

determined, and the percentage of the ingested carotene that disappeared during its passage through the alimentary tract was calculated from twelve experiments with a total of 237 rats.

3. In eleven experiments the amount of carotene that disappeared was greater in males than in females; in one experiment only was the difference in the opposite sense. The mean of the twelve differences in percentage disappearance was 2.44 ± 0.78 .

4. There was no evidence that such factors as the nature of the vehicle in which the carotene was given, the proportions of the dietary components, or the vitamin A status of the rats, significantly affected the differences observed between the sexes.

REFERENCES

- Arnrich, L. & Morgan, A. F. (1954). *J. Nutr.* **54**, 107.
 Booth, V. H. (1950). *Biochem. J.* **47**, xliii.
 Booth, V. H. (1952). *J. Nutr.* **48**, 13.
 Booth, V. H. (1956). *Brit. J. Nutr.* **10**, 241.
 Booth, V. H. (1957). *Carotene, its Determination in Biological Materials*. Cambridge: W. Heffer and Sons Ltd.
 Fisher, R. A. (1950). *Statistical Methods for Research Workers*, 11th ed. Section 21. 1. London: Oliver & Boyd.
 Moore, T. & Sharman, I. M. (1950). *Biochem. J.* **47**, xliii.
 Moore, T., Sharman, I. M. & Ward, R. J. (1951). *Biochem. J.* **49**, xxxix.

The effect of ascorbic-acid deficiency on the concentration of acid mucopolysaccharides in guinea-pig skin and cartilage

By J. M. BOWNESS

Department of Biochemistry, University of Malaya, Singapore

(Received 18 September 1956—Revised 1 January 1957)

Previous histological studies have indicated that mucopolysaccharides are affected during scurvy in guinea-pigs. The evidence about the precise effect of ascorbic-acid deficiency on the metabolism of the mucopolysaccharides is, however, somewhat conflicting. A diminished content of acid mucopolysaccharides was found by Meyer (1944) in the collagen, and by Penney & Balfour (1949) in the early stages of healing wounds, in scorbutic as compared with normal guinea-pigs. Studying healing wounds, Bunting & White (1950) noted that mucopolysaccharide material persisted longer in scorbutic than in normal animals. Bradfield & Kodicek (1951) found mucopolysaccharide to be more abundant round the 'precollagen strands' of old scorbutic wounds than of non-scorbutic wounds (see also Kodicek & Loewi, 1955). Gersh & Catchpole (1949) concluded that acid mucopolysaccharides were depolymerized in the healing wounds of scorbutic guinea-pigs.

In studies with ^{35}S , Friberg & Ringertz (1954) found that the uptake of radioactive sulphate by guinea-pig tissues was reduced during scurvy. They suggested that it was

due to a reduced rate of formation of sulphomucopolysaccharides. Reddi & Nörstrom (1954) recorded a decreased uptake of radioactive sulphate-S by cartilage chondroitin sulphate in scurvy.

In this work, estimates of the concentration of acid mucopolysaccharides in normal and scorbutic guinea-pig skin and cartilage were made by determining the content of uronic acid in polysaccharide fractions of the homogenized tissues. In the cartilage, sulphate was also estimated. A procedure for homogenizing the tissues, utilizing a preliminary softening in strong urea solution, is described.

EXPERIMENTAL

Animals and diets. Four groups of nine young guinea-pigs (six male and three female), weighing 150–300 g at the beginning of the experiment, were studied. Animals in group C ('deficient' group) were fed on a scorbutogenic diet derived from those of Krehl (1951) and of Kirk & Tressler (1941). Marmite was substituted for the dried brewer's yeast and salt given in the diet of the latter authors. The approximate percentage composition of the scorbutogenic diet by weight was: rolled oats 40, wheat bran 26, casein 12.5, whole-milk powder 10, Marmite 5, red palm oil 4, cod-liver oil 1, CaCO_3 1, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5. Each animal in group D ('pair-fed' group) received daily the same amount of the scorbutogenic diet as the corresponding animal of group C, with the difference that the food was given to the group D animal on the following day. In addition, each animal in group D was given 20 mg ascorbic acid daily. Group A animals ('positive' group) were fed on an unlimited amount of the scorbutogenic diet and 20 mg ascorbic acid daily. Group B animals ('restricted food' group) were limited to 3–4 g of the scorbutogenic diet daily and 20 mg ascorbic acid; they received further a vitamin supplement, mixed daily with the food, in the following approximate amounts; thiamine hydrochloride 1 mg, riboflavin 1.6 mg, nicotinamide 8 mg, pyridoxine hydrochloride 1 mg, dried brewer's yeast 100 mg, cod-liver oil 0.1 ml.

