As with the poliovirus-HeLa cell system, low pH and high salt concentration dissociate the virus-receptor complex. It is interesting that lipid solvents destroy receptor activity without releasing receptor bound virus. This indicates that, once receptor protein is bound to the virus, receptor lipid can be dissolved without effecting the receptor-virus complex. Therefore, lipid may not function as receptor material, except to preserve the integrity of the protein moiety in the membrane structure. SDC, on the other hand, releases bound virus without destroying receptor activity. Preliminary experiments suggest that SDC may be solubilizing RBC receptor but further work is required to confirm that the observed inactivation of HA is due to receptor activity.

The receptor material reported on here appears to possess properties common to the hemagglutinating and non-hemagglutinating human enterovirus receptors(3-8), *i.e.*, it resembles poliovirus receptors in its sensitivity to lipid solvents, hydrogen bond disruptors and trypsin, and ECHO 7 receptors in its sensitivity to chymotrypsin. This intermediate position may merely be a reflection of the different cell species used or differences in experimental technique and therefore lack taxonomic significance for the viruses involved. It is of interest that SV2 HA was inactivated by pCMB in a manner similar to human enteroviruses(9,10), *i.e.*, HA was inactivated but no effect was observed on cell receptors.

The bond formed between SV2 and RBC appears to be quite stable, as evidenced by its failure to elute at 37°C even though there is no hemagglutination at this temperature. In this respect it resembles ECHO 13 and 19(11) which also fail to elute or cause hemagglutination at 37°C. The reason for this remains obscure but it may be that the bond formed between SV2 and RBC at 37°C is different from the bond which causes hemagglutination, the former being electrostatic and the latter a temperature dependent bond.

*Summary.* A study was made of the effect of various chemical, physical and enzymatic treatments on the receptor activity of rhesus RBC and PMK for SV2 virus. The results indicate that the receptors are lipoprotein in nature and similar to one another on both types of cell.

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## Assay of Papain Using $I^{131}$-Labeled Albumin.* (30466)

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Albumin and casein, labeled with $I^{131}$, have been reported to be suitable substrates for measurement of the activity of papain and other proteolytic enzymes(1-6). Radiochemi-

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cal assay procedures using these labeled proteins have been described as more convenient and, under certain conditions, more precise than older methods using direct chemical technics(4,5).

This report presents a reliable, rapid method for assay of papain protease using $I^{131}$-labeled albumin. The products of the enzymatic digestion of albumin are soluble in trichloracetic acid (TCA). In our method, the percentage of albumin degraded by the enzyme after a specific time interval is determined from the distribution of radioactivity between the precipitated and supernatant fractions of the mixture after addition of trichloracetic acid, TCA. The rate of enzymatic digestion of albumin is measured under conditions assuring maximum velocity of the reaction. Albumin degradation is then directly proportional to the quantity of enzyme over a wide range of enzyme concentration.

*Materials and methods.* Papain protease, twice crystallized according to the method of Kimmel and Smith(7), was purchased from Worthington Biochemical Corp. or Nutritional Biochemicals Corp. Normal human serum albumin was obtained from Merck Sharp and Dohme or from Cutter Laboratories. $I^{131}$-labeled human serum albumin was prepared by the method of Newerly(8) or obtained from Abbott Laboratories, Inc., or from Squibb and Sons. Nitrogen determinations were performed by a modified micro-Kjeldahl technic(9).

In the routine assay of papain by this method, the number of $\mu$g of albumin degraded per $\mu$g papain per minute is determined at papain concentrations of 6 and 12 $\mu$g/ml with an albumin concentration of 15 mg/ml. The reaction is run for 10 minutes at 37°C. 2,3 Dimercaptopropanol (BAL), 0.2 M, is added as activator. All dilutions are prepared in phosphate buffer, 0.1 M, pH 7.0 $\pm$ 0.15. Total volume of the mixture is 2.5 ml. The amount of albumin-$I^{131}$ in the solution should not exceed 0.1 mg/ml. The quantity of labeled albumin added is calculated to produce a minimum of 50,000 counts per minute for each 0.2 ml portion of the mixture withdrawn for measurement. Solutions containing papain, albumin, and BAL are each separately brought to 37°C in a water bath before mixing. After precisely 10 minutes, the reaction is terminated by transferring 0.2 ml of the mixture to a test tube containing 5 mg carrier albumin in 0.02 ml solution. Two ml of 5% TCA are immediately added with vigorous shaking. The tubes are then centrifuged at 3,000 r.p.m. for 10 minutes. In each experiment a control tube containing all constituents except enzyme is treated in the same fashion. The total gamma counts for the tube are measured in a well scintillation counter for one minute. The supernate is then poured into another tube and counted separately. $I^{131}$ in the supernate of the control tube should not exceed 4% of the total. The percentage of total $I^{131}$ in the supernate of the assay tube as determined from these counts less the percentage of total $I^{131}$ in the supernate of the control tube represents the percentage of albumin degraded in 10 minutes. From this the quantity of albumin digested per $\mu$g papain per minute is calculated. Maximum activity of the enzyme was noted with papain concentrations of 4 to 16 $\mu$g/ml. In this range of enzyme concentration, the mean substrate breakdown was 21.9 $\mu$g (S.D. $\pm$ 3.4) albumin/$\mu$g papain/minute (Table I). The activity of an individual enzyme sample is expressed as a percentage of this figure.

