A Hog Kidney Aminopeptidase Liberating N-Terminal

Dipeptides. Partial Purification and Characteristics

V. K. HOPSU-HAVU, P. RINTOLA and
G. G. GLENNER*

Department of Anatomy, University of Turku, Finland and National Institutes of Health,
Bethesda, Maryland, U.S.A.

An aminopeptidase capable of liberating N-terminal dipeptides was purified from hog kidney microsomes. The purification procedure consisted of solubilization by autodigestion and precipitation with ammonium sulphate followed by an ion exchange chromatography on DEAE cellulose. The molecular weight of the enzymic protein was estimated to be 270 000. The enzyme was optimally active at pH 7.6—7.8 and liberated glycyl-proline from glycyl-DL-prolyl-β-naphthylamide and from glycyl-γ-prolyl-L-alanine as well as from glycyl-γ-prolyl-glycyl-glycine. No glycine was liberated. Alanyl-alanine was liberated from L-alanyl-DL-alanyl-β-naphthylamide at a low rate. Amino acid naphthylamides were not hydrolyzed. The enzymic activity was not effected by sulfhydryl compounds or reagents, or by several metal ions or chelators except at a very high concentration.

D uring our earlier studies on the enzymes hydrolyzing amino acid naphthylamides in hog kidney, antiserum was prepared against a preparation solubilized from hog kidney microsomes and lysosomes. Following immuno-electrophoresis with the antigenic material this antiserum gave several precipitin lines with enzymic activities. One of the precipitin lines appeared to possess no hydrolytic activity toward monoamino acid naphthylamides but hydrolyzed readily L-alanyl-DL-alanyl-β-naphthylamide.[1] Since no hydrolysis of CBZ-L-alanyl-DL-alanyl-β-naphthylamide could be demonstrated the enzyme was believed to be an aminopeptidase with unique substrate characteristics.

We have since been able to demonstrate in a commercial enzyme preparation (Acylase I, Sigma) and in rat liver the presence of an aminopeptidase capable of hydrolyzing another dipeptide naphthylamide, glycyl-DL-prolyl-β-naphthylamide, liberating glycyl-proline and naphthylamine.[2] This enzyme did not hydrolyze monoamino acid naphthylamides or dipeptide naphthyl-

amides with a substituent in the α-amino group. It hydrolyzed several tri-peptides and polypeptides, liberating the N-terminal dipeptides, but did not hydrolyze dipeptides further to the individual amino acids. This enzyme was recently purified from rat liver microsomes and characterized.[3] The purpose of the present study was to determine whether the enzyme could be purified from hog kidney tissue following the usual procedures employed for enzyme purification. It was also of importance to ascertain whether the enzyme found to hydrolyze alanyl-DL-alanyl-β-naphthylamide in the immunoelectrophoretic gel might be identical with the enzyme hydrolyzing glycyl-DL-prolyl-β-naphthyl-amide. This possibility was suggested by the fact that the latter enzyme from rat liver was found also to hydrolyze slowly L-alanyl-DL-alanyl-β-naphthyl-amide.

MATERIAL AND METHODS

Substrates and the activity assay. Naphthylamides of amino acids (L-configuration) were obtained from Sigma Chemical Co. and the naphthylamides of di- and tripeptides were synthesized as previously noted.[2,4,5]

The composition of the reaction mixture was: Buffer 1.0 ml (usually 0.2 M Tris-HCl pH 7.8), substrate solution 0.25 ml (usually 1 mM), enzyme solution 0.25 ml (diluted suitably for measurement of the initial reaction velocity). Incubation was per-formed in a water bath at 37°C for 15 min to 1 h after which 0.5 ml solution of diazonium salt fast Garnet GBC (E. Gurr Ltd., London) was added (0.5 mg/ml in 1 M acetate buffer pH 4.2 containing 10 % Tween 20). The azo dye colour was measured with a Klett-Summerson colorimeter using filter No. 52. A modified Bratton-Marshall method was used instead of diazonium coupling in the presence of reducing substances in the reaction mixture.

