BIOCHEMICAL STUDIES OF CONNECTIVE TISSUE

by

John Peter Bentley

A THESIS

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[redacted]

(Professor in Charge of Thesis)

[redacted]

(Chairman, Graduate Council)

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FOREWORD

The work to be presented in this thesis is part of a continuing study of connective tissue metabolism. It will be presented in three parts, each may be considered a discrete experiment yet each integrates with other work being carried out in the Department of Surgery at the University of Oregon Medical School.

PART I

In 1953 Robertson and Schwartz (43) reported that large amounts of rapidly growing connective tissue could be induced by a subcutaneous injection of carageenin, a seaweed extract. In 1957, Jackson (23) used this carageenin granuloma technique to study the incorporation of C^{14} glycine into collagen. It was decided to use this technique in the work which will be presented as Part II of this thesis. However, subsequent to 1957 other preparations of carageenin became available and it thus became necessary to differentiate between them.

Carageenin is a mixture of polysaccharides extracted with hot water from the red algae Chondrus crispus and Gigartina stellata in which it is a cell wall constituent (45). Smith et al (44, 45) made molecular weight estimates ranging from 210,000 to 360,000 for the material and also found it to consist of two main fractions which they designated Kappa and Lambda. The lambda fraction has a higher molecular weight than the kappa and consists largely of D-Galacto pyranose residues joined by α 1,3, glycoside links, with ester sulphate groups on carbon 4. In the kappa fraction more than 1/3 of the residues are 3,6, anhydro-D-galactose and hence it contains less sulphate groups.

The Company which processes carageenin (Seaplant Chemical Corp., New Bedford, Massachusetts) supplies it to the food industry and to the cosmetic industry where it is used as an emulsion stabiliser in artificial cream topping, toothpaste, shaving cream etc. Consequently, many substances are available all with the generic name carageenin but differing in their origin and the method of their preparation.

It was felt that the different preparations and fractions might vary in their stimulating action upon a developing connective tissue, and since carageenin was to be used as a tool in a study of connective tissue metabolism, it was decided to test some of the available fractions in order to determine which gave the best response.

Materials and Methods

Five commercial samples of carageenin were prepared in 1% solution in 0.9% NaCl as follows: The appropriate amount of dry powder was placed in a screw cap vial with sufficient saline solution to give a concentration of 1%. The loosely capped vials were placed in a boiling water bath for 1 to 2 hours and periodically shaken vigorously. The carageenin very slowly formed a colloidal opalescent solution

(the lambda fraction gelled on cooling to room temperature). Thus sterility and solution were achieved. The five samples tested were labeled by the manufacturer as follows:

Carageenin type 6
Sea Kem type 402
Sea Kem type 402 Kappa Fraction
Sea Kem type 402 Lambda Fraction
Extractive of Gigartina acicularis

Thirty-six albino guinea pigs each weighing about 300 gms. were fed a stock diet (see appendix 2) and supplemented three times a week with 50 mgm. ascorbic acid orally. The abdomens were shaved, the animals placed under light ether anesthesia, and 5 mls. of the carageenin solution injected subcutaneously just anterior to the umbilicus. At intervals of four and eight days after the injection animals were killed and the granulomas dissected out, great care being taken not to remove any of the adjacent skin or muscle. Granulomas from three or four animals were pooled and processed as follows:

Tissues were chopped finely with scissors and weighed. They were autoclaved with 0.01M acetic acid at 15 lbs/sq. in. for one hour, the supernatent gelatin solution removed and the autoclaving repeated twice. The gelatin solution was filtered through clarifying grade filter pads in a Seitz filter, made

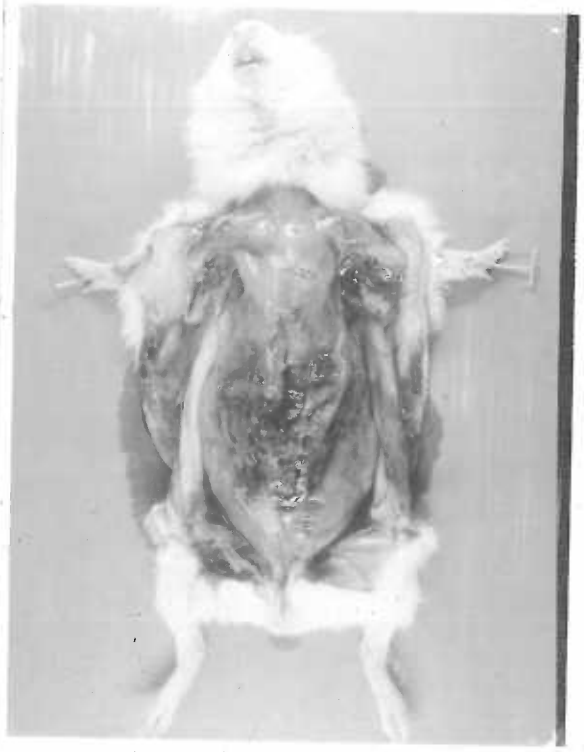
up to known volume and an aliquot evaporated to dryness under a stream of nitrogen and hydrolysed for three hours at 138° C. with 6 N HCl in sealed pyrex tubes. Hydroxyproline was measured by the method of Neuman and Logan (37) as modified by Leach (30).

Results

Table (1) shows that the weight of tissue formed per animal at the two time points varies greatly as does the amount of Hydroxyproline per gram of tissue. The best measure of comparison between the samples would therefore seem to be the total Hydroxyproline produced per animal.

The two types of carageenin extracted from Chondrus crispus, Type 6 and Sea Kem 402 seem to stimulate the production of collagen to about the same degree whilst the extract of Gigartina seems a little less effective. The most striking differences however are observed between the kappa and lambda fractions, the kappa producing an effect which is not sustained and has virtually disappeared by eight days, whilst the lambda fraction gives rise to far more collagen production at both time points than do the unfractionated samples.

Fig. 1



A typical granuloma, seven days after injection of 5 ml s. of 1% carageenin.

TABLE I

Comparison of granulomas formed and collagen produced under the stimulus of five different carageenin samples.

