unit to 0.25 μg of free diethylstilbestrol, the same assay value acquired from a sample of crystalline diethylstilbestrol which was simultaneously submitted to the same hydrolysis procedure.

Forty mg of this sample were dissolved in 200 cc of postmenopausal urine and extracted with butyl alcohol for the recovery of NaPG by the Venning method. No precipitate whatsoever settled out of the aqueous acetone solution. Estrogenic assay of the various fractions revealed that half of the stilbestrol glucuronide had been left in the tenth normal NaOH solution after being re-extracted with butyl alcohol. The other half was present in the residue of the final butyl alcohol extract but, unlike pregnanediol glucuronide, was not precipitated out of 5 cc of water by the addition of 95 cc of acetone. It is quite possible that when considerable amounts of NaPG are present, traces of stilbestrol glucuronide may be carried down by adsorption and even persist through the second precipitation out of water with acetone. That no more than traces are present, however, is apparent from the very slight estrogenic potency of NaPG recovered from pregnant women taking large doses of stilbestrol (v.s). In this connection it may be mentioned that estrogenic activity has also been found by us in hydrolyzed NaPG recovered from the urine of pregnant women who were not taking diethylstilbestrol. The probable explanation is that traces of estriol glucuronide, large amounts of which are present in late pregnancy urine, may also be carried down by adsorption upon the precipitated NaPG. Here again the amount present, although readily demonstrable by estrogenic assay, is too little to influence the figures for pregnanediol recovered as NaPG.

Conclusion. Diethylstilbestrol glucuronide is recovered in no more than trace amounts from human urine by the gravimetric Venning procedure for the determination of sodium pregnanediol glucuronide. In pregnant patients taking large amounts of stilbestrol the quantitative values for urinary pregnanediol were not influenced by this possible source of error. Some other explanation must be found for the fact that values acquired by the Venning method on the urines of such patients do not agree with colorimetric assays for "freed" pregnanediol upon hydrolyzed specimens.

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Activity of Trypsin in Solution Through Membranes.* (17689)

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The concept of specific long range forces of certain biologically active materials has been promulgated by Rothen(1-7). His experiments employing films of antigen and antibody, or films of enzyme and subtrate, separated by inert film barriers indicate the possible existence of such forces. The evidence thus far presented is the increase in thickness of the layered films when homologous antigen-antibody systems are allowed to interact through the barriers. Similarly, trypsin acting through a barrier on bovine albumin prevents an increase in thickness when an antiserum to bovine albumin is subsequently added. Rothen also finds that increasing the number of layers of barrier be-

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1. Rothen, A., *J. Biol. Chem.*, 1944, v152, 679.
2. Rothen, A., *Rev. Scient. Instruments*, 1945, v16, 26.
3. Rothen, A., *Science*, 1945, v102, 446.
4. Rothen, A., *J. Biol. Chem.*, 1946, v163, 345.
5. Rothen, A., *J. Biol. Chem.*, 1947, v167, 299.
6. Rothen, A., *J. Biol. Chem.*, 1947, v168, 75.
7. Rothen, A., *J. Am. Chem. Soc.*, 1948, v70, 2732.

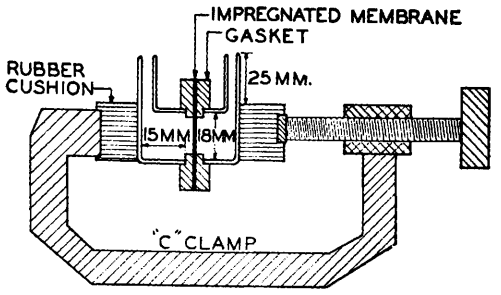

FIG. 1.
Membrane and cell assembly.

yond certain thicknesses obliterates the interacting effect. Increasing the number of monolayers below the barrier reinforces the interaction. A number of systems have all been studied by the same technique, the measurement of increment in film thickness by means of the ellipsometer(2). The findings, however, have met with considerable reservation, principally on the assumption that interaction may take place through minute holes in the film barriers(8,9). Although many substances are known to form films without holes(10) possible imperfections cannot be detected by the ellipsometer. A method for testing interaction, therefore, which might also detect leaks in the barrier could help to determine if the effect is caused by simple diffusion. Such a method, involving a trypsin solution separated by an impregnated paper membrane from a hemoglobin substrate solution, has been devised.

