We now inquire whether calomel half cells with salt bridges can measure an interface potential which may exist across the membrane (Donnan potential).

Let us insert calomel half cells with saturated KCl bridges. The cell is

			A		M	B			
Hg	Hg_2Cl_2	KCl (sat)	I KCl + KR		II KCl	KCl (sat)	Hg_2Cl_2	Hg

We shall assume that the transference numbers of K^+ and Cl^- are equal across boundaries A, B, and M.

In order to obtain the net cell process, we must write the electrode reactions and the transference processes at the interfaces *A, M,* and *B* and obtain their sum. When 2 faradays pass, the following processes occur:

(*a*)	$Hg_2Cl_2 + 2e^-$	$\rightarrow 2Cl^-(I_s) + 2Hg$
(*b*)	$K^+(I) + Cl^-(I_s)$	$\rightarrow Cl^-(I) + K^+(I_s)$
(*c*)	$K^+(II) + Cl^-(I)$	$\rightarrow Cl^-(II) + K^+(I)$
(*d*)	$K^+(II_s) + Cl^-(II)$	$\rightarrow K^+(II) + Cl^-(II_s)$
(*e*)	$2Hg + 2Cl^-(II_s)$	$\rightarrow 2e^- + Hg_2Cl_2$

The subscript *s* denotes that the ion in question is in the salt bridge so that (*b*) corresponds to the transfer of 2 faradays from bridge I to solution I, (*c*) corresponds to the transfer of 2 faradays across the membrane, and (*d*) corresponds to the transfer of 2 faradays from solution II to bridge II. Adding the processes we obtain the net cell reaction,

$$KCl(II_s) \rightarrow KCl(I_s)$$

That is, the net process is simply the transfer of KCl between the two bridges. The ΔF for this process must be zero since KCl is transferred between solutions of the same composition, and hence $E_{cell} = 0$. This same result is obtained even when the transference numbers across the membrane are not the same. If the transference numbers of K^+ and Cl^- across the membrane are t_+ and t_-, then the net cell process becomes,

$$(2t_+ - 1)\,KCl(II) + KCl(II_s) \rightarrow (2t_+ - 1)\,KCl(I) + KCl(I_s)$$

Since the activity of KCl is the same in I as in II at equilibrium, we again find $\Delta F = 0$ and $E_{cell} = 0$.

We are led to the conclusion that if an emf is measured for the cell in the laboratory, the assumption of equal transference numbers at either *A* or *B* or both must be in error. When the transference numbers are not equal at the bridge junction, this means that there is a net transfer of salt from the bridge to the suspension. This transfer is the driving force of the cell, and it cannot have a ΔF which is the measure of the membrane potential, since, as may be demonstrated experimentally, its magnitude depends upon the salt concentration in the bridge.

The arguments above show that even where transference numbers at the KCl bridges are assumed to be equal, the calomel half cell assembly does not measure the membrane potential in a Donnan system. Therefore, if an emf is obtained experimentally for such a cell, it must mean that the transference numbers are not equal at at least one of the bridge junctions. In other words, the nonequilibrium bridge junctions are the only possible seat of an emf.

This finding is in accord with the statements of Gibbs and later of Guggenheim who emphasized that it is impossible to measure interface potentials. However, this is not to deny the probability that such interface potentials may exist. In certain kinds of membrane systems interface potentials can be estimated from theory, provided that the solutions are sufficiently dilute.

The foregoing approach has been extended and applied to membrane systems of other types (permselective membranes). A more complete discussion will be published soon.

References

1. LOEB, J. *Proteins and the Theory of Colloidal Behavior.* New York: McGraw-Hill (1922).
2. JENNY, H., *et al. Science,* **112**, 164 (1950).

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The Mucolytic and Digestive Action of Trypsin in the Preparation of Sputum for Cytologic Study[1]

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Cytology is, at the present time, the most promising approach to the problem of early morphologic diagnosis of lung tumors. The most serious limitation to its widespread application has been the lack, heretofore, of a satisfactory means of concentrating the cellular material found in sputum. As a result, it has been necessary for the pathologist or laboratory technician to select, by macroscopic inspection, a very small proportion of the total specimen for microscopic examination.

