STUDIES ON THE NATURE OF HYDROXY-PROLINE FROM THE MALARIAL SPLEEN (*PL. GALLINACEUM*) IN CHICKS

By A. S. Ramaswamy

(Pharmacology Laboratory, Indian Institute of Science, Bangalore-3)

Received April 15, 1955

Summary

1. A study has been made on the pathogenesis of the malarial spleen in *P. gallinaceum* infection in chicks and data have been [illegible] to indicate the formation of allohydroxy-L-proline.

2. The formation of diasterio-isomers of amino-acids which are toxic to cells has been considered as one of the factors in the [illegible] of malaria.

3. The significance of these findings in relation to several other factors available in literature has been cited and concluded that a basic [illegible] is operating in these varied conditions.

4. It has been concluded that the hydro-thermic stability and [illegible] seem to reside in active (γ) hydroxy-proline and C_β C_γ C_β C_γ interatomic distances seems to be concerned in the directional [illegible] of cytoplasmic materials and water.

The spleen is an organ which has been known for a long time past as pre-eminently suited to deal with the effete products which find their way into the blood stream which is thus restored to its original condition. It has also been definitely established that it is one of the most important places in the body where foreign products, chemical, metabolic or otherwise, are effectively dealt with when the latter are brought to it. Furthermore, the spleen has lately gained considerable importance as the primary centre for the production of antibodies which are absolutely important for the defence of the animal body against microbial or other parasitic invasion. For the performance of the above mentioned functions, the organ is provided with special groups of cellular elements which forms a very characteristic structural unit in the anatomy of the spleen in the cells of the reticulo-endothelial system. These cells are always in sufficiently large number to cope with the usual need of the animal under normal conditions. But under abnormal circumstances either brought about by infections or by the presence of foreign particulate materials resulting therefrom as in malaria or other viral infections or introduced to the body by artificial means as in vital staining, a large amount of extra work is thrown on the spleen which reacts to the increased demand by putting

forth a large number of its vital cellular elements. Such increased output of cells of the reticulo-endothelial system is only possible because of their enormous potentialities of rapid multiplication and in developing a larger amount of cytoplasm in their bodies. While these activities are manifest to cope with an abnormal situation, an adequate supply of nutriments, *e.g.*, glucose or essential metabolites, and oxygen and an equally adequate provision for removal of products of cellular metabolism must be made available and these are brought about by a greater flow both in the arterial and venous systems as well as by dilatation of the existing vascular channels peculiar to the spleen. The net result of all the above changes is an increase in the volume of the organ which is manifested by an enlarged spleen.

It will be seen that the essential underlying basis of splenic enlargements in the majority of instances is a physiological hypertrophy to start with and lasts for a comparatively short or long period according to varying circumstances. If on the other hand, the above factors continue to operate either continuously or repeatedly at frequent intervals the resulting pathological alternations will *pari passu* not only be taking place in increasing intensity, but will become more or less of a permanent nature. The inevitable result of this change is that the splenic capsule and its trabaculæ are increased in number and also in a thickened capsule. Such splenic enlargements, therefore, do not disappear or take a long time to do so, even after the provocating stimulus has ceased to exist.

In order to perform the several functions mentioned the splenic tissue is endowed with a large amount of collagenous tissue interspersed among its structural units. Thus it basically comes to the consideration of the role of collagen component in biological material and its special role in the spleen.

Enormous amount of data on the collagen has been accumulating since the structural studies by W. T. Astbury (1940), Pauling and Corey (1951) and Bear (1952). Further progress became possible with the advent of complete data on the amino-acid composition of mammalian collagen [Bowes and Kenton (1948)] and the corresponding data for fish collagen [Neuman (1949)]. The main difference between collagen of mammals and teleosts is the low content of hydroxy proline (9%) of fish skin collagen—hallibut, [Neuman and Logan (1950)], Shark, Borasky (1955)—compared to the figure of 14% for bovine collagen, apart from minor divergences in amino acids present in small amounts of other hydroxy amino-acids such as serine, threonine and hydroxy-lysine [Vanslyke (1941)] and methionine [Neuman (1949)]. An excellent discussion on these points are to be found in an article by Gustavson (1955).

During the course of study of carbohydrate metabolism of the malarial parasite both in the blood and spleen, the unusual amount of hydroxy-proline were noted in all the carbohydrate fractions which attracted attention. The observations made during the course of this study has been reported here because of its unusual interest and significance.

