Effect of Dilution During Fractionation and Extraction of Serum and Lymph Insulin.* (31580)

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Measurement of serum insulin by immunological methods (immunoreactive insulin, or IRI) yields lower values than those obtained with biological assays (insulin-like activity, or ILA). Many theories on the state of insulin have been derived from this observation and from experiments of serum fractionation and extraction by a great variety of techniques(1).

In this report, insulin in serum and lymph has been studied with gel filtration and acid alcohol extraction procedures. Surprisingly, highly significant rise of ILA was induced simply by dilution with distilled water followed by reconcentration, a step common to both techniques employed here, as well as to many procedures used by others in their studies on insulin and its physiology.

Materials and methods. 1. Collection of serum and lymph. Male Sprague-Dawley rats, weighing between 180 and 200 g, were anesthetized with sodium pentobarbital; lymph was drained from a mesenteric duct, and mixed arterial-venous blood was collected simultaneously from the tail. These specimens were allowed to clot at room temperature for 4 hours, then were centrifuged; the supernatant serum and lymph was collected. Similar amounts of serum and lymph from individual fed and fasted rats were pooled.

Male guinea pigs between 275 and 400 g were decapitated in the morning. Human blood was obtained after an overnight fast by venipuncture.

All samples were kept frozen at −20°C until the day of the assay.

2. Gel filtration technique. Five milliliters of serum or lymph were passed through a Sephadex G 75 (medium grade) column (50 × 3.5 cm) equilibrated with borate buffer (pH 8.0). Two fractions, each of approximately 250 ml, were collected: the "large" protein fraction was a pool of the initial eluates, containing material with an optical density at 280 mμ. The small peptide fraction was a pool of the final eluates. This fractionation lasted 12 hours. Both fractions were then dialyzed in Visking tubing 18/32 against 6 liters of distilled water. The water was changed 3 times over a 24-hour period. After lyophilization, the 2 fractions were either combined or kept separate and dissolved in a buffer with concentrations of cations and anions similar to serum and lymph. The combined fractions were brought to a volume of 5 ml so as to reconstitute the initial serum or lymph. The volume of either the large or small fractions was 2.5 ml. This entire procedure lasted 48 hours and was carried out at 4°C.

To assess the effect of each individual step upon the results obtained with the entire gel filtration procedure, control experiments were conducted with 5 ml of rat serum as follows:

I. Serum was lyophilized and reconstituted with distilled water.

II. Serum was dialyzed against distilled water, lyophilized, and reconstituted with the buffer.

III. Serum was diluted with 500 ml of borate buffer for 12 hours. This procedure was followed by dialysis, lyophilization, and reconstitution, as in control II.

IV. Serum was diluted with 500 ml of distilled water for 12 hours, lyophilized, and reconstituted, as in control I.

3. Acid-ethanol extraction. Guinea pig serum was submitted to a technique adapted from Scott and Fisher(2), in which each ml of serum was extracted in a total volume of 60 ml.

As a control, serum was treated with distilled water in similar proportions. The ex-

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TABLE I. ILA of Serum and Lymph Before and After Gel Filtration and Acid-Ethanol Extraction.*

Animal	Condition	Fluid		Unmodified	After gel filtration C	L	S
Rat	Fed	Pool	Serum	200	—	240	160
			Lymph	50	—	125	45
		Pool	Serum	190	480	280	65
			Lymph	55	200	50	90
		Pool	Serum	270	600	280	105
			Lymph	130	1080	455	160
	Fast 24 hr	Pool	Serum	120	—	600	600
			Lymph	80	—	400	550
	Fast 48 hr	Pool	Serum	180	—	760	760
			Lymph	90	—	1040	600
	Fast 4 days	Pool	Serum	135	680	290	150
			Lymph	40	480	290	145
Guinea pig	Fed	Pool	Serum	30	—	30	160
				Unmodified	After acid-ethanol extraction		
Guinea pig†	Fed	Individual sera		30	220 ± 40		

* Values in μU/ml.

† Mean ± standard error of mean (N = 9).

tracted and diluted sera were dialyzed, lyophilized, and reconstituted with the buffer.

4. Assay of insulin. ILA was measured by the assay described by Renold *et al*(3), adapted to scintillation counting; results were expressed in equivalents of crystalline pork insulin as microunits per ml. IRI was determined by the double antibody technique of Morgan *et al* modified by Soeldner *et al*(4).

Results. The effect of the gel filtration procedure on rat serum and lymph ILA, and the effect of acid-ethanol extraction on guinea pig serum ILA are shown in Table I. The values of ILA after gel filtration were obtained with combined fractions (C) or large (L) and small (S) fraction assayed individually.

Both the gel filtration and acid-ethanol extraction induced a large increase in ILA.

The steps of the gel filtration technique were analyzed for their individual effect upon the observed changes of ILA.

No significant changes in ILA were observed when serum was merely reconstituted after lyophilization (control I) or after water dialysis and lyophilization (control II). On the contrary, when serum was diluted for 12 hours in borate buffer (control III) or in distilled water (control IV) and then reconstituted, a significant rise in ILA was observed, similar to the rise observed after gel filtration.

During the acid-ethanol extraction of guinea pig serum, a dialysis against distilled water was performed with a dilution of 1/60. As a control, distilled water was substituted for acid-ethanol; ILA in this control was found to be as elevated as in the extracted serum. These control data are not shown.

The simple procedure of dilution with distilled water followed by reconcentration was also applied to human serum. The results obtained with serum dilution-reconcentration experiments are summarized in Table II. The effect of different dilutions was assessed as follows: a pool of rat serum with a known insulin-like activity of 340 microunits per ml of unmodified serum was diluted with distilled water 1/10, 1/100, 1/1000; values of ILA in the reconcentrated samples were respectively 800, 880, and 1080 μU/ml.