The hydrolysis of the nonchromogenic peptide substrates glycyl-DL-prolyl-L-alanine and glycyl-prolyl-glycyl-glycine was tested qualitatively using paper chromatography.

Preparation and fractionation of the homogenate. In the initial steps of the fractiona-tion the method of De La Haba *et al.*[6] for purification of cathepsin C was followed.

Fresh hog kidneys were frozen at −30°C overnight, thawed at room temperature and homogenized with a Waring Blendor at 5°C for 1 min (50 g/100 ml distilled water). The homogenate was frozen at −30°C overnight, thawed, rehomogenized as before and centrifuged at 7500 g for 15 min at 2°C. The supernatant was adjusted to pH 3.8 with 6.3 N sulfuric acid and incubated at 37°C for 18 h with constant mixing. The mixture was centrifuged at 25 000 g for 15 min at 2°C and a sample from the supernatant was designated Sample I.

The proteins precipitated from the supernatant using ammonium sulphate (overnight) between 50−85 % saturation were collected and dissolved in 10 ml of 0.01 M Tris-HCl buffer pH 7.2. This fraction was labeled Sample II.

Desalting and exchange of the buffer was performed in a column of Sephadex G-25 (details given later) after which the preparation was subjected to gel filtration in a column of Sephadex G-200 (active fractions pooled, Sample III) or to chromatography in a column of DEAE-cellulose using for elution a continuous linear gradient of NaCl. The enzymically active fractions were pooled and designated Sample IV.

Column chromatography. DEAE-cellulose was obtained from W & R. Balston, England. Column 1.5 × 23 cm, running buffer 0.01 M Tris-HCl pH 7.2, salt gradient 0−0.3 M, fraction volume 3.0 ml, temperature 4°C.

Gel filtration. Sephadex G-25, G-100, and G-200 were from Pharmacia, Uppsala. Column 2.5 × 76 cm, buffer 0.1 M Tris-maleate pH 7.6, fraction volume 5.0 ml, tempera-ture 4°C.

Modifiers. Metal salts (chlorides) and chelators were obtained from E. Merck, SH-inhibitors from A.G. Fluka, E 600 (diethyl-*p*-nitrophenyl phosphate, Mintacol ®) from Bayer, and DFP (di-isopropylphosphofluoridate) from Sigma Chemical Company.

Determination of the molecular weight. Estimation of the molecular weight was carried out using gel filtration in Sephadex G-200. The column was 2.5 × 78 cm and other conditions were the same as during the purification of the enzyme. Egg albumin, crystallized, lyophilized, salt free, M.W. 45 000 (Nutr. Biochem. Corp.), bovine serum albumin, crystalline, M.W. 70 000 (Sigma Chemical Company), γ-globulin, crystalline, M.W. 160 000 (Koch-Light Lab. Ltd.), bovine thyroglobulin, M.W. 650 000 (Sigma Chemical Co.) and Blue-Dextran M.W. over 2 million (Pharmacia) were used as standards for comparison.

Paper chromatography. The reaction products from the naphthylamide substrates as well as from the non-chromogenic peptide substrates were identified using ascending paper chromatography with known peptides and amino acids as standards. This was carried out with Whatman No. 1 paper, and butanol:acetic acid:water (50:30:20) as solvent.

Immunoelectrophoresis. This was carried out according to Grabar and Williams [7] and the immunoserum was that used earlier.[1,8] The plates containing the immuno-precipitin lines were immersed in a solution containing Gly-Pro-NA or Ala-Ala-NA (0.1 mg/ml) as substrate and fast Garnet GBC (0.1 mg/ml) as coupler in 0.1 M Tris-HCl buffer pH 7.0. Enzymic hydrolysis of the substrate was seen as red staining of the precipitin lines resulting from the insoluble azo colour produced by diazo coupling of the naphthylamine liberated.

