more loosely aggregated ground substance in the skin(3). Cortisone appears to increase the rate of formation of certain connective tissue colloids. However, these components have modified physicochemical properties. Stimulating effects of cortisone upon connective tissue have also been demonstrated in nasal mucosa altered by the allergic state(7) and in atopic dermatitis(8).

The rate at which connective tissue is disaggregated and restored following administration of hyaluronidase may be used as an index of its initial state and of its capacity for repair. It is probable that this response varies under the influence of many other hormones and drugs, as well as in disease states. The serial electrometric determination of these changes in connective tissue colloids may serve as a connective tissue "function" test, measuring the ability to maintain homeostasis.

Summary. Reversal of hyaluronidase effects on concentration of negatively charged colloids in the dermis of normal and cortisone treated rabbits was studied by serial measurement of liquid junction potentials. In normal animals, hyaluronidase lowers negative charge density in the tissues; recovery is half completed in about 4 hours. In animals treated with cortisone initial charge density is lower than in normal animals. After hyaluronidase is injected into the dermis, the lowest levels are reached. Half recovery, however, occurs in 2½ hours. Serial electrometric measurements following hyaluronidase injection are suggested as a means of studying hormone and drug effects, and disease states in connective tissue.

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Received January 17, 1957. P.S.E.B.M., 1957, v94.

Lack of Specificity of Insulin-I^{131}-Binding by Isolated Rat Diaphragm. (23075)

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Stadie and coworkers reported an increase in glycogen synthesis by the isolated rat diaphragm exposed briefly to solutions of insulin(1). It was suggested that insulin is bound firmly to tissues at or near sites of enzyme systems and that such fixation is necessary for its physiologic action. Stadie, Haugaard and Vaughn(2) later noted that, following brief immersion of diaphragm into solutions of S^{35}-labeled or I^{131}-labeled insulin, a small fraction of radioactivity remained fixed to the tissue even after repeated washing with saline solution, and with increasing concentrations of insulin there was a tendency for bound insulin to reach a limiting value at about 1½ to 2 μg/g tissue.

In the course of studies with a variety of I^{131} labeled proteins from different sources, *e.g.*, serum albumin, gamma globulin, insulin, glucagon, ACTH, diphtheria toxoid, etc., we observed that, at tracer levels, most of these proteins adsorb nonspecifically to almost any inert surface, and that such adsorption may be minimized by presence of high concentrations of almost any other protein. With insulin, particularly, adsorption to glass(3), and paper(4,5) has been reported previously. It therefore seemed of interest to reevaluate

the specificity of the binding of isotopically labeled insulin to diaphragm *in vitro*.

Methods. I^{131} labeled insulin was prepared from crystalline beef insulin* with specific activity of 1-3 mc/mg by modification of the method of Pressman and Eisen(6). This modification consists of extraction with chloroform of elemental iodine produced in Pressman-Eisen procedure and employment of $CHCl_3$-I_2 solution for iodination of proteins. By this procedure the yield of protein-bound I^{131} is increased to about 30% of the starting radioactivity and the iodinated proteins appear to preserve their biologic integrity(5). All preparations were dialyzed for 18-24 hours until nonprotein bound I^{131} was less than 1% of total radioactivity. I^{131} labeled pooled human serum albumin and gamma globulin were prepared in a similar manner from commercially available sources of these proteins and I^{131} labeled glucagon was prepared from crystalline glucagon.† Assays of radioactivity were performed in a well-type scintillation counter with sensitivity of 1 x 10^6 c/m/μc I^{131} above a background of 200 c/m. Sufficient counts were made in all experiments to reduce the statistical error to less than 3%. The general procedure for determination of insulin-I^{131} bound to diaphragm was essentially the same as that employed by Stadie *et al.*(2). Freshly removed rat diaphragm was rinsed in cold phosphate buffer solution (.04 M sodium phosphate, .087 M sodium chloride, .005 M magnesium chloride, pH 6.8) to remove excess blood. Each hemi-diaphragm was used as such or divided into two parts. The tissues were weighed on Roller-Smith balance to the nearest mg and were then immersed (zero time) for 1 minute in phosphate buffer solution (.04 M, pH 6.8) containing unlabeled protein at various concentrations and tracer amount of I^{131} labeled protein. After 2 preliminary washings of 30 seconds each in 25 ml of phosphate buffer and 1 washing in 50 ml phosphate buffer, the tissue was assayed for radioactivity and was then placed in 50 ml oxygenated buffer solution at room temperature. Subsequent assays for radioactivity were made at intervals thereafter. For each assay the tissue was transferred to clean test tube containing 5 ml buffer solution and counted in the well scintillation counter. After each assay the tissue was again placed in 50 ml buffer solution through which oxygen was bubbled continuously. In 2 experiments the diaphragm was dried in air for 1 hour prior to incubation with insulin-I^{131}, and in 2 experiments diaphragm was immersed in 15% formaldehyde for 20 minutes prior to incubation with insulin-I^{131}. In several experiments a small piece of soft glass replaced the diaphragm but the procedure was otherwise identical to that outlined above.

