Distribution of Zn^{65} 48 h following i.p. injection

Tissue	Cpm per 2.6×10^6 cells[a]		Cpm per 10^9 cells[a]
Mesenterial		0	22.5
lymph nodes		0	12.9
		0	12.0
Thymus		0	24
		0	15
		0	14
Spleen		0	18
		0	29
		0	18
Liver		0	76
		0	53
		0	42
Pancreas		0	22
		0	21
		0	39
Lungs		0	21
		0	30
		0	28
Peritoneal cells (mast cells)	0.011×10^9[b] (0.5×10^6)[c]	70	6,400
	0.013×10^9[b] (0.7×10^6)[c]	69	5,300
	0.014×10^9[b] (0.7×10^6)[c]	68	4,800
Isolated mast cells		38	14,600

[a] The values are corrected for background. [b] Total number of peritoneal cells. [c] Number of peritoneal mast cells in brackets.

To find out whether zinc is released from mast cells under conditions known to cause a release of histamine, rats which had been given 50 μC Zn^{65} i.p. 48 h earlier, were treated with the chemical histamine-liberator, compound 48/80 (400 μg/animal i.p.); other animals received phosphate-buffered saline i.p. The peritoneal fluid was harvested 15 min later, and radioactivity of dried supernatants measured. In controls (672 ± 350 cpm), radioactivity was found to be clearly lower than in animals treated with compound 48/80 (2908 ± 663). The present results suggest that radioactive zinc is incorporated selectively into rat tissue mast cells. And significant amounts of zinc were released by the chemical histamine-liberator, compound 48/80. However, the mechanism of binding of zinc to mast-cell granules, and its role and mode of mobilization in pathophysiological processes needs further elucidation.

Zusammenfassung. Bei der Albinoratte findet sich 48 h nach parenteraler Verabreichung von radioaktivem Zink (Zn^{65}) ein hoher Anteil der Aktivität in den Gewebemastzellen. Nach Verabreichung des chemischen Histaminliberators, Compound 48/80, wird ein erheblicher Teil von Zn^{65} aus den Zellen frei.

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Davos (Switzerland), 30 September 1969.

Collagenolytic Enzymes in Human Serum

The search for collagenases in the tissue of higher animals has led to the isolation of several proteases with acidic and neutral pH optima[1]. Gries and Lindner[2,3] could demonstrate that various mammalian tissues contain enzymes that are capable of splitting soluble peptides from native insoluble collagen under physiological conditions. Animal collagenases that have been shown to cleave the collagen molecule into 2 pieces, representing three-quarters and one-quarter of the molecule, were found some years ago for the first time by Gross et al.[4] and later by Fullmer et al.[5], Evanson et al.[6], Lazarus et al.[7], and Eisen et al.[8].

A new class of collagenolytic enzymes, which are different from the enzymes mentioned above, and which resemble bacterial collagenases in their specificity for the apolar regions of the collagen molecule, have recently been demonstrated by Strauch et al.[9–11] in living cells of higher animals and in invasion zones of human tumuors[12]. The activity of these enzymes was measured by means of the synthetic substrate PZ-Pro-Leu-Gly-Pro-D-Arg (PZ = *p*-phenylazobenzyloxycarbonyl-), recently developed by Wünsch and Heidrich[13].

This substrate, which has a sequence analogous to the repeating sequence -Gly-Pro-X-Gly-Pro-X- (Pro is proline or hydroxyproline and X a variable amino acid) present in the apolar regions of collagen molecule, is split by a collagenase of the bacterial type between the Leu- and Gly-residues. The fragment PZ-Pro-Leu is insoluble in aqueous solutions at acid pH and can be extracted from the reaction mixture with organic solvents and determined spectrophotometrically. The C-terminal arginine makes the peptide easily soluble in water and the D-form makes the compound stable against attack by trypsin, carboxypeptidase and other cell proteases. The substrate was found to be non-toxic for the cells at the concentrations required for the determination. The method is very sensitive and allows the measurement of extremely low collagenolytic activity.

The isolation, the mode of action, and the properties of animal collagenases with the specificity for apolar regions of the collagen molecule will be published else-

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where. This paper reports the investigation of such enzymes in human serum with the aid of the synthetic substrate PZ-Pro-Leu-Gly-Pro-D-Arg.

The incubation of serum with the substrate in a *Tris* buffer of pH 8.0 resulted in splitting of the collagenase-specific bond. The expected PZ-Pro-Leu fragment was identified by thin-layer chromatography on silica gel.

Enzymatic activity showed a linear pattern at 25 °C. At 37 °C a descending curve was obtained, probably caused by denaturation or inactivation of the enzyme. Raising the temperature of the serum to 56 °C led to a rapid loss of enzymatic activity. Leaving the serum at room temperature for several hours likewise resulted in loss of enzymatic activity.