Fraction	Four days			Eight days		
	Wet wt. tissue per animal (gms.)	Micrograms of hydroxyproline per gm. tissue	Mgms. of hydroxyproline per animal	Wet wt. tissue per animal (gms.)	Micrograms of hydroxyproline per gm. tissue	Mgms. of hydroxyproline per animal
Carageenin #6	7.8	423	3.30	14.6	545	7.96
Sea Kem type 402	8.8	264	2.32	13.4	564	7.56
Sea Kem type 402 lambda fraction	14.8	334	4.94	20.0	525	10.50
Sea Kem type 402 kappa fraction	6.6	280	1.85	1.3	515	0.67
Extractive of Gigartina acicularis	16.8	97	1.64	18.7	378	7.07

Tissues from three or four animals were pooled for analysis at each point.

Discussion

The variety of effects noted may be due to the differing proportions of high molecular weight polysaccharides in the samples, or it may be due to the differing amounts of highly sulphated polysaccharides. Both of these attributes are seen in the lambda fraction as compared with the kappafraction whilst the other three samples are presumably mixtures of kappa and lambda in varying proportions.

Kappa fraction is precipitated from commercial carageenin by adding potassium chloride to a concentration of 0.15 M leaving the lambda fraction in solution (44). As Smith et al point out, (45) any polysaccharide impurities present would tend to concentrate in the lambda fraction since potassium salts do not have the marked effect on most polysaccharides that they have on kappa carageenin.

The effects noted may thus be due to some, as yet undetected, impurity which is active in stimulating connective tissue growth. For example, less than 1 microgram of bacterial endotoxin will produce an inflammatory response as great as that produced by 100 micrograms of carageenin. (Jackson and Freihoffer private communication).

Some slight support for the suggestion that degree of sulphation may be instrumental in producing

the effect noted is given by the fact that other polysaccharides (alginic acid, Agar-Agar) produce collagen in granulomas to a greater or lesser extent according to their degree of sulphation, i.e. high degree sulphation leads to a greater production of collagen than a lower degree of sulphation (Jackson and Williams private communication).

Kimoto et al (28) have recently described alginic acid granulomas in rats which were considerably smaller and had a lower collagen content than those produced by the author with carageenin.

It is noteworthy that the naturally occurring animal polysaccharides of high sulphate content, do not have any stimulating effect, e.g. chondroitin sulphate (Jackson and Williams private communication).

Since the lambda fraction gave rise to a greater production of collagen in a developing granuloma it became, on purely empirical grounds, the material of choice for all future work in this laboratory.

PART II

Collagen, the major protein component of connective tissue is classed as a scleroprotein and as such was considered to be insoluble in normal protein solvents. Since 1931, however, many reports have appeared in the literature demonstrating a multiplicity of soluble collagen fractions, claims to the role of biological precursor of collagen being made for most of them.

Nageotte (34, 35) extracted rat tail tendon with dilute acetic acid and obtained a viscous solution of collagen, which on standing in contact with the original fibers, or on addition of salt, precipitated fibrils similar to native collagen fibrils. He postulated that this precipitation from solution was the method by which collagen fibers formed in vivo during the course of wound healing, or of normal growth. Orekhovitch and co workers (38) were able to obtain solutions of collagen by extracting various tissues with citrate buffers at acid pH, and this material, upon addition of salt, precipitated fibrous collagen, which appeared identical to native collagen.

Highberger, Gross and Schmitt (22) and Harkness et al (20), extracting tissue with phosphate buffer at pH 7.4, found a soluble fraction, as did Jackson and Fessler (24) who used 0.2 M NaCl solutions

buffered at pH 7.4. Jackson and Fessler referred to their extract as "Neutral Salt Soluble Collagen". Gross (12) and Gross, Highberger and Schmitt (15) increased the salt concentration of the extraction medium to 0 45 M and were able to solubilise relatively more collagen with this than with 0.2 M NaCl or 0.14 M NaCl. Once extracted and purified however, all these fractions are interconvertible (11). They have the same amino acid composition (7, 4, 25) x-ray diffraction pattern (48), and in solution behave as a single molecular species, a rod shaped particle of 3000 Å in length and 15 Å diameter. The molecular weight is approximately 360,000 (2, 24, 39). Gross (11) named this molecule "Tropocollagen" and considered it to be the basic unit of collagen structure from which the fibers were formed.

It was later found that if a solution of tropocollagen prepared by any of the above methods is warmed to 37° C, fibrils appear having the typical 640 Å spacing of native collagen (24, 15). The gel which is thus formed becomes increasingly soluble with time of incubation first in neutral solution and later in acid (13).

Postulated Precursors

The investigators who first described the various fractions each considered them to be collagen precursors even though their identity was not yet clear. Thus Nageotte (34, 35) considered the soluble collagen which he extracted with acetic acid to be a precursor since the amount extractable decreased as the animal aged. He named the protein "Précollagène". Orekhovitch and Schpikiter (40) gave the name "Procollagen" to citrate buffer soluble collagen since it had a faster uptake of C^{14} glycine than the insoluble skin residue. Harkness et al (20) reported a faster uptake of labeled glycine into phosphate buffer soluble collagen than into procollagen or insoluble collagen and Jackson (23) in a study of the uptake of C^{14} glycine into collagen formed in a carageenin granuloma, compared the metabolic activities of insoluble collagen, "Procollagen", and Neutral Salt Soluble collagen. He found the collagen extracted with neutral salt solution to be metabolically more active than either of the other two fractions and hence deemed it more likely to be the true precursor than Procollagen.

The in vitro gelling phenomena led Gross (14) to conclude that the process of fiber formation is one of aggregation of the Tropocollagen molecules into

a polymer in which the intermolecular cross links are strengthened with time, and from which, varying amounts of Tropocollagen may be extracted according to the dispersing power of the solvents used.

Since collagen became more insoluble as time of incubation at 37° C progressed, Gross felt that the solubility of collagen would be influenced by the age of its constituent Tropocollagen molecules and the degree of cross linking which had occurred between them.