Animals in groups A, C and D were killed and dissected 21–24 days after being placed on the diet. Group B animals were killed and dissected 9–12 days after being placed on the diet. If they were not killed, animals on the scorbutogenic diet alone died after 22–28 days and animals on the limited diet given to group B after 10–16 days.

Breakage of the glass tubes during homogenization of tissues obtained from some animals of groups C and D necessitated repetition of the experiment with three further animals in each of these groups. Breakages, due to a subsequently corrected fault in the alignment of the rotating steel shaft of the homogenizing apparatus (see p. 154), again occurred. For this reason three pairs of animals included in groups C and D in Table 2 are not the same as those included in the corresponding groups in Table 1.

Treatment of the tissues. The skin was freed of muscle and subcutaneous fat, shaved of hair and cut in pieces; costal cartilage was separated from adhering muscle. The cleaned tissues were weighed, treated with four 20 ml. portions of acetone, and dried at 85° for 30 h, when they were found to be virtually constant in weight.

Homogenizing the tissues presented some difficulty; several procedures were tried before the one described now was adopted as the most satisfactory. The use of aqueous solutions of KOH and NaOH as solvents for the tissues is known to effect some

degradation of the mucopolysaccharides (see Kent & Whitehouse, 1955); using these alkalis I found less glucuronic acid and sulphate in mucopolysaccharide fractions obtained from cartilage than when using the procedure described below. The ratio of sulphate to uronic acid found in the mucopolysaccharide material obtained by me from cartilage (see Table 2) agrees well with the theoretical 1:1 molar ratio for chondroitin sulphate. A trial of the procedure, by the addition of chondroitin sulphate (L. Light and Co.) to a mixture of dried skin and cartilage whose glucuronic-acid and sulphate contents were known approximately, showed a recovery of over 80% measured in terms of these two substances.

The dry tissues were cut into small strips and softened in 6M urea solution at 37°. The use of a urea solution as a dissolving and softening agent is based upon its use as a solvent for proteins (see Rudall, 1952). I obtained the most satisfactory homogenates with about 0.7 g dry skin softened in 10 ml. urea solution for 1 day, and about 0.2 g dry cartilage in 5 ml. urea solution for 2 days. The softened tissues with the urea solution were homogenized in an apparatus constructed in our laboratory. It consisted of a corrugated glass tube (for homogenizing skin the capacity was 60 ml. and the internal diameter 3.3 cm, for cartilage the capacity was 45 ml. and the internal diameter 3.0 cm) in which two stainless steel blades (each about 1.5 cm long) were rotated at about 15,000 r.p.m. by a high-speed grinder motor (Wolf Electric Tools Ltd, Type DG. 1A). The blades were attached by their centres to a vertical stainless steel shaft (diam. 6 mm) which passed through a cork stopper in the glass tube, was suspended from above by a bulge on the shaft carried between two sets of ball bearings, and was connected to the vertically mounted motor by a thick rubber tube. There were four corrugations lengthwise in the bottom half of each tube; each corrugation was made by denting the softened glass of the originally round tube inwards to a depth of 3-5 mm.

Separation and estimation of mucopolysaccharide materials. A mucopolysaccharide fraction was obtained from the homogenized tissues by a procedure based on parts of several standard methods used for separating mucopolysaccharides and mucoids from tissue extracts. The use of urea for extraction of submaxillary mucoïd and of trypsin digestion for removal of proteins from mucopolysaccharide fractions is described by Meyer (1945). Digestibility by trypsin, after mechanical disintegration, has been demonstrated for keratin by Routh (1938) and for collagen by Sizer (1949). I found that most of the protein precipitated by acidified ethanol (see below) from the urea-treated, homogenized tissues was digested by trypsin. Ethanol precipitation has been used to separate a number of mucopolysaccharides (see Kent & Whitehouse, 1955).

