teraction of azacyclonol and ADH at this dose range are unwarranted. Further investigation is required to explain the diphasic pattern of action of azacyclonol upon urine output in the rat.

*Summary.* Studies of creatinine and PAHA clearance showed a sufficient reduction of GFR and RPF to explain the antidiuretic action of a large dose (75 mg/kg) of azacyclonol. However, a moderate dose (50 mg/kg) produced antidiuresis in the absence of such changes in creatinine and PAHA clearance. A mild diuretic effect was noted in lower doses (12.5 to 25.0 mg/kg). Azacyclonol in doses from 12.5 to 62.5 mg/kg showed antagonism to the antidiuretic action of vasopressin. It is suggested that this antagonism may be the result of weak ADH-like properties of azacyclonol.

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## Enzymatic Digestion of Mucoproteins.* (28323)

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Mucoproteins are found in blood, in mucus, in tumors and about sites of injury. They are elevated in the blood after injury, in arthritis, tuberculosis, cancer and obstructive biliary disease(1,2,3). They are increased in amount about wounds and in malignant tumors. To determine what enzymes attack mucoproteins is the subject of this investigation. This information may help us to understand how mucoproteins are concentrated and whether enzymes are involved in the changes. There are no previous publications on the susceptibility of mucoproteins although trypsin and chymotrypsin have been used to degrade mucoproteins to study breakdown products(4). Recently Pigman *et al.* have stated that mucins are poorly digested by trypsin(5). The enzymes to be employed consist of some that should attack the carbohydrate moiety of the mucoprotein, some that should attack the protein fraction and some that should attack both.

*Methods.* The seromucoproteins employed were extracted from plasmas of rabbit and human blood by an adaptation of the quantitative method of Winzler, Devon, Mehl and Smyth(6). For this isolation a volume of 0.75 M perchloric acid double that of the plasma was added slowly with shaking. After standing for exactly 10 minutes the precipitate of other proteins obtained was filtered off. Longer contact destroys human mucoproteins (Table I). Then a volume of 5% phosphotungstic acid in 2N hydrochloric acid, equal to the original volume of plasma used was added to the filtrate and shaken to bring down a flocculant white precipitate. After being refrigerated overnight this second precipitate was filtered off. Then just enough 0.2 N sodium hydroxide was added to dissolve the precipitate off the filter paper, washing several times with distilled water to remove the last traces. This concentrated solution of mucoproteins, amino acids and

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TABLE I. Influence of Perchloric Acid on Isolated Mucoproteins.

| | | | |
|---|---|---|---|
| | | Human mucoproteins | |
| 12 ml water<br>3 ml mucoprotein<br>No dialysis<br>30 hr later<br>5 ml + 1 ml<br>Phosphotungstic acid<br>Ppt ml | 12 ml water<br>3 ml mucoprotein<br>Dialysis 24 hr<br>30 hr later<br>5 ml + 1 ml<br>Phosphotungstic acid<br>Ppt ml | 12 ml perchloric acid<br>3 ml mucoprotein<br>No dialysis<br>30 hr later<br>5 ml + 1 ml<br>Phosphotungstic acid<br>Ppt ml | 12 ml perchloric acid<br>3 ml mucoprotein<br>Dialysis 24 hr<br>30 hr later<br>5 ml + 1 ml<br>Phosphotungstic acid<br>Ppt ml |
| 1. .33<br>2. .25<br>3. .25<br>Avg .28 | 1. .27<br>2. .30<br>3. .25<br>.27 | 1. .40<br>2. .35<br>3. .38<br>.38 | 1. .20<br>2. .20<br>3. .17<br>.19 |
| | | Rabbit mucoproteins | |
| Avg .45 | .45 | .55 | .35 |

salts was now dialyzed against running water for 48 hours to leave the mucoproteins inside the dialyzing sack. They were then freeze-dried. The mucoproteins were obtained as a fluffy white powder with a faint greenish cast. Small amounts of substances other than mucoproteins were precipitated by these 2 acids and some undoubtedly remained after dialysis—the globulins, nucleoproteins and ceruloplasmin. Mannose, sialic acid and tyrosine determinations identified the powder as mucoproteins.