An in vivo experiment was thus deemed necessary to complement the in vitro studies.

Methods

Male and female albino guinea pigs weighing about 400 gms. were fed a stock diet (see appendix 2) supplemented with 50 mgms. of ascorbic acid orally every two days. Granulomata were produced as in Section I, using lambda carageenin, and on the sixth day after carageenin each animal was injected intraperitoneally with 35 microcuries of 2-C^{14} glycine in 1 Ml. of 0.9% NaCl solution. At intervals of 8, 10 24 and 36 hours following the injection of glycine, six animals were killed and the granulomata dissected out. A sample of dorsal skin was also removed after shaving. The tissues from six animals were pooled frozen in liquid nitrogen and crushed in a stainless

steel mortar as described by Walser and Bodenlos (46). All the following manipulations were carried out in the cold room at 2° C.

The tissues were suspended for 24 hours in 0.14 M NaCl buffered to pH 7.4 with .02 M Phosphate buffer. The supernatent was removed by centrifugation and pooled with three further extracts made with 0.14 M NaCl. Subsequent extracts were made as above, with 0.28 M NaCl, 0.45 M NaCl, 1.0 M NaCl, 2.0 M NaCl, 0.2 M citrate buffer pH 3.6 followed by autoclaving of the tissue three times with 0.01 M acetic acid at 138° C to convert the unextracted collagen to gelatin. A measured aliquot of each total extract from the 8 and 36 hour time points was dialysed salt free, evaporated to dryness and hydrolysed prior to hydroxyproline determination. A measure of total collagen extractable by each extraction medium per unit weight of tissue was thus obtained.

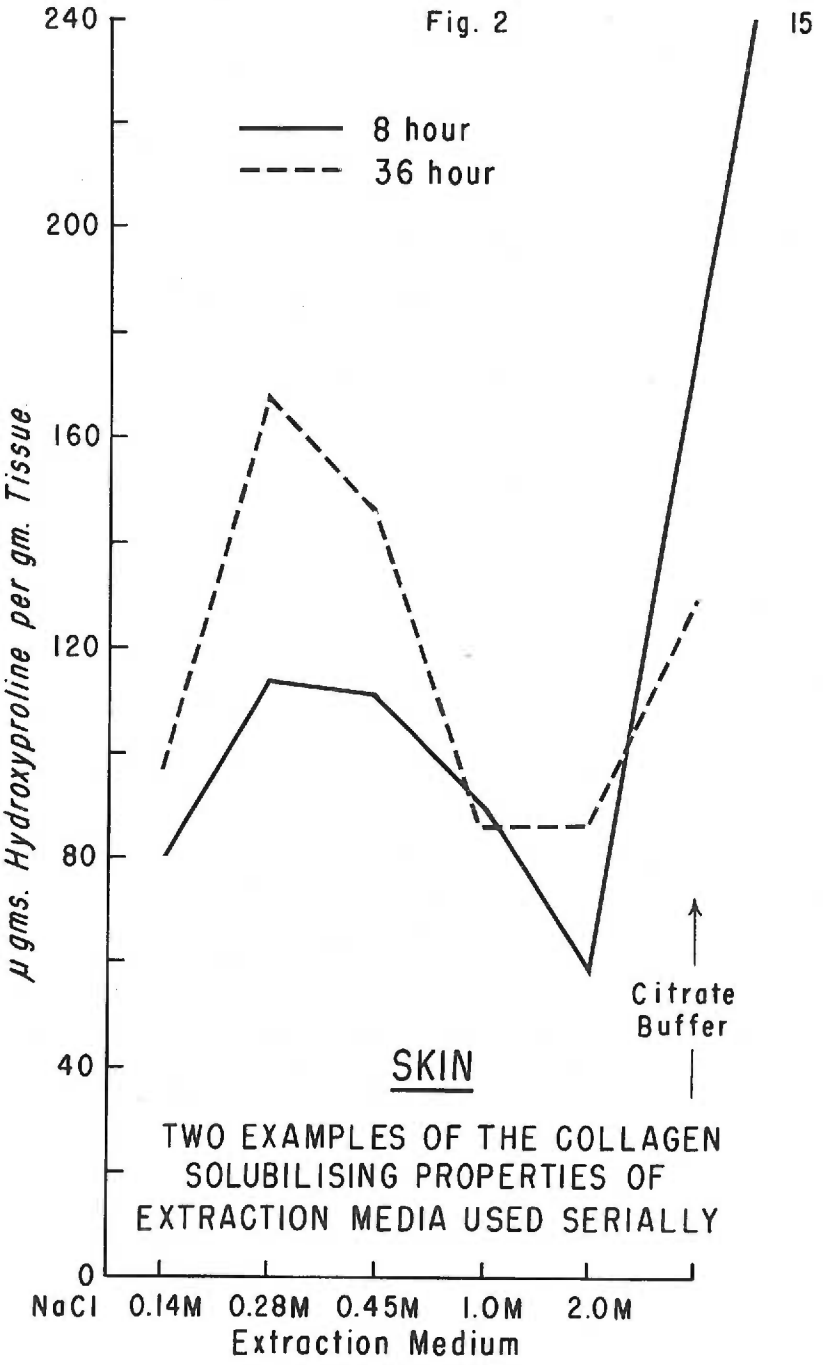
The remainder of each sodium chloride extract was made up to 20% NaCl concentration to precipitate the collagen (25). This was redissolved in 0.01 M acetic acid and precipitated by addition of NaCl to a concentration of 5%. This step was repeated three times followed by solution in 0.01 M acetic acid and drying in vacuo. The treatment of the citrate buffer

extractable material was identical with the above except that only 5% NaCl was needed for the initial precipitation. The gelatin was purified by precipitation of non-collagenous proteins with 5% Trichloroacetic acid, followed by removal of the trichloroacetic acid by dialysis and precipitation of gelatin with acetone (36).

The purified proteins were hydrolysed with 6 N Hcl at 138° C for three hours and the glycine isolated by a modification of the Perrone method (41) (see appendix 1).