MATERIALS AND METHODS

Chicks 8–12 weeks old were used for the purpose of this study. The strains of the malarial parasite *P. vallisnerium* which was obtained from the southern branch of the Malaria Research Institute, Coonoor, was maintained in chicks and the method of maintaining the strain in chicks was the same as reported [illegible] (Ramaswamy *et al.*, 1950).

Paper chromatography. Circular Paper Chromatography as developed by Giri *et al.* (1952) was used using Whatman No. 1 filter paper discs (25 cm. diam.) and chromatograms developed with *n*-butanol-acetic acid-water solvent (40 : 5 : 14) as described by Rao and Wadhwani (1954). This resolves hydroxy proline band well from other amino-acids especially tryptophan. The dried papers were sprayed with 0·1% ninhydrin in 95% aqueous acetone and heated at 60° C to show bands. Isatin reagent (Acher *et al.*, 1950) and diphenylamine reagent for sugars (Buchan and Savage, 1952) and also *p*-anisidine hydrochloride reagent (0·1 M in butanol) was used (Hough *et al.*, 1950) to detect carbohydrate fractions.

Sampling of tissue for chromatography. The animals were sacrificed on the day of high parasitaemia in the (4–5 day) peripheral blood. The spleen was dissected out and materials from two or three chicks were pooled together and homogenised in a small quantity (2 c.c.) of 2% sodium citrate solution and made upto 10 c.c. with N/2 potassium hydroxide and allowed to digest the tissue for 4 days. The lipoids are extracted with ethanol-ether [Dische and Osnos (1950)] and further steps were followed as described by Clegg (1954).

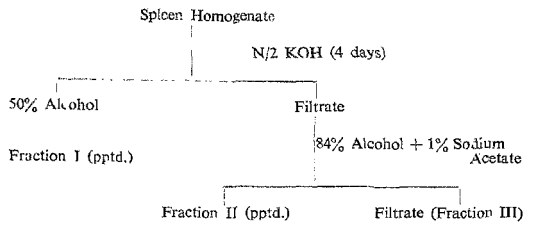
Analytical reagents.—Authentic samples of hydroxy-proline and [illegible] L-proline were used as markers for chromatography and chemical tests.

EXPERIMENTAL

The splenic tissue homogenates were fractionated according to scheme given in Fig. 1. The fractions 1, 2 and 3 were chromatographed to detect [illegible] proline and carbohydrates. Fraction 2 contained greater amount of hydroxy-proline and 1 and 3 contained smaller amounts. The presence of [illegible] in normal spleen was found to be negligible, whereas infected tissue showed nearly 5 times the amount shown in the normal tissue. The ninhydrin spraying reagent showed an uncharacteristic pale yellowish brown colour, which demonstrated that the nature of hydroxy-proline was not natural in its behaviour.

Isolation by paper chromatography.—A sample of fraction 2 was deposited at the centre of a Whatman No. 3 (disc 25 cm.) paper and was saturated to capacity and was developed with *n*-butanol-acetic acid-water solvent. The hydroxy-proline band was cut out by means of guide strips sprayed with ninhydrin as before, to indicate the position of the amino-acid. A number of them were extracted with 75% ethanol, and evaporation of the solvent gave a residue containing hydroxy-proline with traces of threonine and lysine. The amount of material obtainable was of the order of 2–3 mg. only by these methods. Crystallisation of this material

FIG. 1. Schemeta for fractionation of spleen (Chick).



was very difficult but formation of crystals could be found after 7–8 days when kept in a dessicator and dehydrated over calcium chloride. The typical crystals obtained are given in Plate I.

Identification of the amino-acid as allohydroxy-L-proline complex.—The crystals obtained from the spleen was compared with synthetic allohydroxy-L-proline (Robinson and Greenstein, 1952; Radhakrishnan and Giri, 1954) under the microscope. It was found to be identical with authentic sample of allohydroxy-L-proline. The amino-acid was not easily soluble in water, relatively insoluble in absolute ethanol and *n*-butanol. The isolated amino-acid was optically inactive (observed over 20 min. in water). The isolated material gave positive tests for allo(-) hydroxy-proline m.p. 248° C. (sinters).