This method of selection is unavoidably influenced by chance. Nasal and oral materials are always present in sputum; if the amount of true bronchial sputum is minimal, it may be entirely overlooked in the selection of material for screening.

It is now believed that a negative cytological report is not justified unless at least 15 slides have been studied. When 15 slides, from 5 different sputum samples are examined, an accuracy of approximately 92% can be achieved (*1, 2*). If for any reason this series is not complete, accuracy falls off sharply. A satisfactory method of concentration, it is hoped, would not only increase over-all accuracy, but reduce the number of sputum samples required as well, and thus considerably expedite diagnosis.

[1] The trypsin used in this study was Tryptar, kindly furnished by the Armour Laboratories, Chicago, Illinois.

Previous approaches to the problem. It is impossible to centrifuge cellular elements out of sputum because of the viscosity of the medium. Hyaluronidase liquefies the mucus in the sputum, but the process is too slow to be feasible (*3*). The proteolytic enzyme, papain, activated by cysteine, is rapid enough in its action, but the residual precipitate markedly obscures cellular detail (*4*). Another enzyme, the "receptor-destroying" enzyme recovered from filtrates of *vibrio cholera,* as suggested by the work of Burnet and Stone (*5, 6*), and a mucolytic agent, lysozyme (*3*), have also been found unsatisfactory.

Agents other than enzymes have been investigated. Urea and sodium bicarbonate were shown to reduce the viscosity of mucus, but they also cause severe distortion of cell structure (*3*). An ingenious attempt to precipitate the mucus by lead acetate, in conjunction with differential centrifugation, likewise failed to give reasonably dense concentration of cellular material (*3*).

Trypsin in the treatment of sputum. Trypsin is chiefly known for its selective enzymatic digestion of nonviable organic material (*7, 8*). Since the bulk of cellular material in sputum is mature, nonviable squamous cells, normally exfoliated in great numbers from oral mucosa, it was thought that this action of trypsin might be useful in the preparation of specimens of such secretions. By reducing the number of such cells, it might speed the screening process, and increase the number of specimens that could be evaluated daily by the limited number of trained personnel available. Since trypsin, moreover, is known to have substantial mucolytic action, it was decided to aim at a method by which both the digestive and mucolytic actions could be utilized. We believe that such a technique has been achieved.

Technique for the use of trypsin. Early in this work it became apparent that the mucolytic action of trypsin affected sputum only when the mixture was simultaneously heated and vigorously agitated. A Kahn shaker provides suitable agitation, but we have been unable to devise a method of supplying controlled heat with this method. At present, we are using manual agitation with frequent heating under a tap of running warm water.

Another method, using commonly available equipment, has been found satisfactory. Heat is supplied by a serological water bath regulated to 57° C, and the agitation force is supplied by air pressure from a portable compression tank. The tank attachment has multiple faucets which are connected by rubber tubing to glass droppers inserted into centrifuge tubes containing the sputum and trypsin mixture. Pressure is regulated to a forceful stream. Results with this method have been satisfactory, although further experience may indicate a more simple approach.

We have found that 125 mg (125,000 Armour units) of trypsin, dissolved in 12.5 ml of Sörensen's buffer (pH 7.1), is sufficient to liquefy from 8–10 ml of sputum. The sputum and trypsin, in this proportion

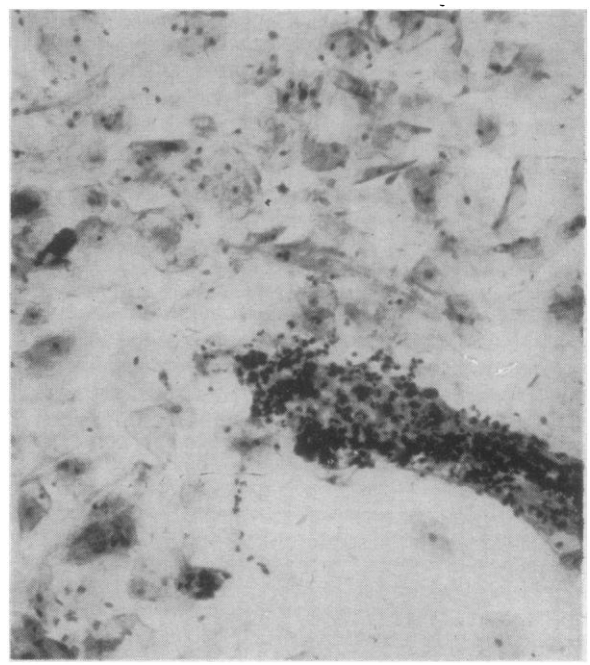

FIG. 1. Before tryptic digestion. Numerous mature squamous epithelial cells and fibrinous strands. × 100.