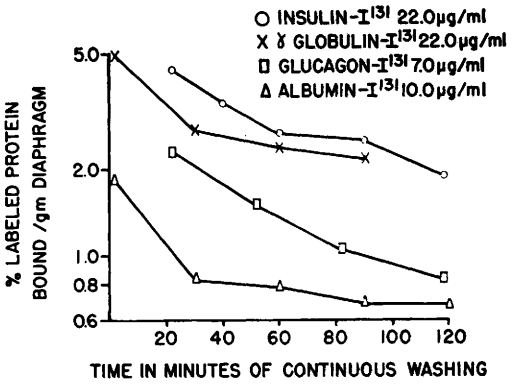

FIG. 1. Binding of various I^{131} labeled proteins to diaphragm and elution on continuous washing.

Results. With all I^{131} labeled proteins, radioactivity "bound" to diaphragm decreased significantly for about 1-1½ hours (Fig. 1). After this time a relative "plateau" was reached in which rate of loss of radioactivity was variable and did not exceed about 50%/hour. In most cases, however, progressive diminution in bound radioactivity occurred as long as observations were continued. In experiments described subsequently, the amount present at 1½ hours is taken as the amount "bound" unless otherwise indicated.

When concentration of insulin in the initial bathing solution was varied, the fraction bound to diaphragm showed no consistent correla-

*We are indebted to Drs. O. K. Behrens and C. W. Pettinga of Eli Lilly Co. for generous supply of crystalline insulin.

†We are indebted to Drs. O. K. Behrens and C. W. Pettinga of Eli Lilly Co. for generous supply of crystalline glucagon lot No. 208-15B-292A.

TABLE I. Influence of Insulin Concentration in Medium on Binding of Insulin by Diaphragm *In Vitro*.

Insulin conc. in medium, μg/ml	% bound per g tissue	μg insulin bound per g tissue
.09	1.6	.007
.30	7.7	.12
.41	15.6	.32
1.6	7.7	.61
2.0	2.24	.22
4.0	1.40	.28
5.6	7.80	2.20
7.0	1.70	.59
8.1	2.90	1.20
13.0	2.34	1.50
15.0	.51	.41
21.6	2.34	2.50
30.0	4.30	6.40
30.0	.26	.47
41.6	3.30	6.90
70.0	1.50	5.20
80.1	2.70	10.8
85.0	.21	1.07
101.6	.90	4.60
142.	1.16	8.20
198.	3.50	34.6
282.	1.30	18.0
302.	2.28	34.4
335.	.38	7.6
422.	1.10	23.2
485.	1.10	26.5
502.	.46	11.6
700.	.81	28.4
800.	.74	29.6
802.	.44	18.0
1005.	1.98	100.
1400.	.92	64.
2004.	.51	51.

tion with insulin concentrations in the range 0.09 - 500 μg/ml but, as a rule, was definitely decreased at higher concentrations (Table I). Consequently, there was no constant increase in absolute amount of insulin bound/g tissue with increase in concentration in the lower concentration range, nor was there a definite limiting value to the amount of bound insulin with increase in concentration up to limits of solubility of insulin at the pH employed (Table I). Such lack of consistency was observed not only among different experiments but frequently also when different pieces of the same diaphragm were tested simultaneously (Fig. 2). Variable adsorption to glassware with loss from bathing solutions was also observed at low insulin concentrations and was probably responsible for absence of reproducible adsorption isotherm; the data for binding as given, are expressed in terms of amounts still remaining in solution at beginning of incubation periods.