The radioactivity was measured on "infinite thinness" samples (21) using a windowless gas flow counter. The "infinite thinness" technique is based upon the fact that absorption of the beta particles by the sample itself during counting is negligible below a certain thickness of sample. For dinitro phenyl glycine the count rates are linearly proportional to sample weight between 20 and 200 micrograms per 2 sq. cm. planchet. Henriques et al (21). These limits were strictly adhered to in the present investigation and consequently no correction for self absorption was applied to the data.

Fig. 2



TWO EXAMPLES OF THE COLLAGEN SOLUBILISING PROPERTIES OF EXTRACTION MEDIA USED SERIALLY

Fig. 3

GRANULOMA

TWO EXAMPLES OF THE COLLAGEN SOLUBILISING PROPERTIES OF EXTRACTION MEDIA USED SERIALLY

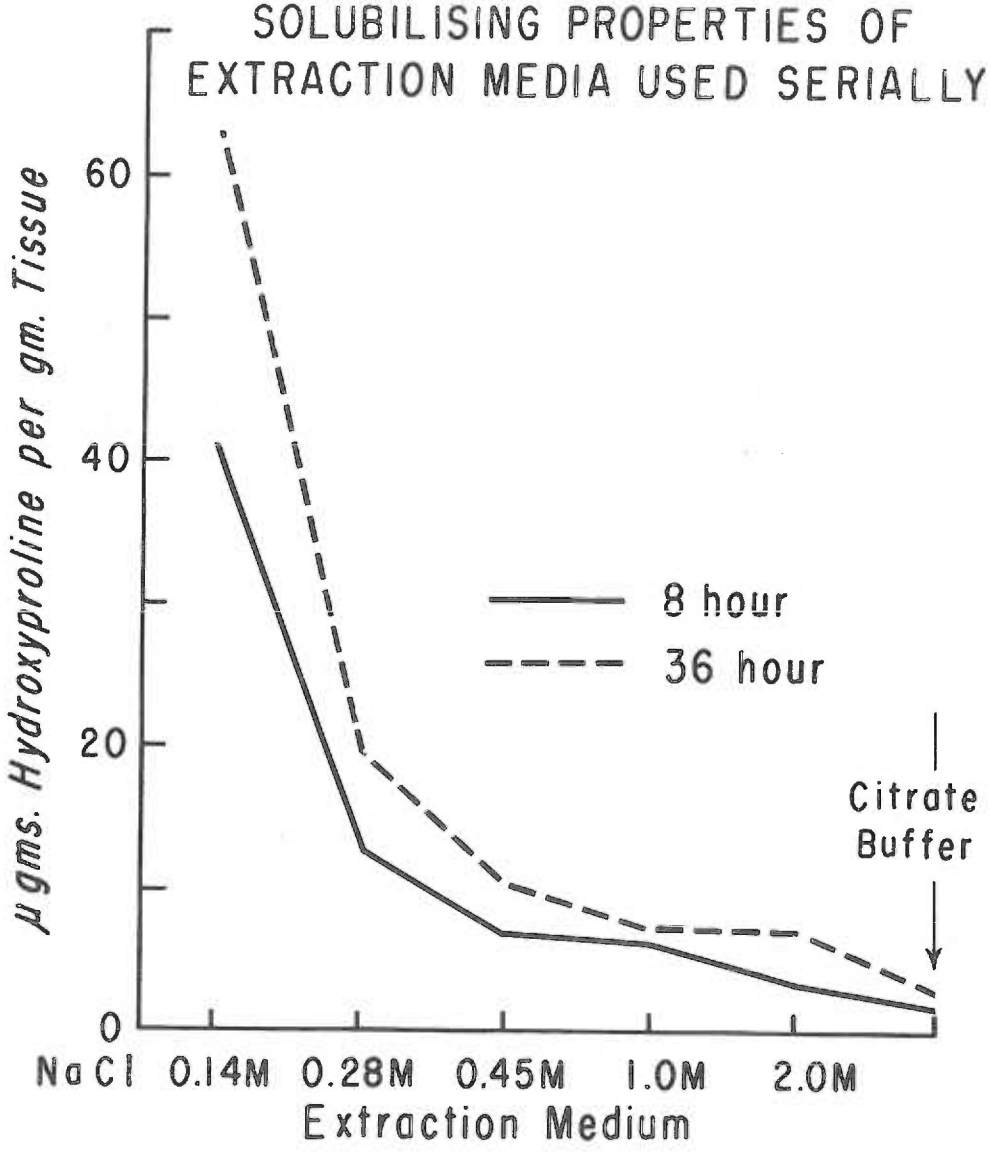


Fig. 4

SKIN

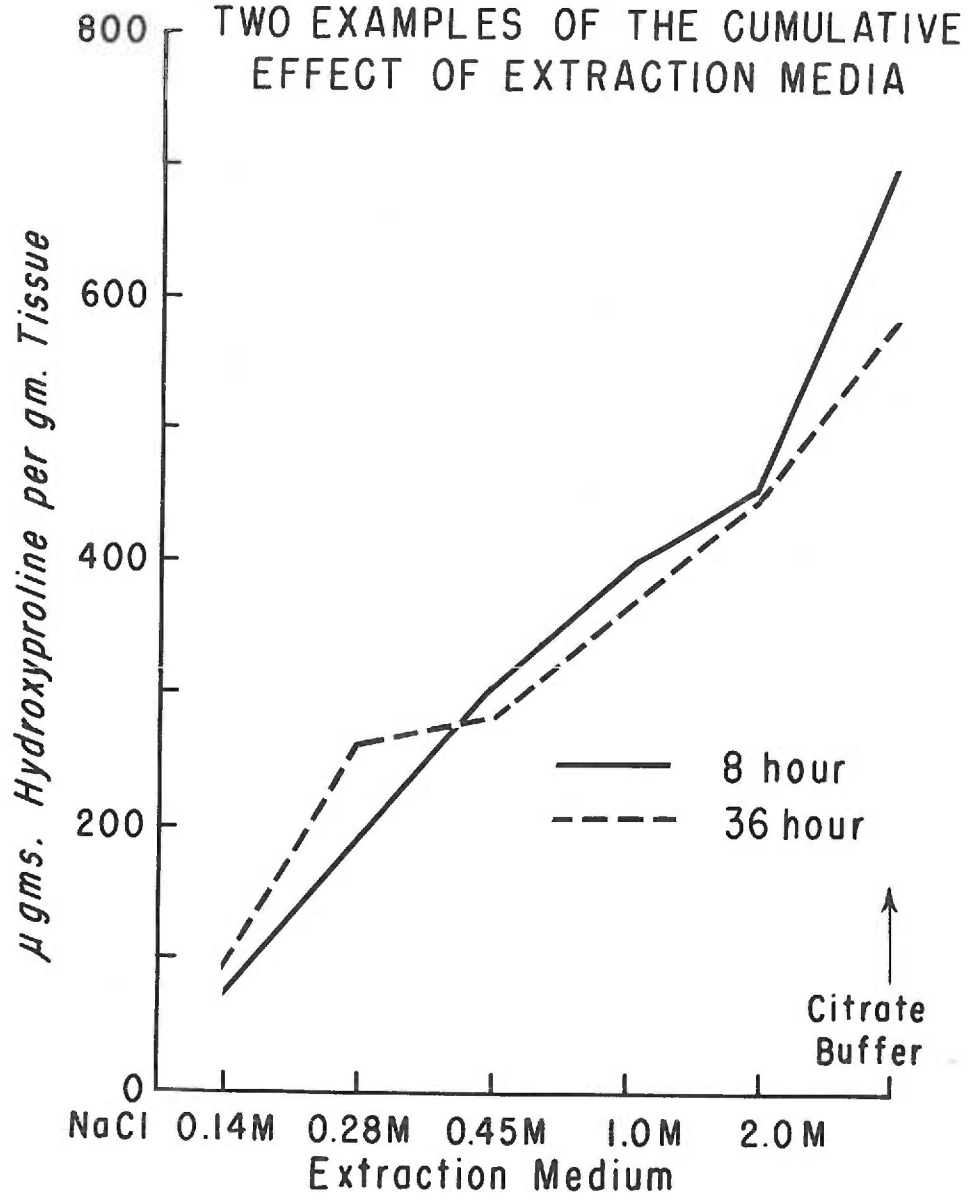

TWO EXAMPLES OF THE CUMULATIVE EFFECT OF EXTRACTION MEDIA
800
600
400
200
0
μ gms. Hydroxyproline per gm. Tissue
8 hour
36 hour
Citrate Buffer
NaCl 0.14M 0.28M 0.45M 1.0M 2.0M
Extraction Medium

Fig. 5

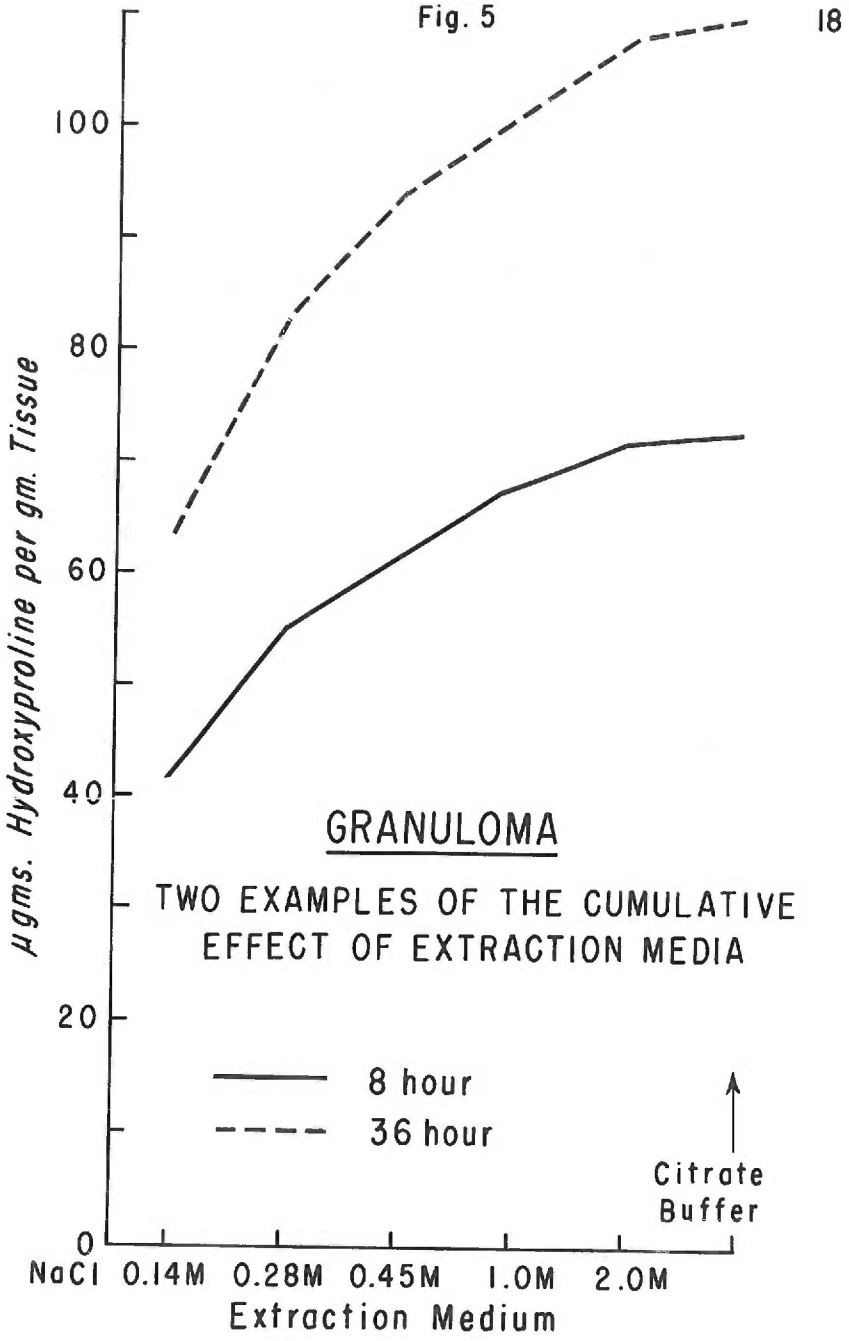

100
80
60
40
20
0
µgms. Hydroxyproline per gm. Tissue
GRANULOMA
TWO EXAMPLES OF THE CUMULATIVE EFFECT OF EXTRACTION MEDIA
8 hour
36 hour
Citrate Buffer
NaCl 0.14M 0.28M 0.45M 1.0M 2.0M
Extraction Medium

TABLE 2

Specific activity (c.p.m./microcule glycine) of collagen fractions from skin and granulomata at various time intervals following the injection of C^{14} glycine.