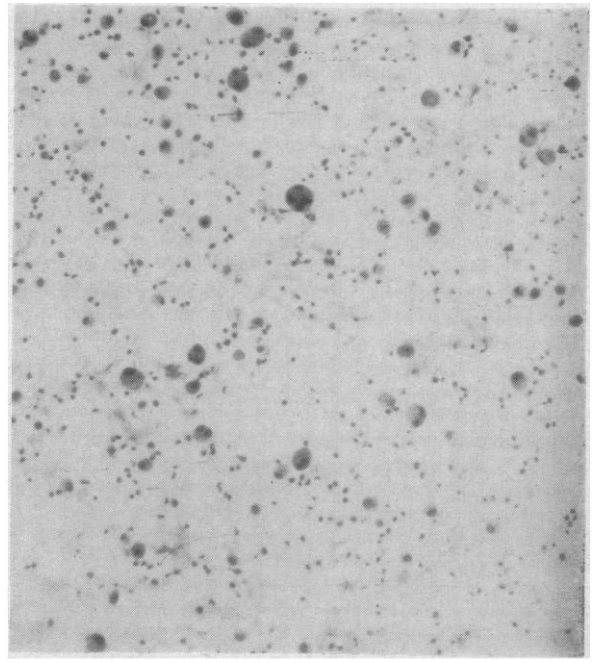

FIG. 2. Same specimen following tryptic digestion. Marked decrease in both squamous cells and fibrin resulting in a thin, homogeneous film of discrete cells. × 100.

are put in a centrifuge tube with a capacity of 2–3 times the total amount of the mixture, so that subsequent agitation will generate sufficient force to break up and dissolve the tenacious strands of mucus and fibrin. One or two drops of caprylic alcohol, depending upon the agitation time, reduces the surface tension,

and thus prevents foaming which commonly occurs when protein solutions are agitated.

The time required to liquefy a specimen thoroughly depends upon its initial viscosity, but the most tenacious specimen usually responds within 15 min. After the specimen has been agitated, a small amount—0.5–1 ml—of human plasma is layered to the bottom of the centrifuge tube with a pipet or capillary dropper. This plasma accomplishes two ends. First, plasma acts, to some extent, as an inhibitor of trypsin activity (*9*) and is a convenient substance to use for arresting the digestive process. Secondly, it acts as an adhesive agent in the subsequent procedures, replacing the liquefied mucus. Unless some such substance is added, cells are readily lost from the slide during fixing and staining.

Results. The evaluation of the mucolytic effect of trypsin was made upon the basis of stained smears made of the sediment recovered after centrifuging. The best criteria of such an effect, we believe, are the homogeneity of the smear, the appearance of the stained cellular elements, and the absence of cell clumps and fibrinous strands of cells. In terms of such criteria, the mucolytic action of trypsin was entirely satisfactory and distinctly superior to the action of any other mucolytic agent we have investigated. In a series of smears made at 5-min intervals (in terms of digestion time), we observed complete disappearance of fibrinous strands and cell clumps, with markedly homogeneous smears at the end of 15 min. There was no further change in the appearance of smears until after 50 min of agitation and digestion when a suggestion of overdigestion, evidenced by a reduction in sharpness of cytoplasmic borders and the appearance of numerous ghostlike fragmentary structures, was noted. These changes were progressively heightened as the agitation and digestion time was increased.

The effectiveness of trypsin in reducing the number of mature, nonviable squamous cells was marked. This was determined by comparing slides prepared with trypsin-treated material with slides from the same sputum specimens which were processed in the usual manner. The apparent effect is a substantial increase in the number of macrophages on the slide made from trypsin-treated material (Figs. 1 and 2), but there has, in fact, been a substantial reduction in the number of mature squamous cells. Trypsin does not alter the standing characteristics of cellular material; all cells maintained their usual reaction to Papanicolaou staining.