Protein determination. This was carried out according to Lowry *et al.*[9] using bovine serum albumin (Sigma Chemical Co.) as standard.

Enzyme unit. One unit of enzyme is the amount of enzyme capable of liberating one μmole NA/min at pH 7.8 and 37°C.

RESULTS

It was shown by separate experiments that the enzyme hydrolyzing Gly-Pro-NA is bound mainly to the microsomes and, therefore, purification was initiated only after autodigestion which liberated the enzyme from the particles. Numerical values throughout the purification procedure are given in Table 1.

Solubilization of the enzyme during autodigestion. It was demonstrated earlier that liberation of the aminopeptidase hydrolyzing amino acid naphthylamides takes place during autodigestion of the homogenate.[8] Therefore, the liberation of the enzyme hydrolyzing Gly-Pro-NA as well as of the amino-peptidase hydrolyzing Ala-NA was followed during the autodigestion procedure. In this experiment the supernatant was separated by centrifugation at 25 000 *g* for 30 min and the enzymic activities in the supernatant as well as

Table 1. Purification of the enzyme hydrolyzing Gly-Pro-NA.

	ml	Units ml	Amount units	Protein (mg/ml)	Protein (mg)	Specific activity	Recovery (%)	Purification coefficient
Sample I	170	0.58	98.5	10.0	1700	0.058	100	1
Sample II	10	9.60	96.0	7.2	72	1.333	97	23
Sample III (G-200)	100	0.64	64.0	0.05	5	12.800	65	221
Sample IV (DEAE)	52	0.26	13.5	0.03	1.6	8.450	14	146

Table 2. The rate of liberation of the aminopeptidase hydrolyzing Ala-NA and that hydrolyzing Gly-Pro-NA during autodigestion of the particle preparation.

		Ala-β-NA as substrate		
		Supernatant	Sediment	Total activity
Incubation	0 h	0.11 units/ml	0.13 units/ml	0.24 units/ml
	1/2 »	0.12 »	0.10 »	0.22 »
	1 »	0.12 »	0.10 »	0.22 »
	6.5 »	0.52 »	0.07 »	0.59 »
	18 »	0.71 »	0.03 »	0.74 »

		Gly-Pro-β-NA as substrate		
		Supernatant	Sediment	Total activity
Incubation	0 h	0.09 units/ml	0.07 units/ml	0.16 units/ml
	1/2 »	0.09 »	0.07 »	0.16 »
	1 »	0.09 »	0.07 »	0.16 »
	3 »	0.34 »	0.05 »	0.39 »
	6.5 »	0.49 »	0.03 »	0.52 »
	18 »	0.63 »	0.02 »	0.63 »

in the precipitate were determined (Table 2). It is seen that both of the enzymes are liberated to the same extent and simultaneously both of them also are markedly activated. This result demonstrates that autodigestion is a suitable step for purification of these enzymes.

Precipitation with ammonium sulphate. It was shown that the precipitate obtained at 50 % saturation with ammonium sulphate contained little enzymic activity toward Gly-Pro-NA and that a practically complete precipitation of the enzymically active protein was accomplished at 85 % saturation. Therefore, these limits could be used for separation of the active enzyme (Sample II).

Gel-filtration on Sephadex G-200. Fig. 1 gives the result obtained by gel filtration. The enzyme active toward Gly-Pro-NA as well as that active
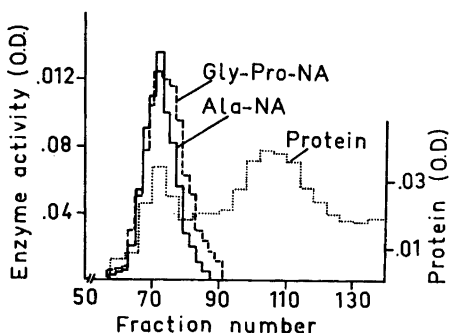