Experimental. Materials and Methods. A small 2-compartment cell was constructed with a membrane separating the compartments (Fig. 1). Each cell half was made from an 18 mm I. D. pyrex test tube. The round bottom of the tube was flattened, and a 25 mm side arm of 4 mm I. D. pyrex tubing was sealed in at a right angle to the test tube and flush with its flattened bottom. The body of the tube was cut off, and the cut edge of the cell half was ground down with carborundum so that the distance from the flat bottom to the ground edge was 15 mm. The capacity was about 3.5 ml. The second cell half was similarly constructed. Two rubber gaskets were used to hold the membrane between the cell halves. They were each constructed of 2 sheets of U. S. Rubber Company Rainbow rubber 1.5 x 35 x 35 mm. Centered circular holes were cut in each of 2 of the pieces of rubber so that the hole in one piece was of the inside diameter of a cell half, while that in the second piece was of the outside diameter. The latter fit the cell half tightly when placed over it. The 2 rubber pieces were washed with benzene and cemented together with rubber cement, the holes placed concentrically. This provided a gasket which fit over the smooth, outside edge, and flush against the ground edge, of the cell half. Another identical gasket was made for the second cell half. When the gaskets were fitted to the cell halves a membrane could be placed between them. The gaskets were conveniently held together with two 1¼-inch screw clamps over their extended edges. The screw clamps thus facilitated placing the assembled cell horizontally in a 2-inch "C" clamp. Two cushions made from rubber stoppers were cemented to the "C" clamp so that the cell could be tightened in the clamp without breaking, the flat ground edges of the cell halves pressing the membrane between the gaskets.

Membranes capable of withstanding even the small hydrostatic pressure of about 2 cm H_2O when only one section of the cell was filled proved difficult to make. Membranes of formvar sufficiently thin to give interference rings (0.1-0.5% in ethylene dichloride) were cast on water and floated onto a supporting screen of 200 mesh stainless steel. However, these were found incapable of supporting the necessary pressure. Similarly collodion and parlodion also failed. It became apparent that a finer mesh for the supporting screen was required, and various types of paper were tried. Several dozen filter paper types had insufficient wet strength or were too thick. It was finally found that Esleeck onion skin typewriter paper had the necessary properties. The paper was washed overnight in distilled water, allowed to dry and cut into 2.5 cm squares.

8. Karush, F., and Siegel, B. M., *Science*, 1948, v108, 107.
9. Trurnit, H. J., *Science*, 1950, v111, 1.
10. Iball, J., *Science*, 1949, v109, 18.

TABLE I.
Activity Through Membranes of Solutions of 0.1% Trypsin on 1.75% Hemoglobin.

Membrane	Solvent	% (wt)	T°C	Mg tyrosine released: Controls: Before	Controls: After	Experimental
Intact:						
Formvar	$C_2H_4Cl_2$	.24	25	0	.002	.160
Cellophane	—	.02 (mm)	25	.002	0	0
Parlodion	C_3H_6O	2.5	25	0	0	.056
Collodion	,,	.19	23	0	.002	.102
,,	,,	.38	23	0	0	.080
,,	,,	1.27	23	0	0	.070
,,	,,	3.40	23	0	0	.032
,,	,,	6.90	23	0	0	.023
,,	,,	10.4	23	0	0	.020
,,	,,	10.8	23	0	.001	.021
,,	,,	11.2	23	0	0	0
,,	,,	12.6	23	0	0	0
—	—	—	23	—	—	2.210
Defective:						
Cellophane	—	.02 (mm)	22	.008	.006	.020
Collodion	C_3H_6O	.19	23	.007	0	.111
,,	,,	10.4	23	.008	.014	.035
,,	,,	11.2	23	.008	.001	.018

Collodion was used predominantly in the experiments. About 100 ml Mallinckrodt U.S.P.XIII collodion was poured slowly into 2 l distilled H_2O with vigorous stirring. The precipitate was washed with several changes of distilled H_2O to remove most of the alcohol and ether. After drying in air overnight, it was cut in small pieces and dried to constant weight in a vacuum desiccator over $CaCl_2$ at room temperature. A series of approximately 10 ml solutions of collodion in acetone varying from 0.19-12.6% by weight was prepared. The solvents for Mallinckrodt parlodion and Shawinigan formvar were acetone and ethylene dichloride respectively. The membranes were made by immersing the paper squares in the solutions overnight. The impregnated paper was removed from the solution, the excess solution was quickly pressed out between two microscope slides, and the membrane was allowed to dry at least 30 minutes before being assembled in the cell. A new membrane was used for each test.

