and requiring added asparagine for growth *in vitro,* incorporated relatively little valine unless sufficient amounts of asparagine were added to the medium.

The significance of the findings is briefly discussed.

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Trichloracetic Acid Soluble Polysaccharide-Protein Complexes.* (30170)

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It was recently reported (Woodside and Kocholaty(4)) that glycogen was firmly bound to both human and bovine protein fractions of thrombocytes and to gelatin. These biopolymeric complexes were distinguished from unbound glycogen by their solubility in 0.83% trichloroacetic acid (TCA)—83% ethanol solutions. The purpose of this study was to extend these observations to other proteins and polysaccharides.

Materials and methods. The glycogen extraction and precipitation techniques, carbohydrate standards, and quantitative carbohydrate determinations were described earlier (Woodside and Kocholaty(4)). Protein-bound polysaccharide values represent the difference between the 30% KOH extractable polysaccharide (total polysaccharide value) and the 0.83% TCA—83% ethanol insoluble polysaccharide (unbound polysaccharide). Protein was estimated by the Lowry protein determination (Lowry *et al*(3)) with bovine plasma albumin as the standard. For protein determination of gelatin solutions, gelatin, U.S.P. (Difco Laboratory, Detroit, Mich.) was used as the standard.

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The following commercially available proteins were used: gelatin, U.S.P., purified pigskin and calfskin gelatin, collagen, casein, edestin, β-lactoglobulin, bovine hemoglobin, guinea pig complement, human poliomyelitis immune globulin, immune serum globulin, poly-L-proline and poly-L-hydroxyproline. Cohn fraction VI, E. R. Squibb and Sons, New York, was also made available to us by Dr. J. H. Pert, American National Red Cross, Washington, D. C. Commercially available polysaccharides were: glycogens, rabbit liver and shellfish glycogens, dextran, (M.W. 80,000) dextrin, amylodextrin, and inulin. Glycogen, C. P., Pfanstiehl Chemical Corp., has been used as the standard glycogen suspension over the past 5 years.

Ten human serum protein fractions separated on a DEAE cellulose anion exchange column according to Fahey *et al*(5) were supplied by Mr. B. D. Ashley of this laboratory.

All protein and polysaccharide solutions were suspended in distilled deionized water unless otherwise specified. Reaction mixtures for polysaccharide-protein binding studies contained 20 mg protein and 0.5 mg polysaccharide in a total volume of 1.0 ml. In

prolonged incubation studies with α-amylase, 1.0 ml reaction mixtures contained 0.5 mg glycogen in 0.85% NaCl, 1.25 γ α-amylase, and either 20 mg gelatin or immune globulin. Equal volumes of either 10% TCA or 60% KOH were added within 15 seconds after the desired incubation periods.

The effect of $CuCl_2$ on the binding capacity of Difco gelatin was determined according to Bello and Vinograd(1) in the presence of 5% TCA, 1% and 3% NaOH, and at pH 7.0. The Cu^{++} : polypeptide ratio was varied from 1:16 to 200:1 in 0.05% gelatin—0.05% glycogen suspensions in 5% TCA solutions. In neutral and alkaline solutions, the ratios of Cu : polypeptide were varied from 1:16 to 1:2. The complexes were precipitated at a final concentration of 83% ethanol.

Results. It can be seen from Fig. 1 that 0.5 mg gelatin bound 0.5 mg glycogen. Gelatin concentrations between 1.0 and 20 mg bound 93 to 98% of the available glycogen. At gelatin concentrations below 0.5 mg, decreasing amounts of glycogen were bound. On a dry weight basis, the binding ratio of gelatin-glycogen mixtures was 1.0. Identical results were obtained with all 3 gelatins.

Rabbit liver and shellfish glycogens were as effectively bound by gelatin as the standard glycogen preparation which has been used over the last 5 years. Gelatin (2%) also bound 96 to 98% of 1.0 mg samples of dextrin, amylodextrin and dextran; however, less than 10% of 1.0 mg inulin samples were bound by 20 mg of gelatin. When 1.0 and 0.25 mg aliquots of glycogen were used, equivalent amounts of gelatin (1.0 and 0.25 mg, respectively) essentially bound (90 to 98%) all of the available glycogen.