SKIN

Extraction medium	8 Hours	10 Hours	24 Hours	36 Hours
0.14 M NaCl	1970	750	-	625
0.28 M NaCl	1060	1410	993	528
0.45 M NaCl	710	-	1145	693
1.0 M NaCl	370	420	904	615
2.0 M NaCl	300	225	332	512
Citrate	62	63	172	88
Gelatin	0	0	0	0

GRANULOMA

Extraction medium	8 Hours	10 Hours	24 Hours	36 Hours
0.14 M NaCl	2785	1072	820	747
0.28 M NaCl	1528	2655	760	1248
0.45 M NaCl	1365	-	1321	831
1.0 M NaCl	1010	2231	2952	825
2.0 M NaCl	895	1451	1348	493
Citrate	-	-	976	552
Gelatin	190	-	-	550

Results

Total Extractable Collagen

More collagen was extracted from the granuloma with 0.14 M NaCl than with any other concentration. The amount extracted fell steadily with increasing ionic strength of the extraction medium (Fig. 3). 0.28 M NaCl was the most effective extraction medium for skin, measured by the total amount of collagen extracted (Fig. 2). The amount fell with increasing ionic strength of extraction medium but rose again when citrate buffer was used. Total gelatin was not measured since it would have been large, and consequently meaningless in comparison with the soluble fractions.

Incorporation of C^{14} Glycine

Eight hours after injection of the tracer, the maximum specific activity in the skin collagen was in the 0.14 M NaCl extract. The specific activity then fell with increasing ionic strength of extraction medium (Table 2). At ten hours the maximum was found in the 0.28 M NaCl extract, at 24 hours in the 0.45 M NaCl extract and at 36 hours in the 0.45 M NaCl extract. No activity was found in the gelatin fractions at any time.

The specific activities of collagen extracted from the granuloma follow a similar pattern, the

peak activity at 8 hours being in the 0.14 M NaCl extract and progressing with time into the 1.0 M NaCl extract. At 36 hours however, the maximum activity was again in the 0.28 M NaCl extract.

Discussion

In aqueous solutions the maintenance of an intact collagen fiber structure is determined by the balance between the swelling pressure and cohesive forces between adjacent molecules. When the swelling pressure exceeds the cohesive forces, the collagen molecules go into solution (16).

Neutral Salt Effect

The effect of neutral salt solutions on collagen is effectively a salting in process (17) and produces comparatively weak disruptive forces upon the fiber. Increasing the ionic strength of the salt solution, will, within limits, "salt in" increasing amounts of collagen and this is borne out in this study since after extraction with any one concentration of salt, further amounts of collagen are solubilized if the concentration of salt is increased. It appears from figures 2 and 3 however, that a solution of high ionic strength is a less effective extraction medium than one of lower ionic strength but this data is on a sequential type of extraction pattern. If for

example 0.45 M NaCl were used without prior extraction of the tissue with 0.14 M and 0.28 M, it would solubilise much more than it does with prior extraction (Figs. 4 and 5).

Swelling Phenomenon

Collagen contains a fair number of strong positively and negatively charged side chains which partially compensate and attract one another. Gustavson (18) gives the following values for these charged groups:

Total basic groups	0.94 mgm. equivalents/gm.
Free carboxyl groups	0.79 mgm. equivalents/gm.
Amide groups	0.47 mgm. equivalents/gm.

When a high concentration of hydrogen ions is present the charge on the corresponding negative groups will be discharged. A number of ionic groups will thus be freed from internal compensation and a Donnan Membrane potential set up inside the fibers. This will lead to an inflow of water from the surrounding medium and the fiber will swell (19).

Thus citrate buffer pH 3.6 applies greater disruptive forces than neutral salt solutions and will bring into solution molecules which were bound more firmly into the fibers.

Even greater disruptive forces can be applied by gelatinisation and fibers which will resist prolonged extraction in citrate buffer pH 3.6 can

usually be brought into solution by autoclaving.

The methods used for the extraction of collagen molecules from the tissue thus present varying disruptive forces to the fiber and Figs. 3 and 4 showing the cumulative totals indicates that, the order of strength of these disruptive forces is citrate $>$ 2.0 M $>$ 1.0 M $>$ 0.45 M $>$ 0.28 M $>$ 0.14 M. One can therefore conclude that the material extracted with 0 14 M represents the molecules which were least firmly bound and that gelatinisation would extract the most firmly bound collagen.

Incorporation of C^{14} Glycine

Eight hours following injection of the labeled glycine, the maximum specific activity of the skin collagen is found in the 0.14 M NaCl extract and it decreases as the ionic strength of the extraction medium is increased.

Thus most of the collagen which was formed during this eight hours, is now bound so loosely in the fiber that it can be extracted with the medium possessing the weakest disruptive forces. It may even be existing in solution in the intercellular space. As time after isotope injection increases the maximum specific activity is found in the fractions

extracted with progressively higher ionic strengths, and one can assume that the newly synthesised molecules are becoming more firmly bound in the fiber as time after their formation progresses. This continuous process will give rise to a continuous spectrum of collagen aggregates with varying degrees of cross-linking. Any of the extraction media will be effective in bringing into solution a cross section of these aggregates which will contain molecules of varying ages. The greater the disruptive properties of the medium used, the greater will be the heterogeneity of age of molecules extracted (if the medium is used without prior extraction of the tissue). Thus Orekhovitch (40) and Harkness et al (20) found apparent precursors in their first extract even though their extraction media differed considerably.

