TABLE I. Temperatures of Saline Solutions with Different Proportions of Freon-11 and Freon-113.

Wt % Freon-11	Wt % Freon-113	B.p. °C
90	10	25.0
80	20	26.4
70	30	28.0
60	40	29.7
50	50	31.7
40	60	34.2
30	70	36.7

Summary. A compact and inexpensive tissue bath enabling the study of isolated biological materials has been developed. Temperature control of the saline solution is achieved by means of a refluxing solvent. When employed at a temperature of 38°C accurate control to ± .03°C is possible. By appropriate choice of solvents it is possible to study isolated tissue preparations over a wide range of temperatures.

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Cytochemical Demonstration of Diphosphopyridine Nucleotide-Diaphorase Activity in Cells of Vaginal Secretions of Mice.* (25190)

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Our previous studies have indicated the presence of at least 2 oxidative enzyme systems in the vaginal epithelium of rodents both of which have been shown to be under the influence of circulating titres of ovarian hormones during normal cyclical events(1,2). The enzyme systems described were the succinic dehydrogenase (SDH) and the diphosphopyridine nucleotide diaphorase (DPN) systems, histochemically demonstrable by using neotetrazolium as an indicator dye. The use of such methods has made localization of oxidative enzyme systems such as these possible at the tissue or histologic level of study. Recently, however, newer more sensitive and suitable tetrazolium salts have been advanced, especially nitro-blue tetrazolium (NBT), which now make possible enzyme localizations at the cellular or cytochemical level of study both with the light microscope(3) and the electron microscope(4). The purpose of this report is to show the cytochemical distribution of one such oxidative enzyme system (DPN) with use of NBT in the cellular population of vaginal secretion during the estrous cycle. It is our intent to show that: (1) cytochemical localization of such oxidative enzymes is possible through use of smear preparations, and (2) that the various cell types resident in vaginal secretions show distinctive enzyme localization patterns each related to degree of cytomorphological change (cornification) in the cell.

Methods and material. Adult, female albino mice (Swiss strain) were used in these studies. The animals were studied through at least 3 consecutive estrous cycles during which time the vaginal lumen was smeared by the use of a cotton-tipped applicator previously moistened in cold Ringer's solution. Smears were prepared immediately on glass slides which were air dried and quickly immersed into the following preheated incubating medium:

.1 M Na phosphate buffer, pH 7.6	14 ml
1.0 M sodium lactate	4 "
Coenzyme I (1 mg/ml aq.)	2 "
Nitro-blue tetrazolium (DAJAC)	20 mg

Following incubation at 38°C for 30 minutes and fixation in 10% neutral formalin, slides were rinsed in water and counterstained in eosin with subsequent mounting in synthetic resin. The insoluble dye, dinitroformazan, precipitated as a result of enzymatic activity has been shown to resist dissolution in the higher alcohols and xylene(3). Under the conditions of these experiments smear preparations incubated in the absence of lactate contained no detectable amounts of dinitroformazan. Parallel studies of each smear were also

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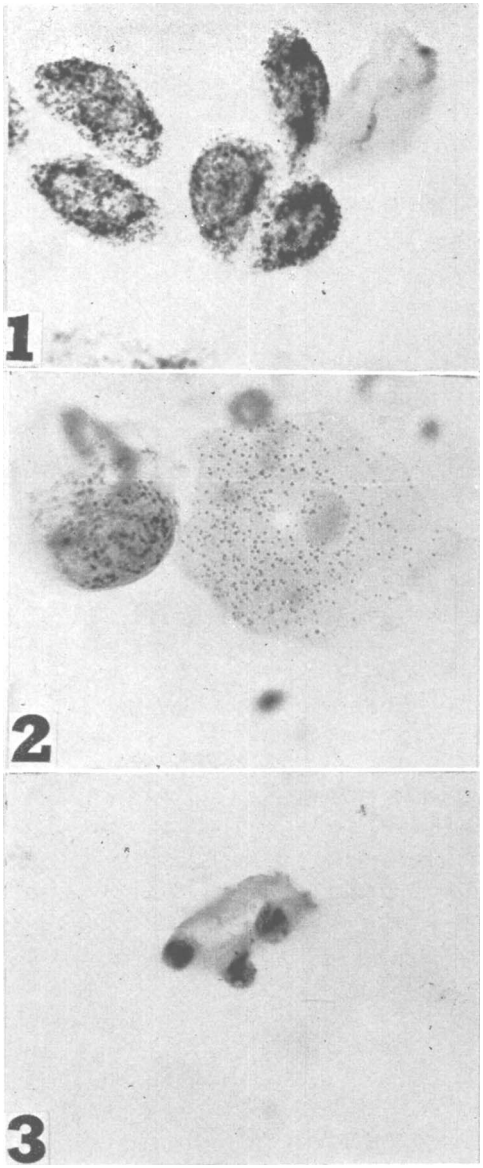
performed using the conventional hematoxylin and eosin method.