*Preliminary investigation.* To determine the optimal conditions for digestion of albumin by papain, experimental conditions were varied and the reaction rate initially studied

TABLE I. Enzymatic Activity Measured by Release of $I^{131}$ at Albumin Concentration of 15 mg/ml with Papain Concentration Varied from 2 $\mu$g/ml to 25 $\mu$g/ml.

| Papain, $\mu$g/ml | Albumin degraded, $\mu$g/ml/$\mu$g papain/min* |
|---|---|
| 2 | 14.5 (2.3) |
| 4 | 21.8 (2.5) |
| 6 | 23.7 (3.3) |
| 8 | 22.8 (3.7) |
| 10 | 22.5 (3.7) |
| 12 | 20.8 (3.3) |
| 16 | 20.2 (3.8) |
| 20 | 18.0 (2.2) |
| 25 | 15.1 (1.2) |

* Standard deviation in parentheses.

by determining the amount of albumin degraded 3, 6, 10, 15, 30 and 60 minutes after mixing substrate and enzyme. The per cent of TCA-soluble radioactivity was then plotted as a function of time. The curves obtained were characteristic of a first order reaction. The zero time slope was determined from the tangent to the curve at the origin (Fig. 1) and the reaction velocity calculated as the product of zero time slope and initial concentration of albumin. It was noted as the investigation progressed that at enzyme-substrate concentrations most suitable for the assay of papain, the reaction velocity determined from the zero time slope closely approximated the value obtained by measurement of the quantity of albumin degraded at 10 minutes. The 10-minute interval was consequently selected for the assay of papain activity.

