The present work with castrates has shown that the effect of cholesterol in males is not controlled by their secondary sex hormones. However, it is of primary importance to elucidate the precise mechanism of the cholesterol-vitamin A effect in male rats before attempting to discover why it is not manifested in females.

SUMMARY

1. The effects of a dietary supplement of cholesterol on liver vitamin A stores have been determined for normal and castrated young male rats.

2. When vitamin A and cholesterol were given in the diet together, liver vitamin A stores were significantly lowered to the same degree in normal and castrated animals. No significant changes in kidney or plasma vitamin A levels were observed.

3. If the animals were given large doses of vitamin A before cholesterol was given in a vitamin A-free diet, liver vitamin A stores were not affected in either normal or castrated rats.

4. Castration significantly decreased the rate of utilization of liver vitamin A in young male rats.

5. In castrates receiving a cholesterol-free diet liver unsaponifiable material was markedly reduced.

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The Chemical Composition of Snail Gelatin

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Early work on the distribution of collagen in the animal kingdom, mainly based on X-ray studies, was considered by Bear (1952) and Rudall (1955). Watson (1958*b*) has reviewed the chemical analyses available for invertebrate collagens, which at that time were restricted to the cuticle of earthworm (*Lumbricus* sp.) (Watson, 1958*a*), the cuticle of *Allobophora longa* (Singleton, 1957), the byssus threads of *Mytilus edulis* (Jackson *et al.* 1953), and the spongin fibrils from the mesogloea of sponges (Gross, Sokol & Rougvie, 1956).

Since Watson's review, analyses have appeared of the cuticle of *Ascaris lumbricoides*, and of the cuverian tubules of sea cucumber (*Holothuria forskali*), both by Watson & Silvester (1959), and a preliminary study was made of the body wall of the garden snail (*Helix aspersa*) by Melnick (1958). More recently, Piez & Gross (1959) have reported the analyses of *Thyone* body wall (Echinodermata), of *Metridium* skin and *Physalia* float (Coelenterata), and of spongin A and spongin B from *Spongia graminiae* (Porifera). This present paper is concerned with the chemical composition of gelatin derived from the intact collagen of the body wall of the garden snail.

EXPERIMENTAL

Materials

Snail gelatin. The 200 mg. (air-dried weight; moisture 12.4%) sample of gelatin used was prepared from the body walls of 30 garden snails by Melnick (1958). The snails were killed by immersion in aqueous 10% (v/v) ethanol for 2 hr. The shells were removed and each body was opened by cutting along the dorsal ridge from the mouth to the collar. The viscera were removed and the body walls soaked in 5% (w/w) hydrochloric acid to coagulate the mucus. The material was then soaked in 0.5% sodium chloride solution for a week followed by a short treatment in saturated lime water. The material was extracted by warming in water for 2 hr. at 70°, 80° and 90°. Each extract was filtered and concentrated *in vacuo* over phosphorus pentoxide. The gelatin used for analysis was that extracted at 90°.

Analytical methods

Hydrolysis. The gelatin (100 mg.) was hydrolysed with 20 ml. of 20% (w/w) hydrochloric acid at 100° in a sealed tube for 24 hr.

Amino acid analysis. The analysis was carried out by the method of Moore & Stein (1951) with the modification suggested by Eastoe (1955); the corrections applied were those used by Eastoe. Hydroxyproline was estimated as by

Table 1. Amino acid composition and related analytical data for snail gelatin

	Amino acid		Amino acid N as % of total N	No. of residues of amino acid per 1000 total residues of amino acids
	(g./100 g. of dry, ash-free sample)	(moles/10 ⁶ g.)		
Alanine	5.69	63.8	5.82	72.3
Glycine	21.24	283.1	24.95	321.0
Valine	2.22	18.97	1.75	21.5
Leucine	3.02	20.74	2.07	23.5
Isoleucine	1.40	10.64	0.97	12.1
Proline	10.57	91.8	8.34	104.1
Phenylalanine	1.45	8.75	0.78	9.9
Tyrosine	1.41	7.78	0.71	8.8
Serine*	5.70	54.2	4.92	61.4
Threonine*	2.90	24.4	2.2	27.7
Cystine	0.00	0.00	0.00	0.00
Methionine†	0.16	1.07	0.13	1.2
Arginine	7.88	45.6	16.43	50.9
Histidine	0.36	2.33	0.65	2.6
Lysine	1.07	7.30	1.33	8.3
Aspartic acid	7.84	58.9	5.37	66.8
Glutamic acid‡	12.85	87.4	7.89	99.1
Hydroxyproline	11.50	87.7	7.95	99.5
Hydroxylysine	1.14	7.2	1.29	8.2
Amide N§	0.69	49.2	4.46	—
Total	99.09	930.9		
Glucosamine	0.17	0.98	0.06	—
Galactosamine	0.48	2.66	0.26	—
Hexose	12.10	—	—	—
Total	12.75			
Total N	15.47 (g./100 g. of dry, ash-free material)			
Mean residue wt.	93.6			
Recovery by wt. (%)	94.7			
Recovery of N (%)	98.3			
Moisture (%)	12.4			
Ash (%)	0.76 (on air-dried material)			

* Corrected for decomposition during hydrolysis (Eastoe, 1955).
† Sum of methionine and methionine sulphoxide peaks.
‡ Corrected for decomposition on the column (Moore & Stein, 1951).
§ Corrected for ammonia formed by decomposition of threonine and serine (Eastoe, 1955).
|| Chibnall (1942).