Fig. 1. Gel filtration on Sephadex G-200 of the enzymically active protein obtained by fractional salt precipitation with ammonium sulphate between 55 and 85 % saturation. Gly-Pro-Na, Ala-NA = Enzymic hydrolysis of Ala-NA. For test conditions see Material and Methods.
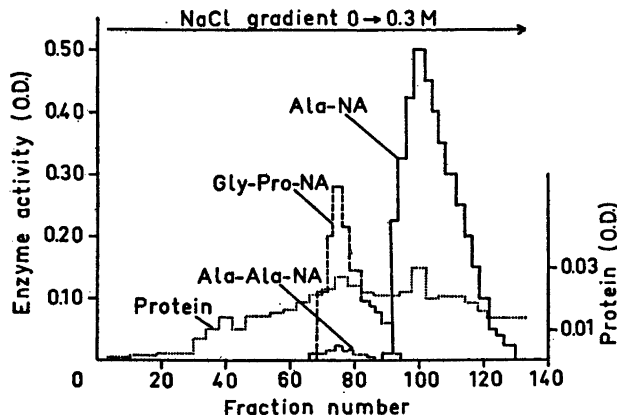
Fig. 2. Column chromatography on DEAE-cellulose of the protein precipitated by ammonium sulphate between 55 and 85 % saturation. For test conditions see Material and Methods.

toward Ala-NA were both eluted in the first protein peak. Therefore, this procedure was not capable of separating the two enzymic activities. Fractions 67—80 were combined for further studies (Sample III).

Column chromatography on DEAE-cellulose. Result of the chromatography of Sample II is shown in Fig. 2. It is seen that the peak activity hydrolyzing Gly-Pro-NA is clearly separated from that hydrolyzing Ala-NA. It also appears that Ala-Ala-NA was hydrolyzed in the same fractions as Gly-Pro-NA. Fractions 70—82 were pooled for further studies. This pooled preparation was used in the following experiments to characterize the enzyme capable of hydrolyzing Gly-Pro-NA (Sample IV).

Characteristics of the enzyme preparation

Molecular weight. The V_e/V_o (elution volume/void volume) of the proteins used as standards and of the enzyme protein is plotted against the log of the molecular weights of the proteins in Fig. 3. It is seen that the relationship is not linear at the range of the molecular size of the present enzyme protein. Therefore, no exact data can be obtained. The molecular weight calculated from these data is about 270 000.

pH-optimum. Gly-Pro-NA was hydrolyzed by the enzyme preparation optimally at pH 7.6—7.8 as seen in Fig. 4.

Substrate specificity. Results of the hydrolysis of several aminoacid and peptide naphthylamides are given in Table 3. It appears that Gly-Pro-NA was the substrate hydrolyzed most rapidly and the substrates hydrolyzed at a slow rate were Ala-Ala-NA and Leu-Leu-NA. The others were not hydrolyzed detectably under the present conditions.
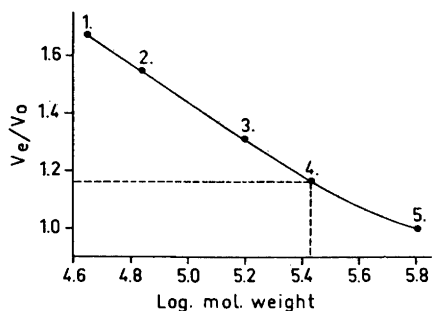
Fig. 3. Whitaker plot of data on gel filtration of the enzymic protein and of the standard proteins on Sephadex G-200. 1 = egg albumin, 2 = serum albumin, 3 = γ-globulin, 4 = purified enzymic protein, 5 = thyroglobulin.
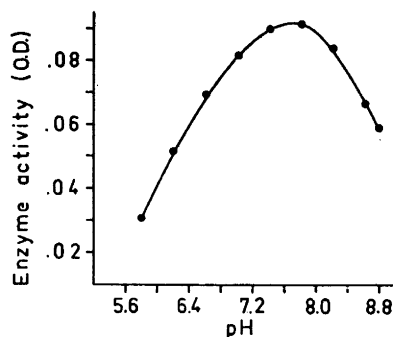
Fig. 4. pH-Dependence of the hydrolysis of Gly-Pro-NA by the purified enzyme preparation. For test conditions see Material and Methods.