The radioactivity data from the granuloma is not as clear cut as that from the skin, probably due to the fact that collagen fibers formed in the granuloma are broken down again (47). Jackson (23) showed that as the granuloma was being reabsorbed the proportion of neutral salt soluble collagen increased and was presumably due to breakdown of collagen fibers into smaller aggregates. The specific activity of soluble collagen derived from natural breakdown would differ from that of newly synthesised material and

would thus obscure the data to some extent.

The apparent disparity between the solvent properties of the same medium in skin and granuloma may be explained by the great difference in age of the two tissues, the granuloma being much younger than skin and consequently containing relatively more recently formed collagen, which is extractable with solutions of low ionic strength. This is in agreement with the fact that the amount of soluble collagen in skin declines with decreasing growth rate of the animal (34, 12).

Collagen Fibrogenesis

From the above biological evidence one can formulate a hypothesis of the process of fiber formation which agrees with that which Gross formulated from in vitro studies (14). Green and Louther (10) have isolated collagen from microsomal fraction of fibroblasts which has an extremely rapid turnover. This suggests that the collagen molecule is formed, as would be expected, inside the cell. Porter (42) Yardley (50) and Fitton Jackson (26) have published electron microscope photographs of collagen fibrils on the surface and possibly inside fibroblasts and actually being extruded from the cell. These protofibrils appear to gather material from the intercellular spaces by accretion (26) probably

acting as templates (40). From the time these fibrils are formed, two things are occurring, the intermolecular cross links are becoming stronger, and more collagen molecules are being laid down on the surface. Extraction with 0.14 M NaCl will bring into solution the very earliest fibrils formed, together with newly synthesised molecules laid on the surface of older fibrils. As time progresses, any one collagen molecule will become buried ever deeper in the fiber and it will become more firmly bound to its neighbors as it moves into the most favorable steric apposition to them under the influence of thermal agitation.

The whole process may be compared to the building of a brick wall. The latest bricks to be laid are easily removable. It becomes progressively difficult to remove bricks in lower courses as time progresses and the "interbrick cross links" (cement) becomes firmer.

PART III

Work is proceeding in the wound healing laboratory of the Department of Surgery at the University of Oregon Medical School on the relationship between inflammation and fibroplasia, and it has been shown that injection of bacterial pyrogens into guinea pigs carrying subcutaneous vinyl sponges causes an increase in the rate of collagen production in these sponges (9). The pyrogen also gives rise to a marked leucocytosis in the order of 4-6 times normal (33). This may be interpreted as lending support to one, or all of the three main theories connecting leucocytes with collagen production.

Algower (1) considers that some leucocytes particularly monocytes are capable of producing a connective tissue network either directly or by becoming transformed into fibroblasts.

Kelsall and Crabb (27) have presented evidence which indicates that lymphocytes act as storehouses of nucleoprotein and other substances which can be transported to a site where active anabolism is proceeding and then be given up to the other, dividing, cells. Carrel (5) felt that the presence of leucocytes has a stimulating effect upon the division and growth of other cells, such as fibroblasts. This theory could of course be

incorporated into the theory of Kelsall and Crabb. These three theories, together with the work of Freihoffer et al (9) prompted the following investigation into the effect of local leucocyte concentration upon collagen production in a subcutaneous sponge in the guinea pig.

Materials and Methods

Twelve guinea pigs weighing 300-350 gms. were fed a stock diet (see appendix 2) supplemented with 50 mgm. ascorbic acid orally every other day. Four tared circular polyvinyl (Ivalon) sponges, 1 cm. diam, and about 2 mm. thick were implanted subcutaneously on the back of each animal (3, 8). For four days after implantation, two of the sponges were injected daily through the skin with 0.2 mls. of leucocyte suspension containing about 50,000 cells/cu.mm. in guinea pig plasma and two control sponges were injected with guinea pig plasma alone. The leucocyte suspension was prepared by the method of Li and Osgood (32) in which erythrocytes are agglutinated by addition of a phyto hemagglutinin extracted from red kidney beans. Slow centrifugation removes the erythrocytes leaving a suspension of nucleated cells.

At intervals of 6, 10, 14 and 18 days, three animals were killed and the sponges dissected out and dried by placing in several changes of acetone.

TABLE 3

Effect of leucocyte injections on collagen formation in subcutaneous sponges.

Collagen concentration is expressed as micrograms of hydroxyproline per 100 mgms. of new tissue.

Animal	Treated sponges	Untreated sponges	Mean Values
	6 days		Six days
A	90.9	47.3	Treated 82.5 ± 19.3
	65.6	71.4	Untreated 83.6 ± 21.4
B	101.0	103.9	
	70.2	101.4	
C	110.3	---	
	57.3	93.9	
	10 days		Mean Values
D	234.3	229.2	
	361.6	406.3	Ten days
E	607.1	404.5	Treated 427.6 ± 149.6
	289.2	316.3	Untreated 426.7 ± 182.1
F	443.8	395.7	
	629.8	808.7	
	14 days		Mean Values
G	905.0	973.6	Fourteen days
	819.3	607.3	
H	748.8	518.0	Treated 740.9 ± 104.0
	579.4	716.4	Untreated 739.2 ± 160.6
I	666.6	703.1	
	726.4	917.0	
	18 days		Mean Values
J	936.4	1096.3	Eighteen days
	941.7	1261.7	
K	686.2	756.7	Treated 855.8 ± 112.3
	958.3	899.3	Untreated 928.1 ± 189.3
L	---	788.0	
	756.4	766.6	

The sponges were dried in vacuo over P_2O_5 and weighed in order to determine the dry weight of tissue in the sponge. Whole sponges were hydrolysed in sealed tubes with 6N HCl at 138° C and the hydroxyproline determined (30). No attempt was made to isolate the collagen since Levine and Gross (31) showed that direct tissue hydrolysis is adequate.

