxic levels of polycations may be responsible for the release of preformed interferon rather than for enhancing the capacity of dsRNA to induce interferon production.

The precise mechanisms by which polycations stimulate binding of nucleic acids and other polyanions to cells have not been rigorously defined. Polycations can serve as agglutinins, presumably by facilitating electrostatic interactions between the negatively charged surfaces of animal cells (26). It seems likely that polycations serve a similar function in binding polyanions to cell surfaces.

The particulate nature of the complex may also play a role in stimulating the uptake of dsRNA. In this regard, the complexing of soluble proteins with antibodies or their aggregation by heat results in a several thousand-fold increase in binding and uptake by macrophages (27). Soluble horseradish peroxidase, which does not bind to the macrophage plasma membrane, is pinocytized at a rate of 0.001% of its extracellular concentration per hour. When complexed with IgG, it is internalized 4,000-fold more rapidly and in proportion to the extracellular load. This is in keeping with the uptake of preformed polycation-polynucleotide complexes.

Cell surface-bound polycations are rapidly internalized by endocytic mechanisms and become unavailable for subsequent binding of added polyanions (references 10, 11; Fig. 8). Competition experiments, such as those described in Table I, demonstrate that the polycation-stimulated uptake of [3H]dsRNA can be blocked by the addition of a competing polynucleotide, such as poly IC, before the addition of [3H]dsRNA. These data, taken together with morphological observations indicating that polycations do not alter the rate of pinocytosis in macrophages, clearly demonstrate that the polycation-stimulated uptake of dsRNA is not a reflection of increased membrane permeability or of a stimulated uptake of bulk fluids from the medium. Rather, they offer strong support for the hypothesis that polycations facilitate the binding of nucleic acids to the cell surface and that it is the polycation-polyanion complex which is removed from the surface by ongoing endocytic processes.

After endocytosis, dsRNA-polycation complexes were concentrated within the vacuolar system of the cell, presumably in secondary lysosomes. Despite their exposure to the acid nucleases and proteases contained within this cellular compartment, both the [3H]dsRNA and the [3H]poly-L-lysine were conserved (Figs. 4, 8, 12) throughout the course of these experiments. Previous work (17) has demonstrated that the [3H]dsRNA used in these experiments is resistant to lysosomal nucleases, but the resistance of [3H]poly-L-lysine to hydrolysis by lysosomal proteases is surprising and remains unexplained.

Virtually all the endocytosed [3H]dsRNA was conserved within the vacular system, as judged by electron microscopic autoradiography. Thus these experiments offer no support for the hypothesis that nontoxic concentrations of polycations stimulate the transport of [3H]dsRNA into the cytoplasmic matrix. Rather, they argue strongly for the orthodoxy view which states that endocytosed macromolecules remain within the vacular system until they are degraded to nucleosides, oligosaccharides, or small peptides (28–30). Admittedly, we cannot rule out the possibility that even at nontoxic concentrations of polycations a small but biologically significant fraction of the [3H]dsRNA reaches the cytoplasmic matrix where it induces interferon production and cellular resistance to a variety of pathogens. Alternatively, it is possible that the biologically important event which triggers interferon production and/or release is the interaction of dsRNA with cellular membranes, and that polycations merely serve to facilitate this interaction; but it is also evident from studies with infectious viral nucleic acids that highly polymerized nucleic acids do enter the cytoplasmic matrix where they initiate the synthesis of progeny virions. In several cases (2, 11) the concentration of polycations required to maximally stimulate viral nucleic acid binding was substantially less than that required to stimulate maximal infectivity. Hence it seems likely that at low concentrations polycations enhance nucleic acid binding to the cell surface while at higher (perhaps minimally toxic) concentrations they facilitate the penetration of nucleic acids into the cytoplasmic matrix.

Whatever mechanism is involved, however, a note of caution seems in order. Polycations, single-stranded RNA (31), and dsRNA have been shown to cause cellular damage in vivo and in vitro, and recently interferon itself has been shown to sensitize cells to the toxic effects of dsRNA (32). Experiments designed to examine the biological effects of these macromolecules on cellular metab-
olism should rigorously exclude their possible cytotoxic effects, especially in the case of interferon, when the parameter being measured is the failure of cells to perform a given function such as supporting the replication of animal viruses.

We thank Cynthia Wang for excellent technical assistance.

This study was supported by grants AI 07012 and AI 8697 from the U.S. Public Health Service.

Received for publication 9 November 1972, and in revised form 9 January 1973.

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