For every sample in which ILA was measured, the corresponding level of IRI was also determined. Neither gel filtration nor acid-ethanol extraction nor any of the steps that formed part of these procedures, significantly altered the amount of IRI when compared to the untreated sample.

Discussion. The increase of ILA by gel filtration of rat serum and lymph or by acid-ethanol extraction of guinea pig serum can similarly be obtained by simple dilution-reconcentration, a step common to both procedures. Therefore, an increment in ILA is

TABLE II. Serum ILA Before and After Water Dilution-Reconcentration.*

Source	Condition	Fluid	Unmodified	After dilution-reconcentration
Rat	Fed	Pool serum 1	280	1250
		2	340	880
		3	380	500
		4	75	500
Guinea pig	Fed	Individual sera	<30	450
			<30	300
			<30	500
			<30	500
			<30	250
			<30	500
Human	Overnight fast	Individual sera	33	135
			90	300

* Results in μU/ml.

to be anticipated whenever diluted serum is dialyzed against distilled water, as occurs in the course of fractionation or extraction procedures. The literature supports these data. ILA, measured by the rat fat pad method in serum protein fractions prepared by electrophoresis (polyvinyl chloride block), is higher than ILA in the corresponding untreated serum(5,6). A similar effect can be noted in other studies, regardless of the fractionation technique or of the bioassay employed(7,8,9).

Extraction of insulin from serum also results in an increment of ILA(10,11). It appears that all techniques of fractionation or extraction involve dilution of serum, serum eluates, or serum protein fractions. Therefore, this discussion will be limited to those aspects of insulin in biological fluids as influenced by the dilution-reconcentration procedure; this procedure could increase serum and lymph ILA by many possible mechanisms:

1. Inactivation of an insulin inhibitor could occur by dilution. This seems unlikely, for no insulin antagonist with an effect on rat epididymal fat is presently known in normal serum, as shown by adequate recovery of crystalline insulin added to serum.

2. Trace elements in distilled water, which would enhance the glucose uptake by the rat epididymal fat tissue: serum dilution at 1/10, 1/100, 1/1000 yields a similar increment in ILA; and, therefore, this hypothesis can be ruled out.

3. Transformation of IRI into other forms of insulin which are immunologically non-detectable but biologically active: any increment in serum or lymph ILA should therefore be associated with a decrease in IRI, which was not observed.

4. Presence of "hidden" ILA in biological fluids, which could be unmasked by dilution: if this were true, the physiological role of such an insulin could be questioned, for no similar dilution occurs *in vivo*.

5. Alteration of circulating proteins by dilution and formation of new compounds with ILA. No data are available concerning the possible alteration of proteins and peptides by dilution with distilled water. Nevertheless, this possibility should be kept in mind in view of the lack of specificity of the insulin bioassays.

Summary. An increase in ILA has been shown after gel filtration of rat serum and lymph and after acid-alcohol extraction of guinea pig serum. This effect was reproduced simply by a dilution-reconcentration step, common to both procedures. It is conceivable that a similar step is responsible for the increment of ILA observed by other investigators using many different techniques. The mechanism by which the dilution-reconcentration procedure enhances ILA is not clear; its physiological role, if any, is dubious, and theories on the state of insulin should be formulated with caution.

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Effect of Hydrocortisone on Secretion of Acid and Pepsin by Heidenhain Pouches.* (31581)

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In a previous study(1) in dogs with Heidenhain pouches, bilateral adrenalectomy reduced significantly gastric acid secretion stimulated by histamine. Small doses of hydrocortisone not only restored acid output to normal but caused a transient elevation to levels above pre-adrenalectomy control. Subsequently acid output returned within the range of pre-adrenalectomy control and remained at this level. This study was undertaken to determine the effect of hydrocortisone on gastric secretion in dogs with their adrenals intact.

Materials and methods. Three mongrel dogs weighing between 14 and 18 kg were used. A pouch of the oxyntic gland area (Heidenhain) was made in each animal and a Gregory cannula(2) was inserted. Studies were started 3 weeks later.

The animals were fasted 18 hours before each test. A continuous intravenous infusion (30 ml/hr) of 0.15 M NaCl was given throughout each experiment. Gastric juice was collected for two 15-minute periods to obtain basal levels of secretion. At the end of this period either gastrin or histamine was added to the saline to give desired dosage. Doses were doubled every 60 minutes for histamine and every 75 minutes for gastrin.

Gastric juice was collected by gravity drainage every 15 minutes. The volume was recorded to the nearest 0.1 ml and the acid concentration determined by titrating 0.2 ml of juice with 0.2 N NaOH to pH 7 using a glass electrode and an automatic titrator (Radiometer, Copenhagen). Pepsin activity was determined by the method of Grossman and Marks(3).

Gastrin extracts were prepared from the mucosa of the pyloric gland area of the hog stomach by the method of Gregory and Tracy (4) carried only through the stage of fractionation with isopropanol. All the gastrin used was from the same batch. Doses of gastrin are expressed in terms of the wet weight of mucosa from which the extract was obtained. Histamine doses are recorded in terms of the dihydrochloride salt.

After control studies, each animal was given 50 mg of hydrocortisone intramuscularly every day and secretory responses to gastrin and histamine again determined.

Results. Effect of hydrocortisone on histamine-stimulated acid and pepsin secretion. During control studies, as the acid output increased with increasing doses of histamine, the pepsin concentration decreased (Fig. 1). Maximal pepsin output occurred with a dose of histamine (1.5 mg/hr) that was submaximal for acid output.

During the period when hydrocortisone was being given the mean maximal acid output

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