When the diaphragms were immersed in solutions containing tracer insulin-I^{131} and significant concentrations of unlabeled serum albumin or gamma globulin, radioactivity "bound" to diaphragm was strikingly diminished (Fig. 3). Similarly the binding of tracer albumin-I^{131} or γ globulin-I^{131} to diaphragm was inhibited by presence of high concentrations of heterologous unlabeled proteins (Table II). Of the 3 proteins, insulin appeared to compete most effectively for the binding of all.

Insulin binding, by air-dried and formaldehyde-treated diaphragms, was no less marked than by fresh diaphragm (Table III).

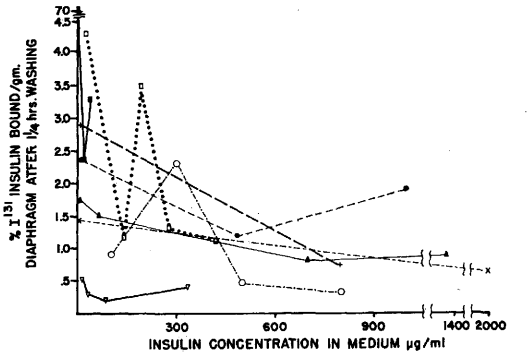

FIG. 2. Binding of I^{131} labeled insulin to diaphragm as function of insulin concentration. For each curve, experiments at different concentrations were run simultaneously with pieces of the same diaphragm.

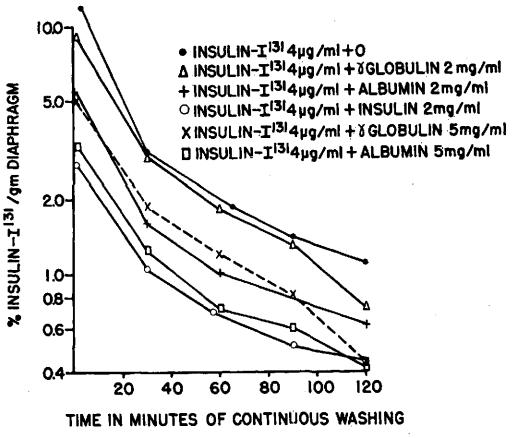

FIG. 3. Effect of high concentrations of homologous and heterologous proteins on binding of I^{131} labeled insulin to diaphragm.

TABLE II. Influence of Presence of Homologous and Heterologous Carrier Proteins on Binding of I^{131} Labeled Proteins by Diaphragm *In Vitro*.

Labeled protein	μg/ml	Carrier protein	μg/ml	% labeled protein bound per g diaphragm
Insulin-I^{131}	9.	0	0	1.70
	9.	I*	1,400	1.06
	4.	0	0	1.40
	4.	I	2,000	.51
	4.	A	2,000	.81
	4.	A	5,000	.60
	4.	γ-g	2,000	1.35
	4.	γ-g	5,000	.80
Albumin-I^{131}	6.	0	0	.58
	6.	I	800	.36
	20.	0	0	.67
	20.	I	2,000	.25
	10.	0	0	.65
	10.	A	500	.76
	10.	A	10,000	.33
	10.	γ-g	650	.65
	20.	γ-g	10,000	.25
γ-glob.-I^{131}	112.	0	0	2.16
	112.	I	2,000	1.01

* I = Insulin; A = Albumin; γ-g = γ-globulin.

TABLE III. Binding of Insulin *In Vitro* by Fresh Diaphragms and by Diaphragms Dried in Air or Treated with Formaldehyde.

Insulin conc. in medium, μg/ml	Condition of diaphragm		% insulin in medium bound per g tissue after washing 1 hr	1½ hr
6	Fresh	a	1.40	1.10
	"	b	1.50	1.28
6	Air-dried, 1 hr	a	2.40	1.80
	Idem	b	2.30	"
4	Fresh	a	2.46	1.85
	"	b	2.10	1.63
4	15% formaldehyde-treated, 20 min.	a	2.08	1.58
		b	"	"

When pieces of glass instead of rat hemidiaphragm were used, results on binding of insulin after one hour washing were quite similar to those in experiments with diaphragm, including evidence for a stronger competitive inhibitory effect of insulin than of γ globulin on binding of insulin-I^{131} (Table IV). However, the degree of inhibition by high concentrations of protein was much greater than in experiments with diaphragm even when the % adsorbed at trace levels was in the same range. In addition, rate of elution of both proteins from glass was significantly slower than from diaphragm.