The following enzymes were employed: carbohydrate splitting a and b amylases and lysozyme; protein splitting-crystalline pepsin, trypsin, chymotrypsin b, ficin, papain, collagenase, plasmin, and Nagarse (*B. subtilis*) and the following enzymes with combined proteolytic and diastase activity—Rhozymes A-14 and P-11. All enzymes were made up in concentrations and solutions of correct pH and ionic concentrations to give them maximum activity against their respective substrates. Pepsin, for example, was acidified to pH 3.0 while trypsin was prepared in a phosphate buffer of pH 7.5.

For each test a modification of the method of Winzler, Devon, Mehl and Smyth(6) was again used. Three solutions of 3 ml each were prepared; #1 contained 6 mg of mucoprotein; #2, 3 mg of enzyme, and #3, 6 mg of mucoprotein plus 3 mg of the enzyme. These 3 solutions in stoppered tubes were incubated at 37°C for 24 hours. Then to each tube 12 ml of 0.75 M perchloric acid was added with shaking. After exactly 10 minutes the precipitate formed was filtered off. The filtrate was now neutralized with 0.2 N NaOH and dialyzed. Dialysis was done at this point to remove split products of digestion, namely the proteoses, peptones or amino acids. If any of these remained in solution they would be precipitated later by addition of phosphotungstic acid obscuring how much mucoprotein remained undigested (Table I). Without dialysis abnormally high values were obtained with the second precipitation (Table I).

After dialysis an aliquot equivalent to 1 ml of the original solution was transferred to a Constable protein tube, and 1 ml of 5% phosphotungstic acid in 2 N HCl was added. After 15 minutes the tubes were centrifuged at 2600 rpm for another 15 minutes. The amount of precipitate was read in millimeters.

The amount and percentage of mucoproteins digested were determined by the following calculation:

$$\frac{\left(\begin{matrix}\text{ml of ppt}\\ \text{Sol. \#1}\end{matrix}\right)+\left(\begin{matrix}\text{ml of ppt}\\ \text{Sol. \#2}\end{matrix}\right)-\left(\begin{matrix}\text{ml of ppt}\\ \text{Sol. \#3}\end{matrix}\right)}{\left(\begin{matrix}\text{ml of ppt}\\ \text{Sol. \#1}\S\end{matrix}\right)}\times 100$$

= % mucoprotein digested.

To test the accuracy of the method of measuring volume of precipitate recovered, increasing amounts of both rabbit and human seromucoproteins were put into solution and reprecipitated immediately with phosphotungstic acid and again dialyzed. The results obtained from the #1 solution indicated the in-

§ Theoretically solution #2 should be included here but the amounts of precipitates obtained from the enzymes were usually very small.

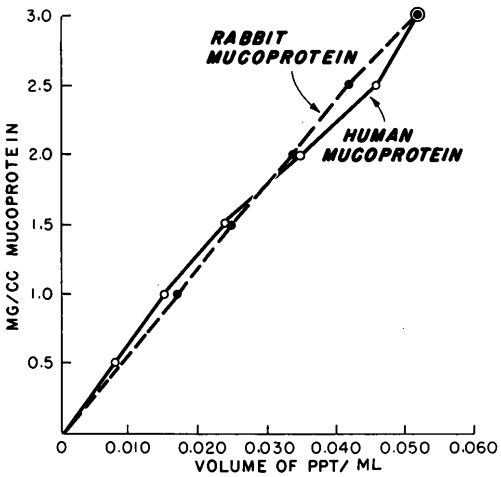


FIG. 1

fluence of incubation on the stability of both mucoproteins. However to determine stability on standing in water or in presence of buffers, solutions containing 1 mg/ml mucoprotein were made up in distilled water and in phosphate buffer pH 7.1, and the volume of precipitate obtained from each determined. Then one-half of each solution was stored at room temperature and the other half in the refrigerator. At intervals for a period of 2 weeks volume precipitates of each were again determined.

*Results.* The volume of both mucoproteins reprecipitated immediately by phosphotungstic acid increased as a straight line with the weighed amounts of each put into solution (Fig. 1). The graphs have identical slopes for both human and rabbit mucoproteins indicating under these conditions both were reprecipitated without loss. However prolonged contact with perchloric acid destroyed about 10% more human than rabbit mucoprotein (Table I). Also, on standing in phosphate buffer, human but not rabbit mucoproteins were affected. Indeed the volumes of precipitate obtained from human mucoproteins held at room temperature for 5 days diminished to one-third of the original amount but remained at this level for the next 9 days. Refrigeration eliminated this change. However, the amounts of precipitate obtained from rabbit mucoproteins remained constant after standing in phosphate buffer at room temperature for some length of time. Standing in distilled water for 14 days at room or refrigerator temperatures did not change the volumes of precipitates obtained from either mucoprotein (Table II).