Results. Various cells are found in the vaginal secretion and the amount and types of these are dependent upon the stage of the estrous cycle during which the secretion is studied. Briefly, during *estrus* high numbers of well-cornified cells either with degenerate pyknotic nuclei or no nucleus are present. These gradually disappear at *metestrus* when leukocytes invade the vaginal epithelium and reside in the luminal contents. Leucocytes are found in highest numbers in the vaginal lumen during *diestrus* when the titre of circulating estrogen is lowest. Cornified cells are absent and the only other cellular constituent is represented by a nucleated epithelial cell derived from the lining of the vaginal canal at this time. In *proestrus* the leucocytes gradually disappear and the nucleated cells are found in rather high numbers. As the vagina comes under the influence of estrogen and estrus approaches, the nucleated epithelial cells show increasing degrees of cornification. As cornification is completed, nuclear degeneration occurs with subsequent pyknosis and final disappearance of this structure.

Thus, in studying the cellular population of vaginal smears during the different stages of the estrous cycle one can discern 4 main categories of cell types: 1. Cornified, nonnucleated epithelial cells; 2. Nucleated, precornified cells; 3. Nucleated, noncornified cells; 4. Leucocytes, mainly neutrophils. We studied these cells for enzymatic activity; a description follows.

1. *The cornified cell.* The completely cornified cells were devoid of any enzymatic activity (Fig. 1). As cornification progressed the DPN activity which was entirely cytoplasmic continued to decline until no reaction was evident.

2. *Precornified cells.* As indicated above there was no nuclear staining while the cytoplasm of these cells varied in intensity of reaction. Cells which showed the least amount of cornification tended to stain rather uniformly throughout, with granules of dinitroformazan showing a relatively even distribution in the cytoplasm (Fig. 2). As cornification proceeded, lack of staining in the peripheral cytoplasm was evident. Fewer granules were



All figures × 720.

FIG. 1. Noncornified epithelial cells (upper center and left) from an animal in proestrus showing high DPN activity in cytoplasm. Cytoplasmic granulation is so abundant as to obscure the nucleus which normally displays no enzymatic activity. Inactive cornified cell, (upper right).

FIG. 2. Cell at left is in an intermediate stage of cytomorphic change between those found in Fig. 1 and the squamous element next to it. Note dispersion of cytoplasmic granules as cell becomes more cornified.

FIG. 3. Leucocytes containing high DPN activity in association with a portion of a degenerating cornified element.

present, usually of smaller size than those found in earlier stages. In many of these cells DPN distribution was limited entirely to a perinuclear zone which was the last region to show a positive reaction prior to complete cornification and destruction of the enzyme system.

3. *Nucleated, noncornified cells.* These were the most active cellular constituents found during the entire estrous cycle. Rather heavy staining was found throughout the cytoplasm which contained dense accumulations of dinitroformazan (Fig. 1). The granulations in these rounded cells were the largest seen in any of the epithelial cells studied. It should be noted, however, that occasionally some of these cells displayed little or no DPN activity.

4. *Leucocytes.* Most of these cells were of the polymorphonuclear neutrophil type with lymphoctyes and possibly monocytes occasionally present. The majority of neutrophils found in the smear contained little or no enzyme, although in certain preparations, areas of active as well as inactive cells were present. Neutrophils giving a positive reaction were generally associated with degenerating cells and were apparently in the process of active phagocytosis. Here, an intense DPN activity was observed (Fig. 3). Some mononuclear leucocytes also demonstrated heavy cytoplasmic staining.