Discussion. Adsorption of 4 different I^{131} labeled proteins to diaphragm has been demonstrated. Evidence for nonspecificity of this adsorption is presented in experiments demonstrating inhibition by heterologous proteins and by a similar pattern of adsorption observed with formaldehyde-treated diaphragms and diaphragms permitted to deteriorate in air. In support are the observations indicating adsorption and competitive inhibition on a biologically inert material, glass. Serum albumin and γ globulin are effective competitive inhibitors for the "binding" of insulin-I^{131} to isolated rat diaphragm and insulin is likewise an effective competitive inhibitor for the binding of either albumin-I^{131} or γ globulin-I^{131}.

It has previously been observed that insulin-I^{131}(4,5) and glucagon-I^{131}(7) adsorb quite firmly to paper, and that such adsorption is not significantly inhibited by the presence of moderate amounts of serum proteins but is inhibited competitively by the presence of high concentrations of homologous proteins. Depending on the nature of the binding sites and the reactive groups of the specific protein, a greater or lesser degree of selection may be observed with different adsorbing systems. In the case of diaphragm, paper and glass, insulin appears to be adsorbed preferentially in the presence of several other proteins but the diaphragm is not more selective than the inert materials.

Furthermore, several of our observations on insulin binding by fresh diaphragm are in dis-

TABLE IV. Binding of I^{131} Labeled Proteins by Glass in Presence and Absence of High Concentration of Homologous and Heterologous Proteins.

Labeled protein	μg/ml	Carrier protein	μg/ml	% I^{131} protein per g glass
γ-glob.-I^{131}	1.6	0	0	1.8
"	"	I*	1,600	.25
Insulin-I^{131}	1.0	0	0	4.2
"	"	I	1,600	.10
"	"	0	0	5.8
"	"	γ-g	10,000	.54

* I = Insulin; γ-g = γ-globulin.

agreement with those of Stadie and coworkers (1,2). We have been unable to demonstrate either the permanent fixation of bound insulin-I^{131} or a limiting value for bound insulin-I^{131} within the range of solubility of insulin.

It is not the intent of the present report to deny that the biologic effects of insulin are exerted through combination with some cell-fixed enzyme system or substrate compound. It is generally accepted that a combination of some sort is necessary for a chemical reaction between substances to take place. However, the present studies do not support the concept that the binding of insulin-I^{131}, observed *in vitro* with isolated rat diaphragm is necessarily of any physiological significance, or that these observations reflect, in any way, a biologic process which takes place *in vivo*.

If a brief exposure of diaphragm to insulin suffices to augment glycogen accumulation on subsequent incubation with glucose(1), the results of the present study would then suggest that survival time of the altered state induced by insulin must persist appreciably beyond the time of reaction with insulin. An evaluation of this survival time would appear to be of interest.

Summary. Serum albumin, serum γ globulin, crystalline glucagon and crystalline insulin were observed to bind to diaphragm *in vitro* and to be eluted slowly on continuous washing with buffered solutions. A lack of reproducibility of binding of insulin at low concentrations is attributable in part to variable adsorption to glassware. A definite saturation of binding sites on diaphragm was not observed at the highest concentrations employed. Competitive inhibition was observed in the presence of heterologous proteins. Binding capacity was not altered by treatment of diaphragms with formaldehyde. Binding of insulin by isolated rat diaphragm *in vitro* is not demonstrably of biologic significance but is attributable to nonspecific surface adsorption of proteins.

We are indebted to Melanie Knopf and Department of Medical Illustration for the figures. We also wish to thank Carl Bacot for technical assistance, and Frieda Steiner and Eve Spelke for secretarial assistance.

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Received January 24, 1957. P.S.E.B.M., 1957, v94.

Susceptibility of Inbred Mice to Group A Streptococcal Infection.* (23076)

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Group A streptococci isolated directly from patients often are rich in M protein but avirulent to mice unless adapted by rapid successive transfer(1). This study was undertaken to compare susceptibility of mice of diverse genetic constitution to infection by representative group A streptococci. The objective of the investigation was to provide a sensitive experimental host-parasite system that could serve as a more adequate model for study of group A streptococcal infections of man and their late sequelae.

* Aided by grants from Natl. Inst. of Allergy and Infectious Disease and from Natl. Heart Inst., of N.I.H., PHS.

Materials and methods. Inbred mice used differed significantly in genetic, anatomic, and physiologic constitution(2). BALB, C58,