The results of the digestion tests are shown in Tables III and IV. The volumes of mucoproteins reprecipitated after incubation in water for 24 hours are shown in solution #1, Column 1. The amounts obtained are equal to those without incubation (Fig. 1), indicating that rabbit mucoproteins were not affected by incubation in water. Individual variations in tests may be seen in this column. On the other hand, for human mucoprotein the average volume of precipitate recovered after incubation in water was only 0.0281 ml/m. These results are all lower than those without incubation (Fig. 1). That human mucoproteins showed greater sensitivity to phosphotungstic acid, to incubation at 37°C in water and to phosphate buffer at room temperature, suggests that some portion of human mucoprotein is less stable. Furthermore this fraction is not found in rabbit mucoprotein, or here it is more stable.

Some of the enzyme solutions yielded precipitates from a trace to .017 ml/ml (Column

TABLE II. Changes in Solutions of Mucoproteins on Standing.

| | Water solution of mucoproteins 1 mg/ml | | | | Phosphate buffer solution of mucoproteins 1 mg/ml pH 7.1 | | | |
|---|---|---|---|---|---|---|---|---|
| | Room temp | | Refrigerator | | Room temp | | Refrigerator | |
| | Rabbit | Human | Rabbit | Human | Rabbit | Human | Rabbit | Human |
| | (ml) | | (ml) | | (ml) | | (ml) | |
| Initial | .015 | .018 | .015 | .018 | .015 | .015 | .015 | .015 |
| 24 hr | .015 | .020 | .015 | .018 | .014 | .013 | .015 | .014 |
| 5 days | .016 | .020 | .017 | .020 | .017 | .005 | .015 | .012 |
| 7 " | .014 | .015 | .015 | .020 | .015 | .005 | .015 | .010 |
| 14 " | .014 | .015 | .014 | .020 | .014 | .004 | .014 | .013 |

TABLE III. Enzymatic Digestion at 37°C of Rabbit Mucoprotein (2 mg).

| Enzyme | mg of | ml of ppt mucoprotein (1) | ml of ppt enzyme (2) | ml of ppt mucoprotein & enzyme (3) | Amt digested | % digested avg 3 tests |
|---|---|---|---|---|---|---|
| a-Amylase | 1 | .036 | 0 | .021 | .015 | 41.0 |
| b-Amylase | 1 | .035 | Trace | .034 | .001 | 2.8 |
| Chymotrypsin b | 1 | .034 | " | .005 | .030 | 86.0 |
| Trypsin (cryst) | 1 | .034 | " | .003 | .031 | 91.0 |
| Pepsin (cryst) pH 3 | 1 | .033 | 0 | .002 | .031 | 94.0 |
| Ficin | 20 | .037 | .009 | .010 | .036 | 78.0 |
| Papain | 20 | .037 | .002 | .006 | .033 | 85.0 |
| Collagenase | 1 | .032 | .003 | .020 | .015 | 43.0 |
| Lysozyme | 1 | .032 | .002 | .033 | .001 | .3 |
| Roz $A_4$ | 1 | .035 | .002 | .007 | .030 | 81.0 |
| Roz $P_{11}$ | 1 | .032 | .002 | .005 | .029 | 85.0 |
| Nagarse | 1 | .033 | Trace | .001 | .032 | 97.0 |

TABLE IV. Enzymatic Digestion at 37°C of Human Mucoprotein (2 mg).