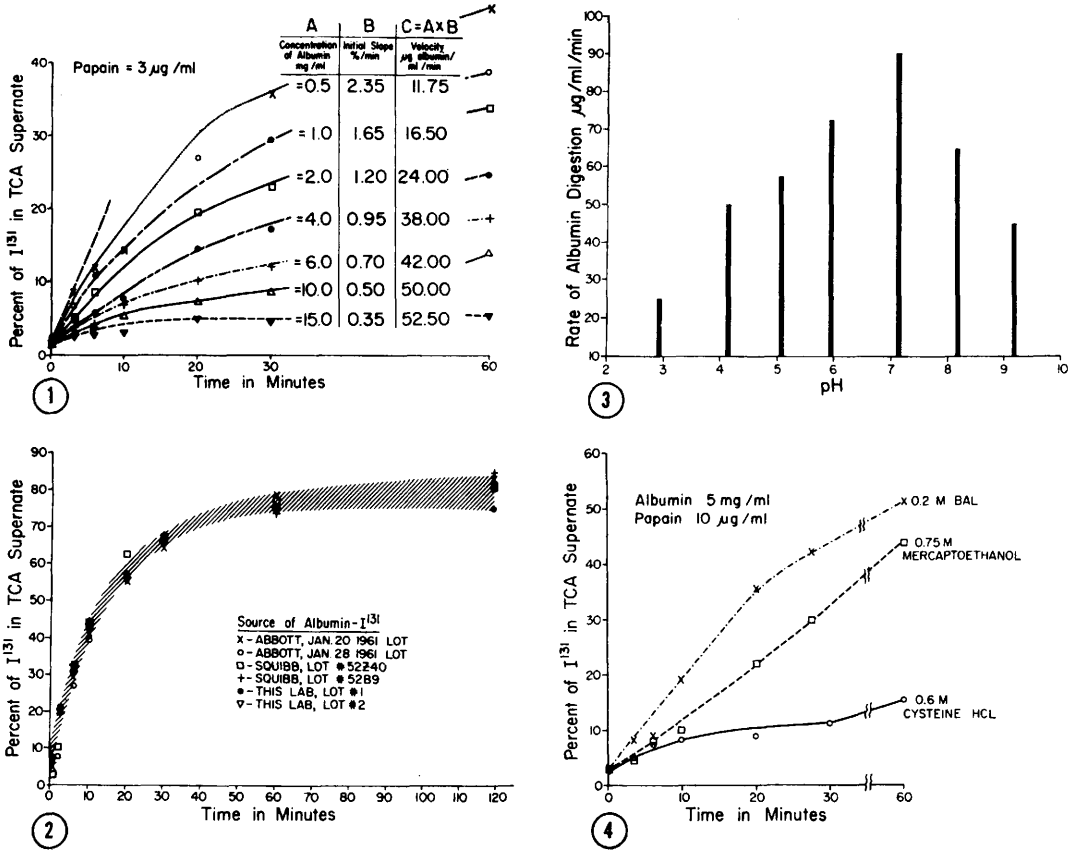


FIG. 1. Velocity of albumin degradation by papain (3 μg/ml) as determined by the appearance of $I^{131}$ in supernate after addition of TCA. Albumin concentration ranged from 0.5 to 15.0 mg/ml as shown. Activator was BAL 0.2 M. From the zero time slope, drawn as the tangent to the curve at the origin, percentage of substrate and μg/ml of substrate degraded per minute were calculated. The slope, drawn in the graph only for the reaction at albumin concentration of 0.5 mg/ml, was measured as 2.35%/min, or 11.75 μg albumin/ml/min. Velocity at the other concentrations of albumin was determined from the initial slope drawn in the same manner as the tangent to each curve.

FIG. 2. Degradation of 6 different preparations of albumin-$I^{131}$ by papain. All tubes contained 5 mg/ml human serum albumin, 4 mg/ml papain, cysteine .006 M, versene .001 M, and albumin-$I^{131}$ at negligible additional albumin concentration.

FIG. 3. Rate of albumin degradation by papain as function of pH. Reaction mixture contained albumin 6 mg/ml, papain 5 μg/ml, BAL 0.2 M. These are results of duplicate determinations at each selected pH.

FIG. 4. Comparison of enzyme activity with 3 different sulfhydryl activators, each at its optimal concentration.

*Reproducibility with various preparations of albumin $I^{131}$.* Two different shipments of albumin-$I^{131}$ from each of 2 commercial suppliers and 2 different preparations made in the laboratory were tested simultaneously using the same enzyme preparation. No significant differences were observed among the different preparations of labeled albumin (Fig. 2).

*pH optimum.* pH was varied over a range extending from 2.9 to 9.2 by adding HCl or NaOH to the phosphate buffer prior to addition of enzyme and substrate (Fig. 3). pH optimum at 37°C was about 7.0, a result similar to that reported by Stockell and Smith, who used benzoyl-L-argininamide as substrate for a study of papain kinetics(10).

*Influence of different sulfhydryl activators.* The level of enzyme activity was compared using as sulfhydryl activator cysteine hydrochloride with ethylene-diaminetetraacetate (versene), .001 M, mercaptoethanol or BAL. Papain was progressively more active with increasing concentrations of cysteine to the limits of solubility of cysteine. At similar concentrations, mercaptoethanol and BAL were much more effective enzyme activators than cysteine (Fig. 4). BAL was consistently more effective than mercaptoethanol. Optimal concentrations were 0.5 to 1.0 M for mercaptoethanol and 0.1 to 0.2 M for BAL. At higher molarities of these 2 activators, marked inhibition of the reaction occurred.

*Comparison of measurements of $I^{131}$ and nitrogen in supernate.* The rate of albumin degradation under conditions established for this assay method was determined with papain concentrations varied from 2 to 25 $\mu$g/ml. Six to 12 experiments using 6 different shipments of papain were performed in duplicate. The quantity of nitrogen in the TCA supernate was also measured by the micro-Kjeldahl technic in most of the same reaction velocity studies. To calculate the quantity of albumin degraded from this nitrogen determination, the conversion factor of 6.25 was employed (Fig. 5). There was close correlation of the results of the 2 methods.