Reaction with ninhydrin and isatin reagents (*Neuberger*, 1945)

	L(-)hydroxy-proline		Allo(-)hydroxy-L-proline	
	In Glacial Acetic acid	In Pyridine	In Glacial Acetic acid	In Pyridine
1. Isatin reagent	Orange red	Red	Orange red	Intense red
2. Ninhydrin reagent	Yellow	Transient red, later turns yellow	Yellow	Yellow colour on warming, alkali stabilises
3. Diazotised sulphanilic acid in alkali	Slowly turns yellow on warming		Quickly turns deep orange red on warming	

In addition to the work presented other tests performed on splenic fraction 2 has been tabulated as in Table I.

TABLE I

Colour tests on alkaline hydrolysate of spleen fractions (chicks)

Colour Tests		Fr. 1	Fr. 2	Fr. 3	Remarks
1. Ninhydrin Reagent	Norm.	—	—	—	Uncharacteristic colour
	Mal.	—	—	—	
2. Isatin Reagent	Norm.	—	—	—	Do.
	Mal.	+	—	—	
3 Fehling's solution	Mal.	Non-reducing	Non-reducing	Non-reducing	Anhydro or Glycol or Glycoseen of sugars
4 Neuman and Logan	Mal.	—	—	—	
Test	Norm.	—	—	—	
5. Diphenylamine Test	Mal.	Broad band	Broad band	Broad band	De-oxy ribose and Hexoses—nil
6. Orcinol Reagent	Mal.	—	—	—	Pentoses nil
7. P-anisidine Reagent	Norm.	—	—	—	
	Mal.	+	Broad band		Sugars present (?)
8. Silver nitrate in alkali	Mal.	—			
9. Idoform Test	Mal.	—			
10. Carotenoides	Mal.	Nil		Nil	

Estimation of collagen as hydroxy-proline by the method of Neuman and Logan (1950) showed very anomalous results. The authentic sample of allo(-)hydroxy-L-proline showed good colour development (uncharacteristic dull orange) according to the method of Neuman and Logan (1950). The method as such for the natural material was not applicable, but was found to give lower values but after hydrolysis of the natural material with 2·5 N sodium hydroxide for 5 min. and later the complexing material was precipitated with acetone and pyridine in the presence of alcohol and the pyridine fraction was found to give positive hydroxy-proline test. The results are given in Table II.

TABLE II

Collagen and hydroxy-proline content in chicks

Chicks (50 gm. B.W.)	Collagen % on nitrogen	Spleen	
		Net wt. (av.) in mg.	Hydroxy-proline γ/1 gm. wet wt.
Normal (3) ..	6–7%	75–90	50–75
Infected (3) ..	..	230–350	400–500

DISCUSSION

The collagen group of proteins is characterised by the presence of proline and hydroxy-proline and the paucity of aromatic amino-acids. Particularly the estimation of hydroxy-proline for the characterisation of collagen has been well recognised (Neuman and Logan, 1950; Robertson, 1950, 1952; Robertson and Schwartz, 1953). The great variation of hydroxy-proline content of collagen from various sources from 6–14% necessitates the consideration of the significance of hydroxy-proline in biological materials.

As a result of the pioneer work of Gustavson (1955) and Takahashi and Tanaka (1953) it is known that the fish collagen forms two distinct groups as regards their hydro-thermal stability. (1) The cold and deep water fishes for which T_s (shrinkage temperature) of skins ranges between 37–45° C. and (2) the warm and surface water fishes between 50–57° C. The bovine and human skin require temperatures of about 65° C. to show thermal shrinkage. The detailed consideration of the amino-acid composition of mammalian collagen (Bowes and Kenton, 1948) and of fish collagen (Neuman, 1949) has revealed interesting data on the structure of collagen itself (Gustavson, 1955). It has been settled in a large measure that the marked differences in the hydro-thermal stability of bovine skin and fish collagen is due to molecular architecture than to differences in amino-acid composition. The results from investigation of numerous species of fishes have shown that hydro-thermal stabilty and hydroxy-proline content of collagen are intimately connected

together (Gustavson, 1955). If the hydroxy-groups of the residues of the hydroxy-amino-acids enter into interchain hydrogen bonding with some other groups for example the CO NH linkage, the stability of the collagen would be expected to increase with the increase in the content of hydroxy [illegible] which is actually is the case (Gustavson, 1953). The data available in literature when considered together with the observations recorded here, seem to reveal a basic factor which is operating in biological materials, than what is demonstrated by hydroxy-proline alone.