The pH dependence of the splitting rate in the Figure (the latter expressed in μM PZ-Pro-Leu/l of serum/h) shows 2 distinct peaks, the first between pH 7.0 and 7.3, and the second between pH 8.0 and 8.2. It is interesting to note that the location of the peaks remains the same for different serum samples, while their height varies. Serum 1 exhibits a low pH optimum at pH 7.0 and a high optimum at pH 8, whereas the opposite is the case for serum 2. We also found sera in which the 2 pH peaks were just as much distinct but were both at the same level. This indicates the presence of 2 enzymes in the serum and corresponds to our other findings[14] on the enzymatic activity in tissue.

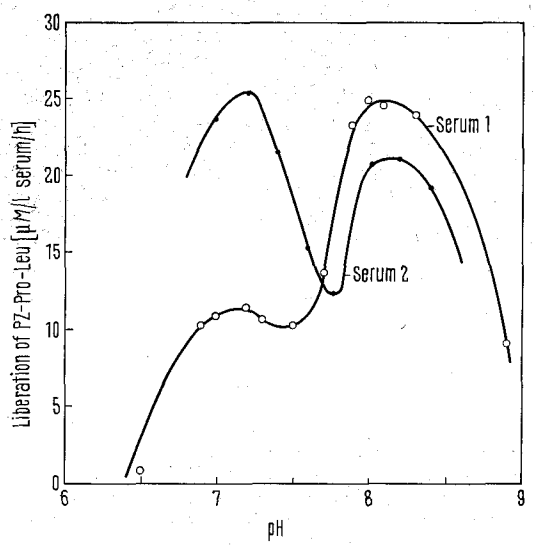
Up to a concentration of 0.5 μM of substrate per sample the substrate concentration is the limiting factor of enzymatic activity. Above 0.5 μM of substrate per preparation, the point of complete saturation of the enzyme with the substrate is reached and the reaction is proportional to the enzyme quantity.

In the light of the above findings we developed the following method of determining the collagenase activity in serum: 1.0 ml of 0.05 *M Tris*-HCl buffer pH 7.2 or pH 8.0 and 1 ml of substrate solution (1.0 μM/ml – 0.812 mg PZ-Pro-Leu-Gly-Pro-D-Arg/ml[15]) in the same buffer solution were added to 0.5–1.0 ml of serum, and the same was incubated for 1 h at 25 °C. Then the reaction was stopped by adding 10% (w/v) citric acid to a pH of approximately 3. The quantity of citric acid required for 1.0 ml of serum at pH 7.2 was 1.0 ml, and for 1.0 ml of serum at pH 8.0, 1.5 ml. The PZ-Pro-Leu fragment split off by the collagenase was extracted from the sample by shaking it with 5.0 ml benzene. After centrifugation for several minutes at about 1000 *g* the organic phase was pipetted off and, if not yet clear, passed through a plaitet filter. The concentration of the PZ-Pro-Leu produced by the action of the enzyme was determined by measuring the adsorption at 320 nm or 440 nm against a control consisting of a sample treated in the same manner described above but without incubation at 25 °C. The concentration of PZ-Pro-Leu was read off from a previously prepared measuring diagram. (PZ-Pro-Leu in μM/5 ml benzene.)

We define 1 collagenase unit (CU) as the quantity of 1 μM PZ-Pro-Leu/l of serum/h, and express the activities measured at pH 7.2 by $CU_{7.2}$ and at pH 8.0 by $CU_{8.0}$. The collagenase activities determined on samples from a number of healthy persons are included in the Table.

Our findings have shown that the use of synthetic substrates of the -Gly-Pro-X-Gly-Pro-X- type provides a suitable means for the rapid, simple, and highly sensitive determination of collagenolytic enzymes.

We hope that this procedure will contribute to the determination of such enzymes in clinical laboratories to get insight into the metabolism and degradation of collagen in the human body.



Dependence of splitting rate on pH. 1.0 ml of serum incubated with 1 μM PZ substrate in 0.05 *M Tris* buffer at 25 °C for 1 h. Each of the measuring points represents the mean value obtained from 6–8 tests.

Collagenolytic activity in sera of normal persons

Name	Sex	Age	$CU_{7.2}$	$CU_{8.0}$
Ke	♀	20	24.9	24.3
Bu	♀	25	21.2	7.4
Li	♀	28	11.1	11.6
Ac	♀	30	21.2	23.9
St	♀	34	33.5	19.2
Ho	♀	39	12.2	6.7
Go	♀	42	26.7	35.6
Gr	♀	42	11.2	15.1
He	♀	46	33.2	24.9
Be	♂	26	23.1	29.7
Wi	♂	27	22.7	28.4
Go	♂	27	–	9.1
De	♂	27	14.8	9.0
Re	♂	28	55.8	35.8
De	♂	28	72.4	22.3
Ch	♂	29	19.4	16.8
Sl	♂	30	25.9	32.0
Ro	♂	31	24.8	41.3
Kn	♂	31	31.2	18.1
Be	♂	31	17.9	16.4
Be	♂	33	29.2	24.0
Wi	♂	36	23.4	23.2
Ge	♂	38	25.5	27.8
Be	♂	39	–	14.5
St	♂	42	29.5	46.5
Gr	♂	46	23.5	21.1

[14] D. PROKOPOVA, L. STRAUCH and K. HANNIG, in preparation. – G. GRIES and J. LINDNER, in preparation.