The mean values in micrograms of hydroxyproline per 100 mgms. of tissue produced are shown in Table 3. They are strikingly similar for the treated and untreated sponges; so much so in fact that no statistical evaluation was considered necessary for any but the 18 day results. These also showed no differences at any tabled level of significance.

Discussion

The entirely negative result of such a small series of experiments is in no way meant to cast doubts upon the monographs of Algower, Kelsall or Carrel, but is at variance with them.

A factor which may influence these results is the extreme difficulty of preventing a certain amount of clumping of cells during their separation. This may affect the viability of these cells or their migration within the sponge. It also precludes the preparation of a uniform suspension.

The cells produced in response to bacterial endotoxin may conceivably differ in their characteristics from normally circulating white cells, and methods of obtaining viable suspensions of these cells are now being sought in this laboratory.

S U M M A R Y

1. The granuloma forming properties of five samples of carageenin were compared. Lambda carageenin gave rise to the greatest production of collagen.

2. Five concentrations of sodium chloride solutions and citrate buffer were compared on the basis of their ability to solubilise collagen from carageenin granuloma and skin.

Uptake of C^{14} glycine into collagen extractable with these solutions was compared at four time points. The hypothesis is suggested that continuous accretion of newly formed molecules upon pre-existing fibrils is the process of collagen fiber formation.

3. The effect of local concentrations of leucocytes on collagen formation in subcutaneous sponges was investigated. No effect was demonstrable with the techniques used.

APPENDIX I

Preparation and Separation of Dinitro Phenyl (DNP) Amino Acids - After Perrone (41)

To N gms. of protein hydrolysate dissolved in 40 N mls. water containing 3 N gms. $NaHCO_3$ are added 3 N gms. of 2, 4 - Dinitro Fluoro Benzene (FDNB) in 240 N mls. of absolute ethanol. This mixture is shaken mechanically in flasks equipped with glass chimneys for two hours at room temperature.

The mixture is diluted with 2 vols. of water and extracted three times with deperoxidised (washed) ether (49), in order to remove excess FDNB. At alkaline pH the DNP amino acids are preferentially soluble in water. The mother liquor is then acidified to congo red paper with concentrated HCl whereupon a distinct change in color appears, from a bright yellow to a pale lemon yellow. This solution is then re-extracted with washed ether until the aqueous layer is relatively colorless. The ether now containing the DNP amino acids is evaporated to dryness, first on a steam bath and then in vacuo. Care must be taken at this stage to protect the solution from excessive heat and from direct sunlight in order to prevent degradation of the product.

The DNP amino acid mixture can be stored dry until the column is prepared, it is then dissolved in a minimum quantity of 10% washed ether in washed chloroform for adding to the column.

Preparation of column:

100 gm. Celite 545 (Johns Manville Corp.) are thoroughly mixed with 65 mls. of 1 M NaH_2PO4 in a large bowl. It is then made into a slurry with 10% ether-chloroform, in which state it may be stored.

Uniform bore thick walled glass tubing is used. The celite is packed down with a perforated metal plunger and thus no constriction at the lower end is needed. Columns of 1.0 x 30 cm., 2.4 x 60 cm. and 3.6 x 75 cm. dependent upon the amount of material to be separated have been used successfully by the author, using plungers of varying sizes. Up to 500 mgm. of hydrolysate may be separated on the large columns.

The celite slurry is poured into a tube, corked at the bottom, and held vertically in a clamp. All air bubbles are expelled by moving the plunger up and down through the slurry. When all the bubbles have been removed, the plunger is moved slowly downwards and a portion of the celite (about 1 cm.) is packed firmly, the corked end of the tube being supported at this stage by the bench.

Further portions are packed until a column of celite about 18 to 25 cm. is obtained. It is most important that even packing be accomplished or uneven separation will result.

Separation of DNP Glycine

The cork is removed from the tube and the solvent allowed to drip out until the top surface of the celite is just dry. The sample, dissolved in ether-chloroform, is now added, in volumes ranging from 2 mls. for the 1 x 30 cm. column, up to 10 mls, for the 3.6 x 75 cm. column. When the surface is again just dry a small portion (2-3 mls.) of solvent is added and allowed to run through. This is repeated two or three times until no color is seen in the added solvent. The column may now be filled with solvent and the run commenced. The mixture separates into three bands, one, the fastest moving is discarded. The second band consists exclusively of DNP glycine and the third, very slow band is a mixture of other amino acids. (It has been found practical to speed up the separation by applying gentle pressure from a nitrogen cylinder to the top of the column.)

Determination of DNP glycine (29)

The DNP glycine is diluted to a known volume with 10% ether in chloroform and its optical density at 450 m compared with a curve obtained from DNP glycine made from pure glycine. The DNP glycine standard may be recrystallised from Dioxan by adding hot cyclohexane (36).

Very small samples are determined by shaking the effluent from the column with 3% ammonium hydroxide which extracts the sample completely from the ether chloroform. The O.D. of this solution is measured at 315 m and compared with a standard curve.DNP glycine has a much higher extinction coefficient in this solvent and hence solutions which are too dilute to measure in the ether chloroform solution can be determined.

APPENDIX 2

STOCK DIET AS USED IN ALL EXPERIMENTS

MacDonald Guinea Pig Basal Diet #5 (6)
(Compounded for us by Ralston Purina Co., St. Louis, Mo.)

Ground Oats	15%
Ground Wheat	13% (See note 1)
Ground Dried Beet Pulp	25%
Linseed Oil meal	12.5% (See note 2)
Skim milk powder	15%
Fish meal	5%
Brewers dried yeast	10%
Bone Char	4% (See note 3)
Salt (iodized)	0.5%

NOTES:

1. 5 lbs. of this may be replaced if desired by an equal weight of molasses for greater ease in pelleting.

2. Purina substitute solvent extracted soy bean meal on an equivalent protein basis.

3. Purina substitute diCalcium phosphate to give equivalent calcium and phosphorous levels.

4. If animals are to be maintained on this diet for prolonged periods they should be given supplemental vitamins A, D and E. It is of course completely lacking in vitamin C.

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