A phenomenon of considerable interest in this connection is the formation of anthocyanin pigments in plants especially in what are called autumnal coloration. The anthocyanin pigment formation has again been linked up with [illegible] environment which is anomalous. The pigment formation bears out the conclusion that increase of anthocyanin is correlated with the [illegible] (Onslow, 1925 and Robertson and Robinson, 1929). Also such winter [illegible] is known to be brought about by mechanical injury or attacks of fungi or insects. The formation of pigment in Cuscuta (Parasitic plant) by Mirande (1899) shows that the reddening of leaves varies in different species, but also in each species according to the host on which it grows. For instance, the same species growing on *Sambucus nigra* (poor in sugar) and *Forsythia virridissima* (sugar rich) becomes green on the former, but very red on the latter. Hence Mirande concludes that the development of the parasite plant and reddening is correlated with good nutrition. Severe frost also has been shown to lead to accumulation of unusual [illegible] which are not normally formed in the beet plant. Raffinose content in beet plant has been reported to be increased by such disturbances as those caused by sudden frost (Armstrong, 1924). Bacon, Baldwin and Bell (1944) found that [illegible] with a chain of 12-glucose units was synthesised by fasting rabbits when glucose or fructose was administered. The administration of galactose or sucrose however led to the formation of 18-unit glycogen. D. G. Steyn (1940) from South Africa also reports poisoning of cattle and sheep when they graze on frost damaged plants. The occurrence of crystalline melezitose in honey dew during seasonal periods of drought has been reported and bees fed on this and nectar from other flowering plants are reported to have led to heavy mortality (Stacey, 1946).

Distribution of hydroxy-proline.—The presence of free hydroxy-proline has been reported to occur in pollens collected by bees (Auclair and Jamieson, 1948), in prunes (Joslyn and Stepka, 1949), in the haemolymph of *Drosophila melanogaster* (Auclair and Dubreuil, 1953) in the blood and malpighian tubes of the larvæ of *Bombyx mori* L. infected with polyhedral disease (Drilhon, Busnel and Vago, 1951). In the combined state it is known to be present in alfalfa proteins (Steward, Thompson and Miller, 1951) protein from vacuum dried *Sarcina lutea* [Belozerskii *et al.* (1943)], in sugar beet protein (Sisakyan, Bezinger and Kuvæva, 1951) in proteins of the insect cuticle [Hackman (1953)], in dentine protein (Losee Neidig and Hess, 1951) and among enzymes in horse-radish peroxidase (Maehly and Paleus, 1950). The hydroxy-proline obtained in natural materials has been definitely identified and

characterised only in two instances known to us. Wieland and Witkop (1940) has isolated from death cap fungus *Amanita phalloides*, a toxic peptide named phalloidine and Giri and Radhakrishnan (1954) from the leaves of *Santalum album*. Both contain l (-)allohydroxy-proline which is quite unusual.

Biological activity has been reported by A. Steinberg in his study on tobacco plants to cause inhibition of flowering and fruiting in *N. rustica* and also reports toxic action on tobacco seedlings (1950, 1951). Whether these factors are operative in inhibiting regeneration of new cells in the malarial spleen and other tissues like bone-marrow require further study.

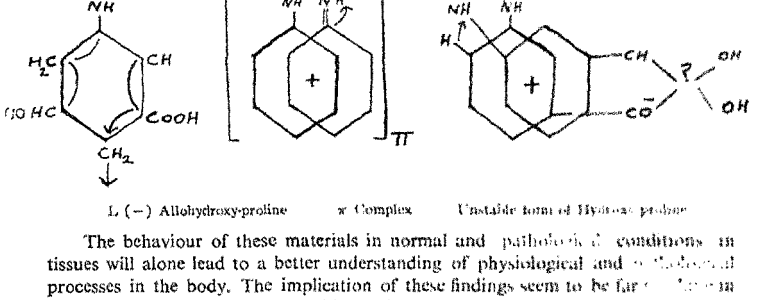
The unequivocal interpretation of the data now available is not simple and often involves additional data which is not readily available. The differences in the various diasterio-isomers of amino-acids as regards biological activity require careful elucidation. Whether there may not be other factors than the mere presence of these non-polar diasterio-isomeric amino-acids, only future research can decide. Experiments recognising this difficulty if successful should permit more exact dilineation of the basic factor working in these varied conditions. The role of the sugar molecule with hydroxy-proline residue shows that the carbohydrate metabolism here is linked in unique manner. It has been tentatively suggested that the hydroxy-proline residue is in the form of a complex with L-ribose complex and the data on which this conclusion is based will be communicated later on.