| Enzyme | mg of | ml of ppt mucoprotein (1) | ml of ppt enzyme (2) | ml of ppt mucoprotein & enzyme (3) | Amt digested | % digested avg 3 tests |
|---|---|---|---|---|---|---|
| a-Amylase | 1 | .026 | Trace | .010 | .016 | 61 |
| b-Amylase | 1 | .026 | " | .021 | .005 | 19 |
| Chymotrypsin b | 1 | .026 | .002 | .002 | .026 | 100 |
| Trypsin (cryst) | 1 | .026 | Trace | .005 | .021 | 81 |
| Pepsin (cryst) | 1 | .021 | " | .005 | .016 | 76 |
| Ficin | 20 | .021 | .019 | .026 | .014 | 67 |
| Papain | 20 | .021 | .006 | .006 | .021 | 100 |
| Collagenase | 1 | .031 | .003 | .010 | .024 | 77 |
| Plasmin (Act) | 10,000 U | .030 | .037 | .055 | .012 | 40 |
| Lysozyme | 1 | .024 | .004 | .025 | .003 | 13 |
| Roz $A_4$ | 1 | .026 | Trace | .006 | .020 | 77 |
| Roz $P_{11}$ | 1 | .026 | .003 | .003 | .026 | 100 |
| Nagarse | 1 | .027 | Trace | .001 | .026 | 98 |

Amylases, crystalline—Worthington. Beta Chymotrypsin—Armour. Trypsin, crystalline—Princeton, Dr. Herman Cohen. Pepsin, crystalline—Worthington. Ficin, purified—Merck. Papain, purified—Bidsawya. Collagenase, *Clostridium histolyticus*—NEB-Lab. Plasmin, human, 25,000 units—Merck Sharp & Dohme. Lysozyme, lysodeikticus—Worthington. Roz $A_4$, conc. *A. oryase*—Rohm & Haas. $P_{11}$ Protease Fungus—Rohm & Haas. Nagarse, *B. subtilis*, 180,000, also elastase activity—Bidsawya.

2). These precipitates were obtained more abundantly from the larger amounts of crude enzyme preparations, for example with ficin and papain, and the tests showed considerable variability. Crystalline enzymes yielded either a trace or very small amounts. Of special interest are those relatively pure enzymes that yielded precipitates suggesting that they contain mucoproteins. Among these are collagenase, lysozyme and chymotrypsin B. Human plasmin that gave the largest reading is obtained from the globulin fractions of the blood and may be a mucoprotein. Indeed some plasmin may have been precipitated when the mucoproteins were isolated from human plasma.

The digestion of the mucoproteins by the various enzymes is shown in Columns 3, 4, and 5. Rabbit mucoproteins were not attacked by b-amylase (2.8%) nor lysozyme (0.3%) and only partially by a-amylase (41%). Most of the combined enzymes attacked the rabbit mucoproteins. They were attacked by all proteolytic enzymes except collagenase (48%). Human mucoproteins were attacked slightly more by b-amylase (18%), lysozyme (12%), and partially also but somewhat more by a-amylase (64%). They were attacked completely by all other proteolytic and combined enzymes except collagenase (71%). On the other hand human mucoproteins were only partially digested (40%) by 10,000

units of human plasmin—the trypsin-like enzyme found in human blood.

In other words, both mucoproteins exhibited the same patterns of susceptibility to the enzymes but human mucoprotein showed less resistance to the 5 enzymes that did not, or only partially, attacked rabbit mucoproteins. In general the mucoproteins were attacked more readily through their protein than through the carbohydrate moiety.

*Discussion.* The instability of human mucoproteins in solutions explains why they appear to be more susceptible to the action of enzymes.

Most of the human bloods were collected in a different manner than those from rabbits before the mucoproteins were separated. Except for the batch used to make Fig. 1, human mucoproteins were obtained from citrated blood that had been discarded after storing, while the rabbit mucoproteins were obtained from freshly drawn heparinized blood and the cells were separated immediately. This standing of human blood with white cells in citrate solution could cause the destruction of some of the more unstable portions of the human mucoproteins.

In the references cited(1,2,3) the mucoproteins of the serum increased quantitatively with diseases and injuries. Markham has suggested that they change qualitatively as well (7). He showed that there are at least 2 mucoproteins and that the $M_1$ fraction is the one that increases in response to a disease. The susceptibility of the types of mucoproteins to enzymes has not been settled by this investigation although when seromucoproteins are increased in amounts, the pattern of susceptibility to enzymes remains unchanged.

Considering that proteolytic enzymes attack mucoproteins, the amounts of mucoproteins present in the plasma should decrease after injury because the amounts of enzymes increase with exudation. Rabbit leukocytes that increase with inflammation contain a potent trypsin-like enzyme(8), but these enzymes are released only when the cell is destroyed, usually locally. The human leukocytes contain only a negligible amount of proteolytic enzymes. Plasmin, the proteolytic enzyme found in human plasma, is present in the globulin fraction, but only partially attacks human mucoproteins. Thus despite the increase in proteolytic enzymes with disease or injury there seems to be no reason why the seromucoproteins should be decreased.