The following proteins and polyamino acids did not bind glycogen under the conditions outlined: poly-L-proline and poly-L-hydroxyproline, either individually or combined, crystalline bovine albumin, bovine hemoglobin, casein, edestin, β-lactoglobulin, α_1-glycoprotein, Cohn fraction VI, and guinea pig complement. A collagen suspension (3.5 mg/ml at pH 7.0) did not bind glycogen. Incubation of the above protein-glycogen mixtures at 25°C for 1 hour did not alter glycogen recoveries. Nine of ten human se-

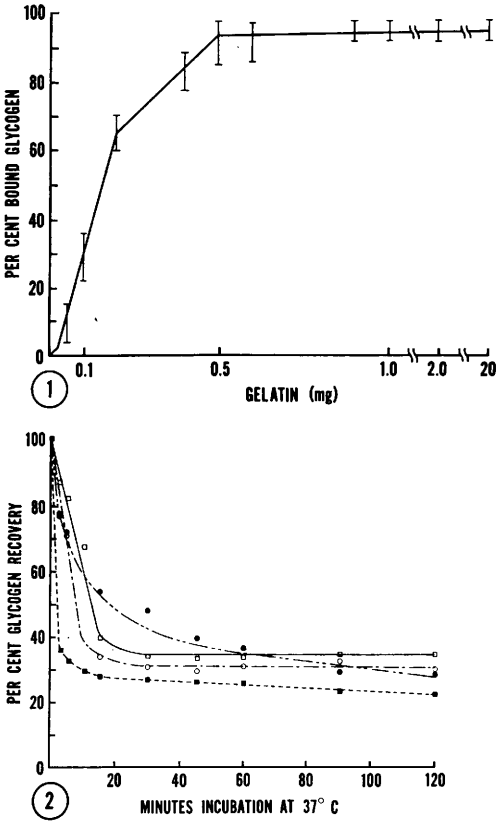

FIG. 1. Glycogen binding capacity of gelatin. All reaction mixtures contained 0.5 mg glycogen. Vertical lines present the extremes of 3 to 12 determinations.

FIG. 2. Glycogenolytic activity of human poliomyelitis immune globulin. All reaction mixtures contained 0.5 mg glycogen. Proteins (20 mg) and 1.25 γ α-amylase were added as indicated: ●- - —- -●, amylase; ○ - — - ○, gelatin + amylase; □——□, immune globulin; ■- - -■, immune globulin + amylase. Glycogen extracted by 30% KOH.

rum protein fractions did not bind glycogen (TCA glycogen values ranged between 97 to 103% KOH glycogen values). With the exception of F-V (albumin), all of the serum protein fractions contained variable amounts of TCA soluble protein which ranged from 0.6 to 28.2% of the total protein content.

In contrast, the human gamma globulin fraction eluted from a DEAE-cellulose column consistently bound 15 ± 3% of the added glycogen. Only 120 γ protein of the 20 mg γ-globulin was soluble in 5% TCA; therefore, the minimal binding ratio was 1.6. In a similar manner, human poliomyelitis immune globulin bound 16 to 20% of the

TABLE I. Effect of Variations in Poliomyelitis Immune Globulin on Glycogen Binding.*

Immune globulin (mg)	80	40	20	10	1.0
% Bound glycogen	33.2	25.0	20.8	6.8	.0

* All experimental manipulations prior to acid and alkali extractions were carried out at 4°C in presence of 0.5 mg glycogen.

TABLE II. Effect of Prolonged Incubation on Glycogen Binding Capacity of Poliomyelitis Immune Globulin.*

	% Glycogen recovery			
	20 mg immune globulin		10 mg immune globulin	
Minutes incubation at 37°C	KOH extraction	TCA extraction	KOH extraction	TCA extraction
0	97.1	79.2	98.4	93.2
30	59.3	45.5	65.0	61.0
60	48.3	42.5	58.4	53.0

* Incubated in presence of 0.5 mg glycogen.

added glycogen. Human poliomyelitis immune globulin released only 400 γ protein into the TCA soluble fraction; consequently, its minimal binding ratio was 5.0.

The data in Table I show that the amount of glycogen bound by poliomyelitis globulin was dependent on the concentration of immune globulin. At concentrations of 80 mg globulin per reaction mixture, 33% of the glycogen was bound, whereas, at 10 mg globulin concentrations, only 7% of the glycogen was bound. At concentrations of 1 mg/ml immune globulin or less, no binding was detected.

In addition to glycogen binding, poliomyelitis immune globulin exhibited a glycogenolytic system. Prolonged incubation of poliomyelitis immune globulin-glycogen mixtures at 37°C resulted in progressive decreases in glycogen recovery as shown in Table II and Fig. 2. Both KOH and TCA extractable glycogen values diminished with increasing incubation periods; in addition, the glycogenolytic rate increased with increasing concentrations of the poliomyelitis immune globulin. In all instances, TCA extractable glycogen values were less than the corresponding KOH extractable glycogen values for any given time interval.

Human poliomyelitis immune globulin hydrolyzed glycogen at constant rates during 15 minutes of initial incubation. At 25 and 37°C the rates of glycogenolysis were 0.87 γ and 1.73 γ glycogen/min respectively. This 2-fold increase in the glycogenolytic rate which accompanied the 12 degree rise in temperature characterizes the glycogen losses as an enzymatic process.