Discussion. The presence of oxidative enzymes in smear preparations of cells was demonstrated by Hirono who used ascites cells and localized SDH using neotetrazolium as an indicator dye(5). More recently, Marcuse(6,7) studied SDH in endometrial and cervical smears employing the same tetrazole. The use of nitro-blue tetrazolium in similar studies should prove more profitable since the formazan of this dye is resistant to the reagents which allow for permanent mounting of the cells. There is possible, therefore, discrete cytochemical localization of the enzyme and a more sensitive indication of oxidative enzymatic activity. The presence of other enzymes in the cells of the vaginal lavage has also been indicated(8,9). These enzymes, such as the phosphatases and esterases, are not as labile as the oxidative enzymes. The retention of DPN activity in cells divorced anatomically and physiologically from the vaginal epithelium is, therefore, an interesting phenomenon. It is not surprising that cells undergoing an increasing degree of cornification should possess less enzymatic activity inasmuch as this morphologic process is in the nature of a degenerative one. Previous studies employing tissue sections have shown that with increasing cornification DPN is progressively lost in the vaginal epithelium(1,2). In addition, during early proestrus, superficial epithelial cells which contain much mucin are strongly positive for DPN. The present study has shown that the activity is still retained even after exfoliation. The occasional absence of DPN in these cells is probably the result of early death after exfoliation.

Contrary to observations on the basal cells of intact vaginal epithelium, these results indicate that the presence of DPN in the vaginal secretion is lowest during the period of the cycle when estrogen is secreted maximally by the ovary (estrus). This finding has been reported earlier for phosphatases by Ayre in a study of human vaginal smears(6). This worker suggests that degree of enzymatic activity in the cells of the vaginal secretion does not directly depend on estrogen levels in the body but rather on the cytomorphological state of the cells induced by ovarian steroids. The findings in these studies for DPN activity can be interpreted similarly.

The intense activity occasionally seen in certain leucocytes is probably an indication of their increased oxidative metabolism during various phases of activity. Apparently, energy yielding oxidations are required for phagocytic ingestion. We also observed such DPN activity in neutrophils and lymphocytes in smears of human peripheral blood.

Studies similar to those described above have been applied to vaginal smears of humans in normal and pathologic states(10,11). Detailed reports will be published.

Summary. Mouse vaginal smears were studied for diphosphopyridine nucleotide-diaphorase (DPN) activity. Cytochemical findings indicate the absence of enzyme in com-

pletely cornified cells while DPN is increased in cells with less cytodifferentiation.

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Fibrinolysis III. Nutritional and Environmental Requirements for Optimum Production of Fibrinolysin from Aspergillus (Aspergillin O). (25191)

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Many fungi are known to produce proteolytic substances as part of their metabolic activity and some of these substances are capable of fibrinolysis. Previously reported studies(1) on fibrinolytic activity of extracts from cultures of 260 fungi have singled out a strain of *Aspergillus oryzae* (B-1273) as the most promising producer of material with strong and relatively selective fibrinolytic activity. In continuing these studies, it soon became apparent that further progress would be greatly handicapped by the inability to obtain high yields of material. This paper describes observations on nutritional and environmental requirements of *Aspergillus oryzae* (B-1273) for increased production of fibrinolytic substance. For convenience, this material has been tentatively designated as "Aspergillin O" to distinguish it from the spore pigment, Aspergillin, previously isolated from *Aspergillus niger* (2). Properties and physiochemical characteristics of Aspergillin O are to be described.

Materials and methods. 1. *Culture media.* Czapek's solution agar slants and malt agar slants were prepared according to standard technics from commercial reagents (Difco). The liquid media consisted of following reagents: Sucrose 7.2 g, Dextrose 3.6 g, KH_2PO_4 13.69 g, KNO_3 2.0 g, Mg SO_4 1.23 g, in double distilled water to 1 liter. Two hundred ml of the media was transferred to 1 liter Roux culture flasks, then autoclaved 15 minutes at 15 lbs steam pressure (121°C). When needed, salts and vitamins were added to the liquid medium. Stock solutions of trace elements were prepared by dissolving 723 mg $FeSO_4$, 440 mg Zn SO_4, and 200 mg Mn SO_4 in 500 ml of double distilled H_2O. Sufficient volume of concentrated sulfuric acid was added to render solutions colorless and the solutions were then brought to 1 liter with double distilled H_2O. Two ml of each stock solution was added to the medium. Stock solutions of vitamins contained 10 mg of pyridoxine hydrochloride in 100 ml of 20% ethanol solution or 10 mg of thiamine hydrochloride in 100 ml of 20% ethanol solution in water. Other media were prepared in which the source of nitrogen was represented by NH_4NO_3, $NH_4H_2PO_4$, (NH_4) 2 HPO_4 and KNO_2 rather than KNO_3. These compounds were used in concentrations yielding a nitrogen equivalent to 2 g KNO_3/L. 2. *Fibrin plates.* Solutions of 350 mg % of bovine fibrinogen (Armour) and of bovine thrombin (Parke Davis) containing 30 NIH units/

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