The homogenized tissues were mixed with three volumes of ethanol and 0.2 m-mole acetic acid and allowed to stand at 4° for 18 h. The sticky precipitate was separated by centrifuging, dispersed in 10 ml. 0.005N-NaOH and digested with trypsin (20 mg, Hopkin and Williams product) for 24 h. In the residue from the cartilage a few unhomogenized lumps remained undissolved during trypsin digestion; they were separated by slow centrifuging of the water-suspended residue, dried, weighed, and their weight was subtracted from the total weight of tissue. Skin was completely homogenized by the procedure.

The digest was centrifuged and the supernatant liquid mixed with three volumes of ethanol containing 0.2 m-mole sodium acetate and 0.3 m-mole acetic acid. After standing for 24 h at 4°, the precipitate was separated by centrifuging and the supernatant liquid discarded. The precipitate was dissolved in water and the uronic-acid content of a portion estimated by the carbazole method of Dische (1947), with minor modifications affecting the applicability to some mixtures of carbohydrates.

The sulphate content of further material from the cartilage was estimated by the method of Bray, Humphris, Thorpe, White & Wood (1952).

RESULTS AND DISCUSSION

Table 1 shows that the mean concentration in wet skin of mucopolysaccharides containing uronic acid was about 20% lower in scorbutic than in normal animals. For dried acetone-extracted skin the corresponding mean difference was about 12%.

Table 1. *Effect of restriction of calories and of scorbutogenic diet on the weights and on the acid-mucopolysaccharide concentration in the skin of groups of nine guinea-pigs**

Group	Weight of guinea-pigs during the experiment (g)		Weight of acetone-extracted dried tissue as percentage of that of wet skin	Glucuronic acid of skin-mucopolysaccharide fraction ($\mu\text{g/g}$ skin)	
	Beginning	End		Dry tissue	Wet tissue
A (unlimited diet)	211 \pm 67	232 \pm 78	31.6 \pm 2.3	864 \pm 93	273 \pm 19
B (restricted diet)	249 \pm 35	186 \pm 25	34.1 \pm 1.2	736 \pm 65	251 \pm 18
C (scorbutogenic diet)	208 \pm 61	164 \pm 24	32.6 \pm 2.2	693 \pm 41	224 \pm 16
D (scorbutogenic diet with ascorbic acid; pair-fed controls to group C)	188 \pm 56	197 \pm 48	33.9 \pm 2.2	799 \pm 37	272 \pm 17
(D - C) pair†	—	—	+1.34	+106	+47.6
differences {t	—	—	2.26	8.09	7.70

* Mean values and standard deviations, except in the two bottom rows.

† The *t* values calculated from the individual pair differences are higher than those that may be calculated from the group mean differences and variances.

Both differences are significant at the 0.1% level. Restricting the daily intake of the diet while continuing to supply adequate quantities of the vitamins, as for animals in group B, also appeared somewhat to reduce the acid-mucopolysaccharide content of skin. The *t* value for the difference in concentration in the wet skins of groups A and B was 2.59, which is significant at the 2% level. However, the difference in mean concentration between groups A (or D) and B was much less than that between groups D and C. On the other hand, the loss in body-weight, which, in the absence of any considerable hydration in the scorbutic animals presumably indicates the severity of the stress to which the animals were subject, was greater in group B animals than in the scorbutic animals of group C.

Table 2 shows that the mean concentration in wet cartilage of acid mucopolysaccharides, as judged by contents of both uronic acid and sulphate, was about 15% less for scorbutic animals than for normal ones. This difference is significant at the 0.1% level.

Table 2. *Effect of restriction of calories and of scorbutogenic diet on the weights and on the acid-mucopolysaccharide concentration in the cartilage of groups of nine guinea-pigs**