The influence of NaCl, acid and alkali on the binding capacity of gelatin are shown in Table III. Neither 0.5 N HCl nor 0.5 N NaOH influenced the binding capacity of gelatin. The inhibition of glycogen binding observed before dialysis of the neutralized alkali and acid mixtures could be attributed to a salting out effect of the glycogen-gelatin complex, since identical results were obtained in 5% NaCl. Exposure of glycogen-gelatin mixtures to 1 M and 6 M urea for 1- and 60-minute intervals at 25°C had no effect on the binding capacity of gelatin.

The glycogen binding capacities of the various gelatins were destroyed by autoclaving at different rates which were dependent on the type of gelatin (Table IV). Autoclaving the glycogen standard for 60 minutes gave identical binding values as obtained with native gelatins. Autoclaving of gelatin solutions resulted in a progressive loss in gelation, viscosity and gelatin binding capacity (Table V).

$CuCl_2$ solutions in ratios varying from 1:16 to 200:1 (Cu^{++}:polypeptide) had no effect on the glycogen-gelatin binding in the presence of TCA (Table VI).

TABLE III. Stability of Glycogen-Gelatin Binding in Various Chemical Environments.

		% Bound glycogen	
		Min incubation at 25°C	
Suspending medium	Salt conc (%)	1	60
Distilled water	.0	99.0	98.0
.85% NaCl	.85	96.5	99.2
5.0% NaCl	5.0	3.3	3.0
5.0% NaCl, dialyzed*	.0	98.0	97.3
.5 N HCl	5.3	3.4	4.8
.5 N HCl, dialyzed	.0	99.2	97.1
.5 N NaOH	5.3	3.4	4.8
.5 N NaOH, dialyzed	.0	99.2	98.8
.5 N KOH	6.8	.5	5.9
.5 N KOH, dialyzed	.0	99.8	97.3
1.0 M urea	.0	98.6	99.0
6.0 M urea	.0	99.8	95.6

* Dialyzed on a multiple dialyzer for three 2-hr periods and one 16-hr period.

TABLE IV. Glycogen-Binding Capacity of Autoclaved Gelatins.*

	Min autoclaved	Gelatin conc, mg/reaction mixture 10	0.25
Difco gelatin	0	95.2 (± 3.7)	97.9 (± 2.4)
	60	96.2 (± .6)	7.5 (± 5.4)
	120	55.6 (± 8.5)	8.2 (± 5.7)
Pigskin gelatin	0	91.7 (± 6.0)	96.6 (± 2.0)
	60	97.3 (± .4)	12.4 (± 5.8)
	120	74.6 (± 9.8)	2.8 (± 1.1)
Calfskin gelatin	0	99.1 (± .8)	98.1 (± 1.5)
	60	2.5 (± 1.7)	8.2 (± 5.1)
	120	6.3 (± 2.3)	6.1 (± 4.6)

* All values expressed as % bound glycogen when using 0.25 mg glycogen in reaction mixtures.

At neutral pH similar results were obtained; however, at alkaline pH, the glycogen, in the presence of gelatin and/or $CuCl_2$ was completely precipitated at 83% ethanol concentrations.

Re-extraction of the alkaline solutions with TCA-83% ethanol (Column B, Table VI) resulted in the partitioning of glycogen into bound and unbound forms.

Discussion. A prerequisite for the occurrence of glycogen protein binding would be the release of at least part of the respective protein into the 5% TCA soluble fraction. Of the various proteins tested, only gelatin and those serum fractions rich in gamma globulin exhibited glycogen-binding capacities. Although other serum protein fractions released considerable amounts of protein into the TCA soluble fraction, they were not capable of binding glycogen. These results imply that the glycogen-binding capacities of TCA soluble proteins are dependent on the nature of the proteins released into the TCA soluble fraction.

The data show that the glycogen binding capacity of gelatin is 5 times greater than that of poliomyelitis immune globulin.

Since gelatin binds dextrin, amylodextrin and a low molecular weight dextran, as well as the 3 glycogens tested, it is evident that the binding of polysaccharides by gelatin is not dependent on the type of glucosidic linkages present in the polysaccharide. The stoichiometric binding ratio for the 1 → 6 linked dextran was identical to that of glycogen (1 → 4 and 1 → 6 linkages). This further substantiates the premise that the types of linkages in these glucose polymers do not specifically contribute to the formation of these macromolecular complexes.