Neuman & Logan (1950). Total nitrogen was estimated by the Kjeldahl method.

Moisture. The loss of weight on drying the gelatin at 105° for 24 hr. was used to calculate the moisture content.

Ash. The gelatin was ignited with concentrated sulphuric acid in a platinum dish, then heated at 550° to constant weight and the residue weighed as sulphated ash.

Estimation of hexose. Hexose was estimated by the anthrone reaction (Dreywood, 1946), with the modification of Bangle & Alford (1954). The hexose is expressed as galactose, this sugar being used for the preparation of the standard curve.

Estimation of hexosamine. Glucosamine and galactosamine were estimated directly from the peaks on the amino acid chromatograms as suggested by Eastoe (1954). Watson (1958a) reports that this method gives rather low and variable values but there was insufficient material available for the independent determination by another method.

Estimation of pentose. Pentose was estimated by the orcinol reaction (Mejbaum, 1939), with the modification of

Albaum & Umbreit (1947). The pentose was expressed as arabinose, this sugar being used for the preparation of the standard curve.

RESULTS

A summary of the amino acid composition and related analytical data is given in Table 1. The number of residues of a given amino acid per 1000 total residues, calculated as by Eastoe & Leach (1958), is also given to enable comparisons to be made with other gelatins and collagens.

When the gelatin was hydrolysed for the determination of amino acids a considerable amount of insoluble humin was formed and the hydrolysate was dark in colour. This is contrary to the observations made by Melnick (1958), who obtained light-coloured hydrolysates and assumed the gelatin samples to be largely free of polysaccharide.

The hydrolysate was filtered to remove the humin and the analysis was carried out on the filtrate. The presence of humin suggested large amounts of polysaccharide components (Watson & Silvester, 1959). The presence of polysaccharide was confirmed by the detection of hexosamines, common constituents of mucopolysaccharides, on the amino acid chromatograms, both galactosamine and glucosamine being detected on the short column of the amino acid separation (Eastoe, 1954), and also by the detection of a peak of red material at about fraction 42 on the long column of the amino acid separation, indicating the emergence of carbohydrate-decomposition products (Dustin, Czajkowska, Moore & Bigwood, 1953).

DISCUSSION

A common chemical feature of gelatins derived from vertebrate and invertebrate collagens is the high number of glycyl residues. This number, 321 in every 1000 amino acid residues, was also found in snail gelatin. A number of chemical features characteristic of vertebrate gelatins were found. The prolyl and hydroxyprolyl contents are of the same order; there is one hydroxylic side chain in every five residues compared with one in six in vertebrates.

The most striking feature, however, is the presence of hydroxylysyl residues. The hydroxylysine content is in fact higher than that of most vertebrate collagens and gelatins. Eastoe & Leach (1958) surveyed the amino acid analyses available for vertebrate collagen and gelatin and found that those samples on which hydroxylysine estimations had been carried out all contained hydroxylysyl residues in amounts varying from 3.5 to 12.2 residues per 1000 total residues. Of the invertebrate collagens and gelatins reviewed by Watson (1958b) only the collagen derived from the cuverian tubules of the sea cucumber had been found to contain hydroxylysyl residues (4.7 per 1000 total residues). Piez & Gross (1959), however, detected hydroxylysine in all the invertebrate gelatins that they studied, *Metridium*, *Physalia* and spongin B having 25, 30 and 24 hydroxylysyl residues per 1000 residues respectively, the highest yet reported for any collagen or gelatin. The amount found by them in *Thyone* gelatin and spongin A, 11 and 12 residues per 1000 residues respectively, is of the same order as that found in snail gelatin.

Although snail gelatin is similar to vertebrate gelatins in amino acid composition it resembles the invertebrate gelatins in the low total nitrogen content. This may be explained by the presence of considerable amounts of polysaccharide. The polysaccharide distribution is comparable with that found in some other invertebrate collagens and

gelatins (Watson, 1958a; Watson & Silvester, 1959), hexoses being present in considerable amounts together with a smaller amount of hexosamines. A small amount (0.66 g./100 g. of dry, ash-free material) of pentose was also estimated, but by the orcinol method, which is not entirely specific. There was insufficient material left to confirm the presence of pentose or for the determination of uronic acids.