With paper chromatography it was shown that Gly-Pro was liberated from Gly-Pro-NA and Ala-Ala from Ala-Ala-NA while no liberation of free amino-acids could be demonstrated. The liberation of Gly-Pro and Ala from Gly-Pro-Ala was demonstrated chromatographically as shown in Fig. 5. It was also shown that Gly-Pro-Gly-Gly was split to Gly-Pro and Gly-Gly by the same enzyme preparation. No free glycine could be demonstrated.

Determination of K_m. The K_m-values for the hydrolysis of Gly-Pro-NA and of Ala-Ala-NA by the enzyme were determined. Lineweaver-Burk plots of the data are given in Fig. 6 for Gly-Pro-NA and in Fig. 7 for Ala-Ala-NA. The K_m-value for Gly-Pro-NA was found to be 0.3 mM and for Ala-Ala-NA 1.3 mM.

Effect of modifier substances. The effect of several modifier substances on the enzymic hydrolysis of Gly-Pro-NA by the enzyme preparation was tested.

Table 3. The rate of hydrolysis of several amino acid- and di- or tripeptide naphthylamides by the enzyme preparation.

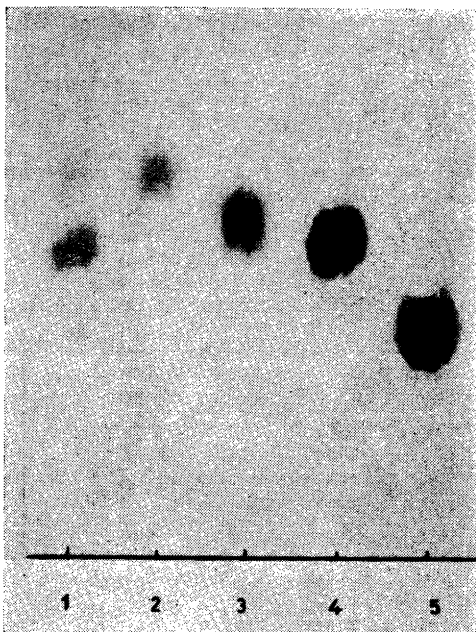| Substrate	Hydrolysis rate (μmole NA/ml/min)	Substrate	Hydrolysis rate (μmole NA/ml/min)
L-AlA-NA	0	L-His-NA	0
L-Ala-DL-Ala-NA	2	L-Leu-Leu-NA	1
Cbz-Ala-DL-Ala-NA	0	L-Norleu-NA	0
L-Cys-NA	0	L-Phe-NA	0
α-L-Glu-NA	0	L-Pro-NA	0
γ-L-Glu-NA	0	L-Thr-NA	0
Gly-L-Phe-Na	0	L-Try-NA	0
Gly-L-Pro-NA	44	L-Tyr-NA	0
Gly-L-Ala-DL-Ala-NA	0	L-Val-NA	0

Fig. 5. Paper chromatographic analysis of the reaction products of the hydrolysis of Gly-Pro-Ala by the enzyme preparation. Sample 50 µl. 1 = Reaction mixture (incubated), 2 = Gly-Pro-Ala, 3 = Gly-Pro, 4 = Ala, and 5 = Gly.
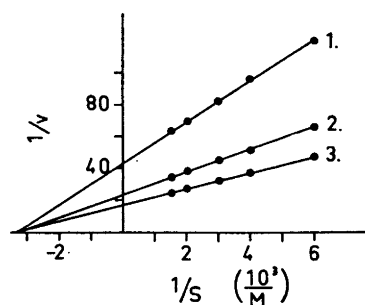

Fig. 6. Lineweaver-Burk plots of the hydrolysis of Gly-Pro-NA by the enzyme preparation. 1 = incubation time 10 min, 2 = incubation time 15 min, and 3 = incubation time 20 min. For test conditions see Material and Methods.