Group	Weight of guinea-pigs during the experiment (g)		Weight of acetone-extracted dried tissue as percentage of that of wet cartilage	Mucopolysaccharide fraction		
	Beginning	End		Glucuronic acid (mg/g cartilage)		Sulphate (mg/g wet cartilage)
				Dry tissue	Wet tissue	
A (unlimited diet)	211 ± 67	232 ± 78	37.9 ± 4.3	53.5 ± 4.3	20.1 ± 1.3	10.0 ± 0.7
B (restricted diet)	249 ± 35	186 ± 25	40.5 ± 3.7	52.5 ± 3.7	21.1 ± 1.2	11.5 ± 0.8
C (scorbutogenic diet)	201 ± 50	166 ± 25	36.9 ± 3.2	47.2 ± 5.7	17.4 ± 1.3	9.1 ± 1.1
D (scorbutogenic diet with ascorbic acid; pair-fed controls to group C)	178 ± 43	183 ± 39	38.9 ± 3.3	52.7 ± 5.6	20.4 ± 1.5	10.9 ± 1.3
(D-C) pair†	Mean	—	+2.01	+5.49	+3.02	+1.81
differences	<i>t</i>	—	4.99	5.35	6.02	6.16

* Mean values and standard deviations, except in the two bottom rows.

† The *t* values calculated from the individual pair differences are higher than those that may be calculated from the group mean differences and variances.

For dried, acetone-extracted cartilage the corresponding difference between the mean concentrations for the normal and the scorbutic animals was only about 10%. Animals with a restricted food intake showed the same concentration as normal animals.

Part of the decrease in the concentration of acid mucopolysaccharides in the wet scorbutic cartilage may have been connected with the decrease in the percentage of the tissue remaining after acetone extraction and drying; the *t* value in Table 2 shows the latter decrease to be significant at the 1% level. The work of Persson (1953) indicates that the water content of cartilage is increased during scurvy. It seems likely that a similar increase in water content could have been responsible for the decrease in percentage of dried and acetone-extracted matter in the cartilage of my scorbutic animals. In view of the significant difference in mean concentration of acid mucopolysaccharides between the scorbutic and the normal animals, evident even in the dried acetone-extracted cartilage, an increase in the water content of the tissue could account for only part of the observed decrease in acid-mucopolysaccharide content of the wet scorbutic cartilage.

The results of this work establish that acid mucopolysaccharides, though reduced in quantity, still occur in the skin and cartilage of scorbutic guinea-pigs. The precise identity of the acid-mucopolysaccharide fractions of both these tissues cannot, however, be regarded as entirely settled. Hyaluronic acid and chondroitin sulphate are, at present, the only mucopolysaccharides containing uronic acid known to occur in skin (see Sylven, 1956). Insufficient material was available for the estimation of sulphate contents of skin, and it was not found practicable to separate hyaluronic acid and chondroitin sulphate for the purposes of estimation. The figures given for skin thus relate to the concentration of a mixture of the acid mucopolysaccharides. In normal ox

cartilage, chondroitin sulphate appears to be the predominant, if not the only, mucopolysaccharide constituent (see Partridge, 1948). It seems likely, therefore, that in my experiments the estimated sulphate and uronic-acid contents of normal guinea-pig cartilage were directly related to the concentration of chondroitin sulphate. It is still possible that the acid mucopolysaccharide of scorbutic guinea-pig cartilage is not identical with that in normal cartilage, but the fact that the ratio of sulphate to uronic acid was virtually the same in both suggests that their chemical composition is similar.

The 15% decrease in the concentration of sulphated mucopolysaccharide of scorbutic cartilage observed by me is rather small compared with the 65% decrease in the radioactive sulphate-S taken up by chondroitin sulphate from scorbutic cartilage found by Reddi & Nörstrom (1954). It appears either that the uptake of radioactive sulphate-S measures not the rate of formation of the whole chondroitin-sulphate molecule but the rate of exchange of the sulphate group, or that the rate of formation, and possibly also the rate of destruction, of chondroitin sulphate is so slow that a decrease in rate as great as 65% does not greatly affect the concentration of the mucopolysaccharide in cartilage during the limited time for which scorbutic guinea-pigs survive.

SUMMARY

1. Four groups of nine guinea-pigs were fed on a scorbutogenic diet: one group received food *ad lib.*; a second group was pair-fed with the first group and received in addition 20 mg ascorbic acid daily; a third group received food *ad lib.* and 20 mg ascorbic acid daily; a fourth group was limited to 3–4 g food daily with 20 mg ascorbic acid and a supplement of other vitamins.

2. After 21–24 days the animals were killed and the mucopolysaccharide content of the skin and cartilage was estimated by determining the uronic acid in an alcohol-precipitated and trypsin-digested homogenate of these tissues. For the cartilage, sulphate was also determined.