[15] Manufactured by Fluka AG, CH-9470 Buchs, SG (Switzerland).

Zusammenfassung. Mit Hilfe des synthetischen Kollagenasesubstrates PZ-Pro-Leu-Gly-Pro-D-Arg wurden im humanen Serum kollagenolytische Enzyme nachgewiesen, deren Spezifität der Clostridiopeptidase A entspricht. Die Spaltungsgeschwindikeit zeigt in Abhängigkeit von pH ein Optimum bei pH 7,2 und bei pH 8,0. Das spricht für die Existenz zweier Enzyme. Zur quantitativen Bestimmung der Enzymaktivitäten wurde eine Methode ausgearbeitet, mit der eine Reihe von normalen humanen Seren analysiert wurde.

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A Sensitive Bioassay for Corticosteroids

Several authors[1-4] reported that the duodenum of the chick embryo is a target organ of adrenocortical steroids.

In the present paper, a bioassay for the quantitative estimation of corticoids in submicrogram amounts has been designed on the basis of the histological changes already described by Hayes[5] on the duodenal mucosa of 16-day-old chick embryos cultivated with cortisone. The results indicate that this method can be employed with advantage in a tissue culture laboratory, for it combines specificity, simplicity, a good precision and simple equipment.

Material and methods. The duodenum of chick Hy-line embryos at 16 days of development was aseptically dissected, and cut longitudinal and transversally in order to obtain pieces of 0.5 mm². They were explanted according to the Wolff and Haffen's technique[6], placing the muscular face down on the medium. The culture was obtained by mixing 1 vol. of embryonic extract (from 9-day-old chick embryos), 1 vol. of Eagle's medium with the addition of lactoalbumin hydrolysate (0.61% w/v), and 2 vol. of agar (1% w/v) in Hank's solution. The explants were cultivated during 48 h at 37 °C, with air as the gas phase.

The following compounds were assayed: corticosterone, cortisol, 11-desoxy corticosterone (DOC), aldosterone, 17-hydroxy 11-desoxy corticosterone (compound 'S'), estradiol, testosterone, adrenaline hydrochloride and noradrenaline bitartrate.

At the end of incubation period, the explants were fixed in Bouin's fluid for 24 h, paraffin embedded, serially cut at 6 μ and stained with hematoxilin-eosin, for the histological study. A series of sections of each explant was performed and one showing a cut perpendicular to the mucosal surface was selected. In this section, the height of the mucosa, taken from the limit with the muscular layer to the free border of the villi, was measured in 4 different zones, with the aid of a reticulated eyepiece; the average reading was computed as the final height of the duodenal mucosa (Figure 1).

Standard statistical methods were employed for the evaluation of the data[7,8].

Results. The duodenum explants treated with corticosterone and cortisol exhibited edema in the connective tissue of the mucosa and dilated 'vascular channels' in the deepest layer of the mucosa near the limit of the muscular layer (Figure 1). Aldosterone and DOC produce edema, but not very evidently the aforementioned 'vascular channels'. In both cases the histological modifications were registered as an increase in the height of the mucosa. Figure 2 shows the linear response of duodenal height to corticosterone in doses ranging from 0.015 to 8.0 μg/ml. (F for linearity: 1.77; $p > 0.05$.) An index of precision (λ) equal to 0.22 was calculated with the means of 35 explants for each dose. Cortisol (Figure 2), aldosterone and DOC (Figure 3) also exhibited linear responses in the dosage range studied. The slopes obtained for these compounds did not differ significantly from that obtained with corticosterone (F: 1.27; $p > 0.05$). Each figure shows also the dose which produces the maximal effect; 0.5 μg/ml for cortisol aldosterone and D.O.C. and 2 μg/ml for cortisol. Corticosterone was still active at 8.0 μg/ml. Within the range of linear response, corticosterone, aldosterone and cortisol are equally active, while DOC is 4 times less potent than any one of the other compounds.

In 9 different experiments the slopes (b) of the dosage-response curves for corticosterone ranged between 0.07 and 0.11.

Fig. 1. Section of a chick embryo duodenum at 16 days of development cultivated in a medium containing corticosterone (4 μg/ml) for 48 h. Hematoxylin-eosin. × 150.

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