Trypsin had no noticeable effect upon the appearance of malignant cells. In comparison with routinely processed material, however, the concentration of malignant cells in the trypsin series was notable. It was estimated that smears prepared by this method contained from 4–6 times as many malignant cells as slides made in the usual way from the same sample of sputum. It is also important to note that malignant cells from an adenocarcinoma do not lose, in the process of being concentrated, their distinctive clumping—an important criterion in evaluating this type of malignancy.

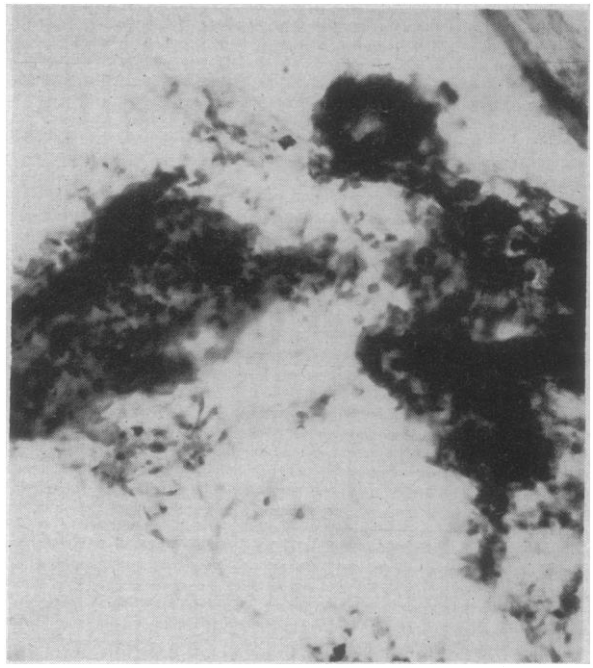

FIG. 3. Overdigestion with trypsin. Large clusters of malignant squamous cells. Other cellular elements appear as an amorphous debris. × 100.

In short, the use of trypsin, as described above, appears to be a practical and feasible answer to the problem of concentrating the cellular elements of sputum,

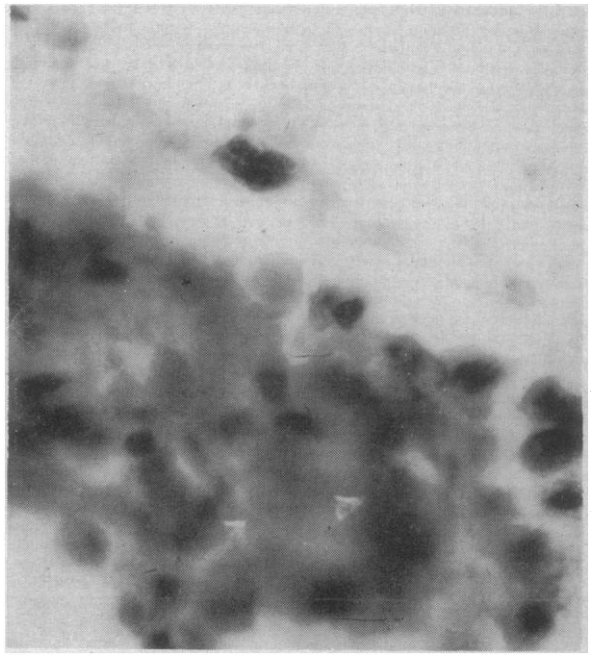

FIG. 4. Higher magnification of Fig. 3, revealing preservation of malignant cellular detail. × 430.

and in addition, it eliminates approximately 60% of the material which is not significant to diagnosis. In almost every case, all cellular material available from a specimen of sputum could be smeared on one or two slides. This technique has not been in use long enough for accurate evaluation, but there is every reason to hope that a substantial increase in efficiency will be achieved, as well as an increase in accuracy of diagnosis.

Overdigestion phenomenon. A specimen collected from a patient with a known epidermoid carcinoma of the lung and coexisting tuberculosis was overdigested by 1½ hr exposure to trypsin in a running tap-water bath of aproximately 60° C. Stained slides made from the sediment, as was expected, revealed a background of hazy, nondescript cell fragments. Quite unexpectedly, however, numerous malignant cells were seen in an excellent state of preservation and appeared in large clumps. The nuclei of these malignant cells retained their typical dense hyperchromicity and the cytoplasm remained brilliantly orange, as is usual for the highly cornified malignant squamous cells (Figs. 3 and 4).