Further studies on the nature of the polysaccharide-protein binding were performed with gelatin suspensions since such studies with immune globulin fractions would be further complicated by the presence of amylolytic activity in these fractions. By the definition of soluble bound glycogen, it is obvious that TCA does not disrupt the bonds which are responsible for glycogen-gelatin complex formation. Furthermore, one-hour exposures of glycogen-gelatin mixtures to either 0.5 N HCl or KOH has no effect on this macromolecular-macromolecular complex. Heating to 100°C for 10 minutes does not denature this complex. The resistance of bound forms of glycogen to diluted acid, alkali and heat suggest the existence of an extremely stable biopolymeric complex.

Attempts to dissociate the complex with 6 M urea and high NaCl concentrations were also negative.

However, autoclaving of the various gelatins resulted in simultaneous alterations in their physical properties and binding capacities. The concomitant losses in viscosities, gelation, and polysaccharide binding capac-

TABLE V. Effect of Autoclaving on Some Physical and Chemical Properties of 2% Gelatin Solutions.

	Min autoclaved	Gelation at 4°C	Relative viscosity	pH	% Bound glycogen*
Difco gelatin	0	+	3.237	3.95	96.7 (± 4.4)
	120	—	1.246	4.03	83.4 (± 3.3)
Pigskin gelatin	0	+	3.678	4.30	79.4 (± 5.3)
	120	—	1.347	4.68	86.9 (± 3.3)
Calfskin gelatin	0	+	2.460	3.62	95.5 (± 4.8)
	120	—	1.150	3.75	3.0 (± 1.2)

* 20 mg gelatin + 0.25 mg glycogen per reaction mixture.

TABLE VI. Effect of pH on 83% Ethanol Precipitation of Glycogen-Gelatin Complexes in the Presence and Absence of Cupric Chloride.*

	$CuCl_2$ ($\times 10^{-3}$M)	1% NaOH A	1% NaOH B	pH 7.0 C	pH 7.0 D
Glycogen only	.0	107	103	99.0	98.2
Gelatin "	.0	2.3	.0	2.9	.0
Glycogen & gelatin	.0	99.2	6.1	8.8	1.2
	.6	96.2	35.2	2.3	.2
	1.2	93.1	53.8	3.6	.2

* Expressed as % precipitated (free) glycogen from reaction mixtures containing 0.5 mg glycogen and/or gelatin. A: precipitated from alkaline solution; B: reprecipitation of "A" from 0.83% TCA—83% ethanol; C: precipitated at neutrality; D: reprecipitation of "C" from 0.83% TCA —83% ethanol.

ities suggested that the polysaccharide binding sites of gelatins may be analogous to the intramolecular binding by the gelatin polypeptide chains presumably responsible for the gelation of gelatin (Harrington and Von Hippel(2).

According to Bello and Vinograd(1), formation of the biuret complex of gelatin with as little as 0.03 M $CuCl_2$ in 5% gelatin at pH 10-12 caused complete suppression of the gelation of gelatin. Partial inhibition of the binding capacity of gelatin was evident in alkaline solutions only. This similarity between inhibition of gelation and inhibition of glycogen-binding capacity of gelatins in alkaline solutions only further suggests that the intramolecular sites responsible for gelation and polysaccharide binding of gelatins may be similar.

Summary. Gelatin is capable of binding glycogen and dextran in a ratio of 1:1 on a dry weight basis and various other polysaccharides with α-1, 4 and/or α-1, 6 glucose linkages to a diminished degree. Gammaglobulins also display this property, although to a lesser extent. The glycogen-gelatin complex is stable in 0.5 N HCl, 0.5 N NaOH and 6 M urea for one hour, and to boiling water for 10 minutes. The glycogen-gelatin complex is salted out of TCA-ethanol solutions at a concentration of 3% NaCl. Dilute alkaline $CuCl_2$ solutions and autoclaving of gelatin solutions result in inhibition of glycogen-gelatin complex formation. Autoclaved gelatins exhibit decreased viscosities, with concomitant destruction of the gelatin phenomenon.

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PMS-Induced Ovulation in the Immature Rat Following Unilateral Castration. (30171)

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Unilateral ovariectomy in the mouse or rat results in a compensatory hypertrophy of the remaining ovary(1,2,3,4) and does not appear to affect litter size(1,2,3,5). Evidence indicates that the remaining ovary compensates for loss of follicles resulting from removal of the contralateral gonad by forming a greater number of mature follicles(3,5) and corpora lutea(6). This is confirmed by the observation that a 2-fold increase in ova released from the remaining ovary occurs following unilateral castration(7,8,9,10). It has also been shown that compensatory hypertrophy of the remaining ovary of hemicastrated rats can be blocked by administration of certain steroids. Indeed the unilaterally ovariectomized rat has been suggested as the animal of choice for determination of gonadotropin-inhibiting potency of such hormones (6).

The current investigation was undertaken to examine the effect of unilateral castration on PMS-induced ovulation in the immature rat and to determine whether a constant