*Discussion.* The technic of assay outlined here differs significantly from the method of

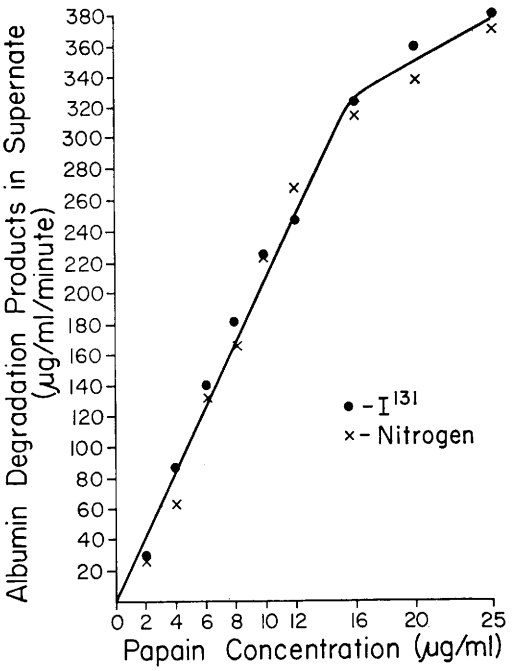


FIG. 5. Quantity of albumin degradation products in supernate in $\mu$g/ml/minute determined from percent albumin $I^{131}$ and by direct measurement of nitrogen by micro-Kjeldahl technic. Albumin concentration was 15 mg/ml in BAL 0.2 M, phosphate buffer 0.1 M, pH 7.0.

Potter and his associates who were the first to advocate the use of albumin-$I^{131}$ for assay of papain(6). They activated the enzyme with .005 M cysteine and were consequently performing their studies at much less than optimal velocity. The maximum rate of substrate breakdown as calculated from their published data was about $2.5 \times 10^{-2}$ $\mu$g albumin/$\mu$g papain/minute, which was comparable to our findings when cysteine was used as activator. This is 900 times slower than that obtainable with BAL-activated papain. Enzyme assay by these investigators was based upon average activity over a period of one hour, a method which does not allow for the possibility of product inhibition.

*Summary.* A sensitive, rapid method for assay of papain using albumin-$I^{131}$ as substrate is reported. Maximum velocity of albumin degradation by papain activated with BAL 0.2 M at pH 7.0 was found to be 21.9 $\mu$g albumin/$\mu$g papain/minute. Activity of the enzyme under assay conditions is expressed as a percentage of this maximum.

Uniform results were obtained with batches of albumin-$I^{131}$ obtained from various sources. The rate of albumin degradation as determined by this method of tracer measurement was closely parallel to the degradation rate obtained by measurement of non-protein nitrogen by the micro-Kjeldahl technic.

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## Assay of Sendai Virus by Immunofluorescence and Hemadsorbed Cell-Counting Procedures. (30467)

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The fluorescent cell-counting technique originally introduced by Wheelock and Tamm (1) has been successfully extended to several viruses such as adenovirus(2), human cytomegalovirus(3), and psittacosis virus(4). In this work, the technique was extended to Sendai virus by infecting L cells. Since L cells infected with egg-grown Sendai virus produce progeny infectious to egg embryos but not to L cells(5,6), the fluorescent cell-counting technique was considered to be suitable for enumerating infectious virus in the inoculum without being annoyed by the spreading of progeny virus from cell to cell through the medium. Possible use of the hemadsorbed cell-counting technique in this virus cell system was also examined, and it was shown to compare favorably with the fluorescent cell-counting technique.

*Materials and methods. Virus.* The Fushimi strain of Sendai virus was used. A seed virus was prepared by an allantoic inoculation of 10-day-old chick embryos with 0.2 ml of the stock virus diluted to $10^{-5}$ in phosphate buffered saline (PBS)(7). At the end of incubation period at 35°C for 40 hours, the allantoic fluids were harvested and stored at −25°C after centrifugation at 3,000 rpm for 15 minutes. This was used within 2 weeks after preparing. The 50% egg infectious dose ($EID_{50}$) of the allantoic harvests, calculated by the Reed and Muench method (8), ranged between $10^{9.7}$ and $10^{10.0}$, the hemagglutinin titer (HAU), $10^{3.3}$ and $10^{3.5}$ per ml.

*Medium for cell growth and infected coverslip cultures.* The medium used for cell growth contained 0.5% lactalbumin hydrolysate and 0.1% yeast extract in Earle's solution with 0.45% glucose and supplemented with 10% unheated bovine serum (growing medium). After infection, the cells were kept in a maintenance solution (MS) in which 2% inactivated horse serum was substituted for the bovine serum.

*L cell cultures.* A Petri dish (90 mm in diameter) with 12 coverslips (6 × 30 mm) received 20 ml of L cell suspension in growing medium at a concentration of $3 \times 10^5$ cells per ml and was incubated at 37°C in an atmosphere of 5% $CO_2$ in air for 3 days. By this time, a complete monolayer formed