Hydroxy-L-proline
λ 250 $m\mu$ −ve

Allo-Hydroxy-L-proline
λ 250 $m\mu$ +ve

The basic fact which emerges from a careful consideration of the data presented seems to involve C_2—C_4 and C_1—C_4 atoms of hydroxy-proline in a specific manner. The inter atomic distances between C_2—C_4 and C_1—C_4 atoms of the hydroxy-proline molecule is the deciding factor which is responsible for the manifold phenomenon exhibited by the collagen. Whether this conclusion can be valid in the case of other molecules only further research on this matter will decide. However, the C_2—C_4 and C_1—C_4 inter atomic distances are affected under different conditions of temperature, hydration with association of molecular water, hydrogen-ion concentration and the presence or absence of nutriments like glucose, calcium or/and enzymes and vitamins and also in the presence of electrolytes especially potassium. Support for such an assumption seems to be possible as from the chemical data (unpublished observations) and physical data as carbon—carbon bond distances has been known to be dependent on the environments (Herzberg, Patat and Verlager,

1937). When abnormal metabolites are formed they seem to be thrown out of the system as electrically neutral molecules as in the case of allo-oxyproline. It is quite possible that allohydroxy-L-proline presents a certain phase of activity of the hydroxy-proline molecule in natural materials. Such a possibility is not inconsistent as glucose is known to exist in the γ-form in blood. Hence it is tentatively suggested that hydroxy-proline can occur in three forms [illegible] in nature, as given below. Here seems to be a unique material which shows the intimate nature of chemical constitution and biological activity.



L (−) Allohydroxy-proline π Complex Unstable form of Hydroxy proline

The behaviour of these materials in normal and pathological conditions in tissues will alone lead to a better understanding of physiological and [illegible] processes in the body. The implication of these findings seem to be far [illegible] in importance as it is likely to provide a fuller and better understanding of the basis of sensation and its ramifications are co-extensive.

ACKNOWLEDGEMENTS

The author wishes to thank Prof. M. S. Thacker, the Director, for his keen interest and Drs. M. Sirsi and K. P. Menon for helpful discussions and criticisms and to Prof. K. V. Giri and Dr. A. N. Radhakrishnan for the gift of authentic samples of L(-)hydroxy-proline and L(-)allohydroxy-proline from their collection. Author's grateful thanks are also due to Prof. R. S. Krishnan and Dr. P. S. Narayanan of the Department of Physics, for the infra-red data and discussions on the subject. His grateful thanks are due to Mr. S. K. Sreepathi Rao for micro-photographs.

REFERENCES

1. Astbury, W. T. .. *J. Int. Soc. Leather Trades' Chemists*, 1940, **24**, 69.
2. Acher, R. Fromageot, C. and Jutisz, M. *Biochim. Biophys. Acta*, 1950, **5**, 81.
3. Armstrong, E. F. .. *The Carbohydrates and the Glucosides*. Longmans, Green and Co., London, 1924, 2nd Edition.
4. Auclair, J. L. and Dubreuil, R. *Can. J. Zool.*, 1953, **31**, 30.