The trypsin used was a dried, commercial preparation. Since the enzyme best maintains its activity in the dry state, it was made into solutions of 1 mg/ml in the same vehicle as the substrate just prior to use. The vehicle consisted of 8.0 ml 1N NaOH, 40 g urea, 82 ml H_2O and 10.0 ml M KH_2PO_4, pH 7.5(11). The activity was such that color was liberated equivalent to 2.210 mg tyrosine/ml in the presence of "phenol" reagent after digestion for 30 minutes at 23°C with the hemoglobin substrate (Table I). The enzyme activity was measured with a Klett-Summerson colorimeter at 600 mμ employing the "phenol" reagent of Folin and Ciocalteau(13) and was read from a tyrosine standard curve. Hemoglobin substrates of a final concentration of 1.75% in the same vehicle as that used for the trypsin, were prepared by urea denaturation according to Anson(11,12).

Controls for determining whether or not the membranes were intact were run on each membrane just before and after every determination. These are described in the following section. However, since only 0.5 ml of the control solutions (*vs.* 2.5 ml hemoglobin solution) could be tested, a further test for the detection of membrane leaks was made. This consisted in placing enzyme and sub-

11. Anson, M. L., *J. Gen. Physiol.*, 1938, v22, 79.
12. Northrop, J. H., *Crystalline Enzymes*, Columbia University Press, New York, 1939.
13. Folin, O., and Ciocalteau, V., *J. Biol. Chem.*, 1927, v73, 627.

strate on opposite sides of a 1.27% collodion membrane in the assembled cell. After 30 minutes, 2.5 ml of substrate were removed via syringe and mixed with 8.5 ml of fresh substrate. Had enzyme leaked and been present in the substrate, it would have been expected to continue to liberate color producing substances so that the tyrosine value would increase with time. Periodically 2.5 ml of substrate were removed and tested with phenol reagent. However, in 2 experiments with different membranes there was no increase in the tyrosine value up to 180 and 250 minutes.

Procedure. After assembling the cell with the desired membrane, it was tightened in the "C" clamp which was supported horizontally on a ring stand. One cell half was filled with about 3.0 ml control buffer solution so that it reached a few millimeters into the side arm. The solution was introduced by means of a hypodermic syringe with stainless steel cannula. The other cell half was similarly filled with enzyme solution to the same level. The cell was allowed to stand 30 minutes when the control solution was removed by syringe to be later tested for possible enzyme which might have leaked through the membrane. The now empty half cell was filled with the hemoglobin substrate to the same level as the enzyme solution with a clean syringe and cannula. After 30 minutes during which interaction could take place, it was removed to be tested for color producing compounds. The residual hemoglobin in the cell half was gently rinsed out and discarded, and the cell half was again filled with buffer. This was allowed to stand for 30 minutes and was then removed via syringe to be tested with the first control for enzyme activity. The whole procedure was carried out at room temperature, which was maintained at 23° ±0.5°C. To 0.5 ml of each of the control samples in a 20 x 150 mm test tube, 2.5 ml of fresh hemoglobin substrate were added. The two controls were allowed to incubate for 30 minutes at room temperature to test for enzyme which might have leaked through the membrane. At this time to 2.5 ml of the hemoglobin which had been in contact with the membrane in the same size tube, 0.5 ml of buffer was added. This brought all three solutions to the same concentrations. A reagent blank consisting of 2.5 ml of hemoglobin substrate and 0.5 ml of buffer had also been prepared at the time of introduction of hemoglobin substrate into the cell. At the conclusion of the 30 minute digestion period, 5.0 ml 0.300 N trichloracetic acid were quickly added to each of the 4 samples, and they were filtered to remove the precipitated hemoglobin. To 5.0 ml of each filtrate were added 10.0 ml 0.5 N NaOH and 3.0 ml of "phenol" reagent. The samples were read in 2-15 minutes in the colorimeter with the instrument set at 0 with the reagent blank. The standard deviation, σ, for the tyrosine standard curve was 0.0050 mg tyrosine per ml.