This phenomenon needs careful evaluation, but it suggests the possibility of a radically new approach to cytology in all its phases. If this observation is confirmed, trypsin could be of great service in the cytologic diagnosis of gastric cancer, where the number of oral squamous and other nonsignificant cells are a great problem. It might be useful as a vaginal douche in cases where it is impossible to obtain satisfactory smears by the usual technique because of postradiation fibrinous and nonviable debris. Trypsin might also be used to lyse clotted pleural and ascitic fluids for cytologic study and, in sufficient concentration, to digest the abundant fecal debris from lower bowel and rectal washings, and thus permit cytologic study of such material.

Summary. By the addition of a solution of trypsin, thick, tenacious sputum can be liquefied and concentrated without loss of significant cellular detail. This compound also digests most of the mature squamous cells which normally make up a large proportion of the cellular elements in sputum, as well as other nonviable materials. As a consequence, it is generally possible to smear all significant cellular material upon only one or two slides. Cell structure is at least as clearly visualized as by the older method, and in the case of pulmonary malignancy the number of malignant cells which can be seen on the slide is enormously increased.

Overdigestion with trypsin was employed on a limited number of specimens. It was found that malignant cells remained intact and easily identifiable after all other cells in the specimen were obliterated by enzymatic digestion.

The utilization of the digestive action of trypsin in the preparation of materials other than sputum for cytologic study has many possibilities, although study of a larger number and variety of cases is necessary before a definite conclusion is warranted as to the range of applicability of this method.

References

1. FARBER, S. M., *et al. Diseases of the Chest,* **14**, 633 (1948).
2. FARBER, S. M., *et al. Radiology,* **52**, 511 (1949).
3. TRAUT, H. F., FARBER, S. M., and ROSENTHAL, M. *Six Months Report of the University of California Cytology Laboratory,* July (1948).
4. ROSENTHAL, M., and TRAUT, H. F. *Cancer,* **4**, 148 (1951).
5. BURNET, F. M. *Australian J. Exptl. Biol. Med. Sci.,* **26**, 71 (1948).
6. BURNET, F. M., and STONE, J. D. *Ibid.,* 218.
7. MADDEN, J. F., and RAVETS, H. G. *J. Am. Med. Assoc.,* **149**, 1616 (1952).
8. RESIER, H. C., PATTON, R., and ROETTIG, L. C. *Arch. Surg.,* **63**, 568 (1951).
9. NORTHRUP, J. H. *Harvey Lectures Ser.,* **21**, 42–49 (1925–26).
10. PAPANICOLAOU, G. N., and TRAUT, H. F. *Diagnosis of Uterine Cancer by the Vaginal Smear.* New York: Commonwealth Fund (1943).

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2,3,5-Triphenyltetrazolium Chloride as a Rapid Indicator of Viability in Cottonseed

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Use of 2,3,5-triphenyltetrazolium chloride for the rapid detection of viability in seeds, first proposed by Lakon (*1*), is based on the capacity of the stain to dye only those parts of the seed embryo that are alive and will grow (*2–5*). The stain, colorless in aqueous solution, is reduced to a red insoluble formazan in the presence of living tissue upon which it is deposited (*4*). A number of investigators have applied this staining technique to cottonseed for the detection of viability, with limited success. Flemion and Poole (*6*) used whole excised embryos and observed a light staining of the entire embryo or most of it. Their results seemed to agree substantially with Flemion's rapid viability test (*7*) in which excised embryos are germinated on moist filter paper in Petri dishes at room temperature. Raggio and de Raggio (*8*) reported results with tetrazolium chloride in good agreement with germination tests. Poe *et al.* (*9*), using whole kernels and an infrared lamp to apply heat and accelerate the reaction, reported that the tetrazolium chloride test agreed with the standard germination tests in only 57% of the individual samples examined. They concluded that the stain could not be relied on to give accurate results for routine laboratory experi-

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[2] One of the laboratories of the Bureau of Agricultural and Industrial Chemistry Agricultural Research Administration, U. S. Department of Agriculture.