3. The acid-mucopolysaccharide concentration in skin and cartilage was found to be significantly lower in scorbutic than in normal animals, the mean differences in concentration being, in wet tissues about 20% for skin and 15% for cartilage, and in dry tissues about 15% for skin and 10% for cartilage.

I wish to express my thanks to Professor J. W. H. Lugg for much helpful advice and to Mr Lim Yen Heng for valuable assistance in the animal feeding.

REFERENCES

- Bradfield, J. R. G. & Kodicek, E. (1951). *Biochem. J.* **49**, xvii.
 Bray, H. G., Humphris, B. G., Thorpe, W. V., White, K. & Wood, P. B. (1952). *Biochem. J.* **52**, 412.
 Bunting, H. & White, R. F. (1950). *Arch. Path.* **49**, 590.
 Dische, Z. (1947). *J. biol. Chem.* **167**, 189.
 Friberg, U. & Ringertz, N. R. (1954). *Exp. Cell Res.* **6**, 529.
 Gersh, I. & Catchpole, H. R. (1949). *Amer. J. Anat.* **85**, 457.
 Kent, P. W. & Whitehouse, M. W. (1955). *Biochemistry of the Aminosugars*. London: Butterworth.
 Kirk, M. M. & Tressler, D. K. (1941). *Food Res.* **6**, 395.
 Kodicek, E. & Loewi, G. (1955). *Proc. Roy. Soc. B*, **144**, 100.
 Krehl, W. A. (1951). Quoted by Bliss, C. I. & György, P. in *Vitamin Methods*, Vol. 2 [P. György, editor]. New York: Academic Press.

- Meyer, A. (1944). *Z. Vitaminforsch.* **14**, 322.
 Meyer, K. (1945). *Advanc. Protein Chem.* **2**, 249.
 Partridge, S. M. (1948). *Biochem. J.* **43**, 387.
 Penney, J. R. & Balfour, B. M. (1949). *J. Path. Bact.* **61**, 171.
 Persson, B. H. (1953). *Studies on Connective Tissue Ground Substance*. Uppsala: Almqvist and Wiksells.
 Reddi, K. K. & Nörstrom, A. (1954). *Nature, Lond.*, **173**, 1232.
 Routh, J. I. (1938). *J. biol. Chem.* **123**, civ.
 Rudall, K. M. (1952). *Advanc. Protein Chem.* **7**, 253.
 Sizer, I. W. (1949). *Enzymologia*, **13**, 288.
 Sylven, B. (1956). In *The Biochemistry and Physiology of Bone* [G. H. Bourne, editor]. New York: Academic Press.

A comparative study of the diets, and results of some blood analyses, of children living in different environments in Jogjakarta (Central Java)

BY YAP-KIE-TIONG

Yap Ophthalmic Hospital, Jogjakarta, Indonesia

(Received 15 October 1956)

The majority of the people living in the Jogjakarta area (Central Java) suffer from a type of chronic malnutrition which generally does not interfere with their ability to perform heavy labour. Studies of the eye changes in this type of chronic malnutrition have led to a comparative study of the diets and blood chemistry of children living in different economic environments. The chemical values can only be considered within certain limits as reliable in the appraisal of the nutritional status, owing to the possible presence of chronic infectious diseases such as malaria and intestinal worms, yet it seemed useful to carry out this comparative study in order to obtain a 'normal' local pattern. The investigation comprises (a) a study of the food intake of groups of children living in different economic environments, and (b) the determination of the concentration of serum albumin and globulin, and the levels of haemoglobin, vitamin A, carotene and calcium in the blood.

EXPERIMENTAL

Subjects. The boys and girls who were the subjects of this study were drawn from three groups: (1) twenty-five doctors' children, aged 6–17 years (mean 12 years); (2) thirty-three children living in an orphanage (not all orphans), aged 6–18 years (mean 14 years); (3) twenty-one children, aged 6–18 years (mean 13 years) living in a poor subdistrict of Jogjakarta, where the people show obvious stigmata of malnutrition. All children were of Indonesian origin.

Diet. The staple food of all the children was rice, the doctors' and the orphanage children having about 350 and the poor-area children about 175 g daily. The customary diet of the doctors' children included legumes, nuts, soya-bean products,