5. Auclair, J. L., and Jamieson, C. A. — *Science*, 1948, **108**, 357.
6. Belozerskii, A. N. and KirceenKova, E. G. — *Mickrobiologiya*, 1943, **12**, 31 (cited from *C.A.*, 1948, **42**, 8875).
7. Bear, R. S. .. — *Adv. Protein Chem.*, 1952, **7**, 69.
8. Bowes, J. H. and Kenton, R. H. — *Biochem. J.*, 1948, **43**, 365.
9. Bornsky, R. .. — (Cited from 15).
10. Buchan, J. L. and Savage, R. I. — *Analyst.*, 1952, **77**, 401.
11. Bacon, J. S. D., Baldwin, E. and Bell, D. J. — *Biochem. J.*, 1944, **38**, 198.
12. Dische, Z. and Osnos, M... — *Fed. Proc.*, 1950, **9**, 165.
13. Drilhon, A., Busnel, R. G. and Vago, C. — *C.R. Acad. Sci. Paris.*, 1951, **232**, 360.
14. Giri, K. V. and Rao, N. A. N. — *Nature*, 1952, **169**, 923; *J. Ind. Inst. Sci.*, 1952, **34**, 95.
15. Gustavson, K. H. .. — *Nature*, 1955, **175**, 70.
16. ———— .. — *Svensk Kem. Tidskr.*, 1953, **65**, 70.
17. Glegg, R. E., Eidinger, D. and Leblond, C. P. — *Science*, 1954, **120**, 839.
18. Herzberg, G. Patat, F., and Verleger, H. — *J. Phys. Chem.*, 1937, **41**, 123.
19. Hackman, R. H. .. — *Biochem. J.*, 1953, **54**, 362.
20. Joslyn, M. A. and Stepka, W. — *Food. Res.*, 1949, **14**, 489.
21. Hough, L., Jones, J. K. N. and Wadman, W. H. — *J. Chem. Soc.*, 1950, 1702.
22. Losee, F. L., Neidig, B. A. and Hess, W. C. — *Proc. Soc. Exp. Biol. and Med.*, 1951, **76**, 783.
23. Mirande, M. .. — *Rescherches Physiologiques et anatomiques Sur le Cuscutaces*, Paris, 1899.
24. Mehly, A. C. and Paleüs, S. — *Acta Chem. Scand.*, 1950, **4**, 508.
25. Neuman, R. E. .. — *Arch. Biochem.*, 1949, **24**, 289.
26. ——— and Logan, M. A. J. — *J. Biol. Chem.*, 1950, **184**, 299.
27. Neuberger, A. .. — *J. Chem. Soc.*, 1945, 429.
28. Onslow, M. W. .. — *The Anthocyanin Pigments of Plants*, Cambridge Univ. Press, 2nd Edition, 1925.
29. Pauling, L. and Corey, R. B. — *Proc. Nat. Acad. Sci. U.S.*, 1951, **37**, 272.
30. Ramaswamy, A. S., Rama Rao, R. and De, N. N. — *Proc. Ind. Acad. Sci.*, 1950, **32 B**, 126.
31. Rao, N. A. N. and Wadhwani, T. K. — *Curr. Sci.*, 1954, **23**, 359.
32. Robertson, Van B., and Schwartz, W. — *J. Biol. Chem.*, 1953, **201**, 689.

33. Radhakrishnan, A. N. and Giri, K. V. — *Biochem. J.*, 1954, **58**, 87.
34. Robertson, Van B — *J. Biol. Chem.* 1950, **187**, 673, [illegible], 198, 401.
35. Robertson, A. and Robinson, R. — *Biochem. J.*, 1929, **23**, 35.
36. Stacey, M. — *Adv. in Carbohydrate Chemistry*, 1946, **2**, Academic Press, N.Y.
37. Stevn, D. G. — *Farming in South Africa*, 1930, [illegible].
38. Steinberg, A. — *Am. J. Botany*, 1950, **37**, 647.
39. ———— — *Bull. Torrey Bot. Club.*, 1951, **78**.
40. Sisakyan, N. M., Beringer, E. N. and Kuvaeva, E. B. — *Biochemistry Leningr.*, 1951, **16**, [illegible].
41. Steward, F. C., Thompson, J. F. and Millar, F. K. — *Plant Physiology*, 1941, **26**, 123.
42. Takahashi, T. and Tanaka, T. — *Bull. Jap. Soc. Fish.*, 1953, **19**, 641.
43. Van Slyke, D. D., Hiller, A. and MacFadyen, D. A. — *J. Biol. Chem.*, 1941, **141**, 681.
44. Wieland, H. and Wilkon, B. — *Liebigs Ann.*, 1940, **543**, 171.
45. Williams, R. J. — *Biochemical Institute Studies*, No. 4, University of Texas publication, Texas, 1951.

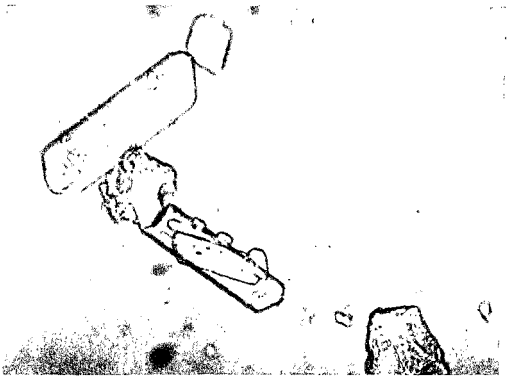


FIG. 1. Crystals of Hydroxy-proline, × 120.

FIG. 2. Crystals of Allo-hydroxy-proline, ×120.

FIG. 3. Crystals of Hydroxy proline obtained from a [illegible]
(X [illegible] 120).