Generally, vertebrate collagens and gelatins have low polysaccharide contents. The cuticle of *Ascaris lumbricoides* (Watson & Silvester, 1959) is similar in this respect, but collagens from earthworm cuticle (*Lumbricus* sp.; Watson, 1958a) and the cuverian tubules of *Holothuria forskali* (Watson & Silvester, 1959) contain large amounts of polysaccharide. There is insufficient evidence available to show that the polysaccharide found in these materials and in snail gelatin forms an integral part of the collagen from which they were derived.

SUMMARY

1. A gelatin fraction isolated from the intact collagen of the body wall of *Helix aspersa* was submitted to chemical analysis.
2. The analysis showed that the gelatin contains a high proportion of glycyl and hydroxyprolyl residues. The proportion of hydroxyproline is similar to that of proline. There are also a number of hydroxylysyl residues present. The amino acid composition of snail gelatin resembles vertebrate gelatins more closely than the invertebrate gelatins previously studied.
3. The remainder of the gelatin is polysaccharide and yields galactose and smaller amounts of pentose and hexosamines on hydrolysis.

The author is indebted to Mr S. C. Melnick for the sample of snail gelatin. The author is very grateful to Mr A. A. Leach of The British Gelatine and Glue Research Association for advice on the Moore & Stein (1951) technique and for much valuable discussion.

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A Native Cobalamin-Polypeptide Complex from Liver: Isolation and Characterization

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In mammalian liver vitamin B₁₂ and related substances occur mainly as weakly bound protein or polypeptide complexes. These bound forms should probably be regarded as the biologically functional units rather than the free cyanocobalamin (Smith, 1958). Very little information, however, is as yet available about the nature of these complexes.

In a preliminary note from this Laboratory, the isolation of a cobalamin polypeptide from a liver concentrate (Organon WBC) was described (Hedbom, 1955). Because further supplies of this particular raw material were not available, we have now developed a method for obtaining a similar concentrate from fresh ox liver. From this it has been possible to isolate a cobalamin polypeptide, with essentially the properties previously described, in quantities which enable us to perform a more detailed examination.

This paper deals mainly with the isolation procedure and characterization of the cobalamin polypeptide.

EXPERIMENTAL AND RESULTS

Isolation of the cobalamin-polypeptide complex

No method of assay which is specific for the vitamin B₁₂ conjugates in question is yet known. Therefore, the isolation could be guided only by determination of the total vitamin B₁₂ content.

In the starting material and the first fractions from the isolation procedure, the vitamin B₁₂ activity was detected and estimated by microbiological assay. Various extraction methods were tested in order to find the optimum conditions for the liberation of cyanocobalamin from the

samples without destruction, and different micro-organisms requiring vitamin B₁₂ were used and compared for the estimation. A modification of Burkholder's (1951) *Escherichia coli* tube method was found to be satisfactory for the purpose.

In more concentrated fractions the vitamin B₁₂ content was estimated by absorption spectrophotometry, or by cobalt determination, as a check on the microbiological determination.

Microbiological assay

Preparation of samples. The vitamin B₁₂ was released from the protein moiety by papain digestion. A sample (1 g.) was suspended in 10 ml. of 0.1M-sodium acetate buffer, pH 5.0, containing 50 mg. of papain (E. Merck, Darmstadt) and a trace of cyanide. The mixture was incubated for 3 hr. at 40° and subsequently diluted with water to a total volume of 50 ml. After filtration and appropriate dilution, samples of the solution were assayed for vitamin B₁₂ activity as described below.

Test organism. This was *Escherichia coli* mutant 113-3 (Davis & Mingioli, 1950). Stock cultures were stored and the inoculae prepared as described by Burkholder (1951).

Basal medium. The medium (in fivefold concentration) contained, in 200 ml. of water: K₂HPO₄ 7.0 g., KH₂PO₄ 3.0 g., trisodium citrate 0.5 g., MgSO₄·7H₂O 0.1 g., (NH₄)₂SO₄ 1.0 g., glucose 10.0 g., thiomalic acid 0.1 g., L-asparagine 4.0 g., L-arginine 0.1 g., L-glutamic acid 0.1 g., glycine 0.1 g., L-histidine 0.1 g., L-proline 0.1 g., DL-tryptophan 0.1 g.

The pH was measured with a Beckman glass-electrode pH meter, and adjusted, if necessary, to 7.0, by addition of KOH. In each tube 1 ml. of medium was used.

Vitamin standard. This was a water solution containing 0.200 µmg. of cyanocobalamin/ml., prepared fresh before each assay from a stock solution containing 2 µg. of cyanocobalamin (on a colorimetric basis) and 2 mg. of KCN/ml. The standard solution was added in duplicates to assay tubes in the volumes 0, 0.2, 0.5, 1.0, 1.5, 2.0 and 4.0 ml. per