Results. Table I illustrates the results obtained with several different membrane materials made at various concentrations. In the experiments employing 0.24% formvar, 2.5% parlodion and 0.19-10.8% collodion membranes, the trypsin acted on the hemoglobin substrate to liberate color producing substances. There was no indication that the membranes allowed trypsin to leak into the control solutions during the 30 minute control periods before and after enzyme and substrate had been in contact with the membranes. These membranes are labeled "intact." The values for several "defective" membranes are included in Table I. Since the standard deviation for the tyrosine standard curve was 0.0050, experiments in which the control values were greater than this were considered to have defective membranes. Fig. 2 illustrates the effect of increasing the collodion concentrations in a series of membranes from 0.19-12.6%. It was found that the tryptic action on the hemoglobin follows a decreasing, non-logarithmic, curvilinear pattern up to 11.0% collodion. At this point the effect abruptly drops from 0.020 mg tyrosine per ml to zero.

Discussion. Interpretation of the results reported here is necessarily centered about the controls since evidence for interaction across an inert membrane must preclude the possibility of enzyme and substrate diffusing into one another through imperfections in the membrane. That they do not diffuse through

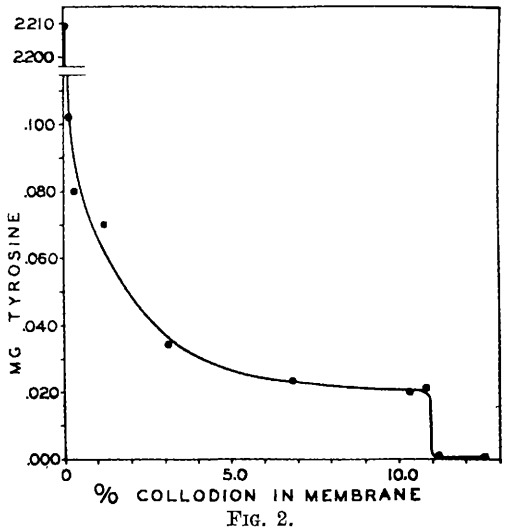

FIG. 2.

Decrease in trypsin activity as the concentration of collodion in the membranes is increased. Trypsin activity is expressed as mg of tyrosine.

the membranes is shown by the controls.

The high degree of sensitivity of the controls is illustrated in Table I from the differences between the same kind of intact and defective membranes. Thus, in the case of the cellophane membranes, the intact membrane transferred no tryptic activity, nor did the controls indicate leaking. However, the defective membrane showed a tryptic activity value of .020, while its controls jumped to .008 and .006 indicating leakage. Similar results were obtained for intact and defective 0.19, 10.4 and 11.2% collodion membranes. Therefore, experiments yielding no leak values but yielding transfer values from .020 all the way to .160 must require the interpretation that tryptic activity had been transferred across the membranes in some manner. In Rothen's experiments the thickness of the films cast on smooth surfaces may be measured with considerable accuracy by the ellipsometer. The thickness of the films in the interstices of the impregnated membranes is purely a matter of conjecture. However, both methods indicate transference of tryptic activity across thin membranes. Rothen's findings indicate that interaction takes place in systems wherein enzyme, substrate and barrier are immobilized as solid films. The experiments here described indicate that the effect also takes place when enzyme and substrate are in solution and are separated by a solid membrane. Whatever the mechanism for the transfer of the activity may be, the latter experiments show that this effect would have to be ascribed to some phenomenon other than simple diffusion through the membranes.

Summary and Conclusions. Formvar, parlodion and collodion membranes have been prepared by impregnating paper with solutions of these substances. A suitable cell of two compartments was constructed in which solutions of trypsin and hemoglobin could be separated by the membranes.

Interaction between solutions of trypsin and hemoglobin separated by formvar, parlodion or collodion membranes has been demonstrated.

Examination of a series of membranes impregnated with collodion of different concentrations showed that interaction decreased in a curvilinear fashion as the collodion concentration in the membranes was increased.

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Antigenic Property of Renal Cortex.* (17690)

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The experimental production of chronic renal disease in rats by means of heteronephrotoxins obtained from rabbits has been studied by Smadel, Swift and Farr(1-3), and later

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1. Smadel, J. E., and Farr, L. E., *J. Exp. Med.*, 1937, v65, 527.