The enzyme was first preincubated in the presence of the modifier ion for 15 min before the addition of substrate. The results are presented in Table 4. It is evident that none of the modifiers tested had any marked effect on the enzyme, although EDTA at a concentration of 0.01 M did produce an inhibition of 40 %.
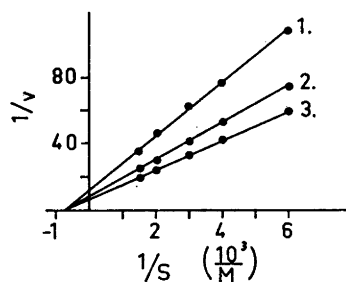
Fig. 7. Lineweaver-Burk plots of the hydrolysis of Ala-Ala-NA by the enzyme preparation. 1 = incubation time 10 min, 2 = incubation time 15 min, and 3 = incubation time 20 min. For test conditions see Material and Methods.

Table 4. Effect of several modifier agents on the hydrolysis of Gly-Pro-NA by the enzyme preparation. For details see Materials and Methods.

	Concentration (M)	Activity (%)
Zn^{2+}	10^{-3}	40
Co^{2+}	10^{-3}	96
Mn^{2+}	10^{-3}	100
Ca^{2+}	10^{-3}	100
Hg^{2+}	1.67×10^{-6}	60
EDTA	10^{-2}	60
PCMB	10^{-3}	105
Cysteine	10^{-3}	100
Mercaptoethanol	10^{-3}	100
Iodobenzoic acid	10^{-3}	90
Iodoacetamide	10^{-3}	100
Iodobenzoic acid	10^{-3}	84
N-Ethylmaleimide	10^{-3}	100
Hydroxylamine HCl	10^{-3}	100
8-Hydroxyquinoline	10^{-3}	82
KCN	10^{-3}	100
E 600	10^{-3}	100
DFP	10^{-3}	—

Immunoelectrophoresis. By immunoelectrophoresis the precipitin line shown earlier to contain an enzyme hydrolyzing Ala-Ala-NA [1] also appeared to possess hydrolytic activity toward Gly-Pro-NA. Moreover, the staining of the precipitin line (resulting from diazo coupling of the naphthylamine liberated) was much faster with Gly-Pro-NA as substrate than with Ala-Ala-NA. This suggests that both of these substrates are split by the same enzyme and that it hydrolyzes Gly-Pro-NA much more rapidly than Ala-Ala-NA.

DISCUSSION

The kidney enzyme, just as the corresponding rat liver enzyme, was found to be particle bound and was readily liberated by autodigestion which also caused a marked activation of the enzyme. This activation may be due to liberation of enzyme sites which have been hindered because of binding to the particles. It is also possible that destruction of effective inhibitor substances takes place during autodigestion.

The present enzyme as well as the very active particle bound aminopeptidase were solubilized during autodigestion. The latter enzyme is capable of hydrolyzing a number of peptide substrates and amino acid naphtylamides,[8,10] and it was demonstrated in the present study with Ala-NA as substrate. If this latter aminopeptidase is present in the final preparation even in low amounts, an essentially different result is obtained with substrate specificity studies, since it readily hydrolyzes the dipeptides liberated by the present enzyme. In this case both free glycine as well as proline could be demonstrated

as reaction products of the hydrolysis of Gly-Pro-NA and also liberation of several amino acids from the oligopeptide substrates used. A hog kidney peptidase preparation with this kind of substrate specificity has been described by Nordwig and Mitsui.[11] The essential step in the purification procedure, therefore, was the separation of these two enzymes. Separation of these two enzymes was not possible with gel filtration in Sephadex G-200 since both of the enzyme proteins are large molecules with molecular weights well over 220 000, the practical resolution limit of Sephadex G-200 gel. Separation could, however, be readily accomplished by chromatography in DEAE-cellulose. The separation of the aminopeptidase present in rat liver was much simpler since the aminopeptidase there is in a soluble form in the homogenate instead of being particle-bound.[3]