During the generative phase of healing proteins are synthesized by cells that must be protected against the proteolytic enzymes. Cell membranes furnish this protection, of course, but the build up of mucoproteins locally in the last 2 days of the exudative phase of healing and in the first few days of the generative phase suggests that this increase is in some way associated with the anabolism of proteins.

*Summary.* 1. Both rabbit and human seromucoproteins isolated by Winzler's method exhibited the same patterns of digestibility to enzymes. 2. They were digested completely by the common proteolytic enzymes, but only partially by collagenase, and human mucoproteins were only partially digested by activated human plasmin. 3. They were not digested by the amylases nor lysozyme. 4. However, mucoproteins were digested by enzymes combining proteolytic and diastase splitting activities. 5. The mucoproteins as conjugated proteins seem to be less susceptible to attack through the carbohydrate than through the protein moiety. 6. Some portions of human mucoproteins, unlike the mucoproteins from the rabbits, were found to be less stable on standing in phosphate buffer pH 7.1 at room temperature, to incubation, and to prolonged contact with 0.75M perchloric acid. 7. Quantitatively the mucoproteins that increase in serum after injury should be reduced in amount by the proteolytic enzymes that increase also during exudation. However human seromucoproteins are not too susceptible to the action of plasmin present in serum and the trypsin-like enzymes found abundantly in leukocytes are not found in the human and in the rabbit are only discharged locally.

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## Cancer Induction in Hamsters by Human Type 12 Adenovirus. Effect of Route of Injection.* (28324)

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We have recently reported induction of a high incidence of undifferentiated sarcomas, in short latent period, at the site of intrapulmonary injection of human adenovirus, type 12, into newborn hamsters(1,2). In our original systematic testing of the human adenoviruses for oncogenic effect in hamsters, the intrapulmonary (transthoracic) route of injection was selected as probably the most suitable for this group of predominantly respiratory viruses. Having obtained the initial positive results by this route of injection of adenovirus, type 12, the following comparison was then made of several routes of injection of this virus.

*Methods.* Human adenovirus, type 12, prototype strain Huie, was obtained originally from the American Type Culture Collection and propagated in our laboratory in HeLa cells as previously described(1). The virus was injected into newborn hamsters within 24 hours of birth by the intrapulmonary (through the chest wall), intrapleural, intraperitoneal, intracranial, intravenous, or subcutaneous routes, or was instilled intranasally. For intrapulmonary injection, the needle was inserted through the chest wall into the peripheral part of the lung. In most cases a small amount of the inoculum leaked from the nose or mouth, via the bronchi and trachea. For intrapleural injection the needle was inserted into the pleural cavity only, and not into the lung. Subcutaneous injection was made on the dorsum. Intravenous injection was by way of facial veins. All injections were made with a 30 gauge needle. All other materials, methods and precautions were as previously described(1).

*Results and Discussion.* Those hamsters injected with 50 times the minimum 100% tissue culture infectious dose ($MTCID_{100}$) of virus by the intrapulmonary, intrapleural, or intraperitoneal route, developed a high incidence of tumors at the site of injection in from 33 to 77 days (Table I). Three hamsters injected subcutaneously with the same virus fluid have not developed tumors in from 508 to 636 days to date. However, at the 500 $MTCID_{100}$ dose of virus, the subcutaneous route appeared as effective as any route tested. Of 17 hamsters injected with 500 $MTCID_{100}$ of virus by either the intrapulmonary, intrapleural, intravenous or subcutaneous route, all but 2 developed tumors within 98 days. By the intrapulmonary route, as little as 5 $MTCID_{100}$ produced tumors in 5 of 14 hamsters by 98 days. Of 25 hamsters injected intracranially with doses of from 2 to 200 $MTCID_{100}$ of virus, tumors arose in only one animal, of the 7 at the highest dose. This animal had no tumor at the site of intracranial injection, but had multiple abdominal tumor masses leading to death by 93 days. Hydrocephalus developed in 3 of the intracranially injected hamsters only, in from 36 to 58 days. Huebner *et al.*(3) have observed

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