Within the limits of the experiments carried out, the substrate specificity of this hog kidney enzyme seems to be identical to the specificity of the enzyme purified from the rat liver.[3] Gly-Pro-NA was the best substrate among the naphthylamide derivatives tested, and the monoamino acid naphthylamides were not hydrolyzed measurably. The N-terminal glycyl-proline group was liberated also from the nonchromogenic peptides tested, and no further hydrolysis of the dipeptides could be demonstrated. This suggests that the enzyme prefers as substrate peptides with an N-terminal X-Pro-group (X = amino acid). The requirement of proline is, however, not absolute since Ala-Ala-NA was also shown to be hydrolyzed by the same enzyme though at a much lower rate. The much lower affinity of the enzyme toward Ala-Ala-NA is illustrated by the much higher K_m-value measured for Ala-Ala-NA than for Gly-Pro-NA. These studies demonstrate that the moiety coupled to this N-terminal group may vary, i.e. it may be naphthylamine, amino acid or oligopeptide. The presence of only one enzyme in the final preparation active on both Gly-Pro-NA and Ala-Ala-NA was proven by the fact that the enzymes hydrolyzing these substrates could not be separated from each other, and further by the fact that both of the substrates were hydrolyzed by enzyme present in the same immunoprecipitin line. This enzyme is identical to that described earlier by immunoelectrophoresis.[1]

The studies with various modifiers demonstrated that the enzyme is apparently not dependent on any ion or cofactor which could be easily destroyed, or removed by chelators. This was also found to be true with respect to the rat liver enzyme having the same substrate specificity. Further studies will be necessary to show whether any more tightly bound cofactors are present by employing more drastic conditions for their separation.

These studies clearly demonstrate the presence in hog kidney of an enzyme similar to that demonstrated earlier to be present in rat liver. Further studies on the distribution of the enzyme in various organs as well as on its enzymic characteristics are required before the physiological function of the enzyme can be elucidated. A possible role in collagen metabolism is suggested by the fact that aminoacid sequences of the type glycyl-proline are the essence of the collagen molecule.

REFERENCES

1. Vanha-Perttula, T., Hopsu, V. K. and Glenner, G. G. *Histochemie* **5** (1965) 448.
2. Hopsu-Havu, V. K. and Glenner, G. G. *Histochemie* **7** (1966) 197.
3. Hopsu-Havu, V. K. and Sarimo, S. *Z. physiol. Chem.* **348** (1967) 1540.
4. Hopsu, V. K. and Glenner, G. G. *J. Histochem. Cytochem.* **11** (1963) 520.
5. Glenner, G. G., Cohen, L. A. and Folk, J. E. *J. Histochem. Cytochem.* **13** (1965) 57.
6. De La Haba, G., Cammarate, P. S. and Timasheff, S. N. *J. Biol. Chem.* **234** (1959) 316.
7. Grabar, P. and Williams, C. A. *J. Chromatog.* **17** (1965) 245.
8. Vanha-Perttula, T., Hopsu, V. K. and Glenner, G. G. *Histochemie* **14** (1966) 314.
9. Lowry, O. H., Rosebrough, N. H., Farr, A. and Randall, R. J. *J. Biol. Chem.* **193** (1951) 265.
10. Pfleiderer, G., Celliers, P. G., Stanulovic, M., Wachsmuth, E. D., Determan, H. and Braunitzer, G. *Biochem. Z.* **340** (1964) 552.
11. Nordwig, A. and Mitsui, T. *Abstracts, 3rd Meeting Fed. European Bichem. Soc., Warsaw, April 4th -7th 1966*, p. 297. Academic, London, New York.

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