LXXXVII. THE VITAMIN C CONTENT OF SHEEP-LIVER; WITH OBSERVATIONS ON THE EFFECTS OF FREEZING AND STORAGE.

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PREVIOUS workers have shown that liver may contain an appreciable amount of vitamin C; *e.g.* rat-liver [Parsons, 1920]; chicken-liver [Hart, Steenbock, Lepkovsky and Halpin, 1925]; calf-liver [Thurston, Eckles and Palmer, 1929]; but no investigation of the antiscorbutic activity of sheep-liver has hitherto been carried out. The results of the experiments described below show this to be considerable, and in view of the fact that large quantities of livers are imported into this country in the frozen state from overseas it was of interest to investigate the effects of freezing and of storage on the vitamin C content. In the course of the work conclusions have been reached as to the influence of freezing in inactivating the vitamin.

1. VITAMIN C CONTENT OF SHEEP-LIVER.

Preparation of material. It proved impracticable to feed the liver directly to guinea-pigs receiving a scorbutic, but otherwise complete diet, since severe digestive trouble and loss of appetite rapidly ensued and the liver intake could not be controlled. Aqueous extracts of the liver did not have this effect, and were employed instead, the preparation being carried out as follows. Portions of sheep-liver were minced and passed through a fine wire sieve. The finely divided liver was then weighed into a bottle, and an equal weight of water added. The mixture was next shaken for 20 minutes in a mechanical shaker, the air having first been replaced by carbon dioxide to prevent possible inactivation of vitamin C by oxidation. The mixture was freed from particles of connective tissue by filtration through flannel and the filtrate centrifuged. The supernatant fluid was then decanted off and fed quantitatively from a pipette to guinea-pigs immediately after preparation.

The maximum liver equivalent of the aqueous extract was calculated as follows. To 100 g. of liver were added 100 cc. of water. Since the water content of sheep-liver is 71.3 % [Plimmer, quoted by McKillop, 1930] the total water content of the mixture is 171.3 g. Assuming that the water-soluble vitamin C is dispersed in the aqueous phase and not adsorbed on the solid surfaces on centrifuging, 1 cc. of the supernatant fluid corresponds with $\frac{100}{171.3}$ g. liver and therefore 10 cc. of the supernatant fluid are equivalent in vitamin C content to 5.77 g. liver.

Feeding experiments. Two different methods were employed to measure the antiscorbutic activity of the aqueous extract of sheep-liver.

(1) Curative action on guinea-pigs of graded doses of the extract in comparison with that of cabbage.

(2) Determination of the smallest amount of extract added daily to the basal diet necessary to prevent histological scorbutic changes in the teeth of normal guinea-pigs [Höjer, 1926].

In the former experiment, the animals (male guinea-pigs, selected and grouped according to weight and previous growth) were fed on a scorbutic basal diet composed as follows: Sussex ground oats, 3600 g.; bran, 400 g.; dried egg-yolk, 200 g. 1 % of cod-liver oil was added daily and the diet mixed to a friable mass with water. At the onset of scurvy and sudden cessation of normal growth, daily doses of 10 cc. and 5 cc. of liver extract were administered.

For comparison, two other groups of animals were given 1 g. and 0.5 g. cabbage *per diem* respectively, and in addition 5 cc. autoclaved liver extract (120° for 1 hour, sufficient for destruction of vitamin C) to control any ill effect of the extract upon digestive processes *etc.* Control animals received (1) a fully curative dose of 5 g. cabbage in addition to the basal diet; (2) basal scorbutic diet only.

The composition of the basal diet and the arrangement of the groups were identical in the second experiment. The animals were killed on the 14th day of the experiment, histological examination of the teeth and ribs being then made.

The results are shown in the relevant columns of Table I. From this it is seen that 5 cc. of the extract compares closely in its effect with 1 g. of cabbage, while, by Höjer's method, it is seen that the minimum protective dose lies between 5 and 10 cc. of the extract.

2. EFFECT OF FREEZING AND STORAGE IN THE FROZEN STATE UPON THE VITAMIN C CONTENT OF SHEEP-LIVER.

Freezing produces an irreversible change in the structure of the liver, which is shown by the fact that the tissue, on thawing, is spongy to the touch and loses a considerable amount of free fluid. It is essential, therefore, to consider separately the effects on the vitamin C content of (1) freezing the liver, (2) storage in the frozen state.

A comparison was accordingly made of the antiscorbutic properties of

- (a) fresh sheep-liver;
- (b) control portions of sheep-liver frozen at an average temperature of -19° for 6 days (*i.e.* sufficiently long to ensure thermal equilibrium);
- (c) sheep-liver frozen at an average temperature of -19° and stored at this temperature for 6 months.

Fresh sheep-livers were halved, placed in a chamber at -19° until the surface had hardened, sealed in greaseproof paper to prevent evaporation, and stored at the low temperature. The storage was started at periods of

6 months and 6 days before the commencement of the feeding experiments and continued so as to provide a constant daily supply of frozen half livers stored for the required periods. These were then thawed for 24 hours at an average temperature of 15°, and after disintegration through a sieve and addition of the collected "drip," were treated in the same way as the fresh liver for the preparation of aqueous extracts. Feeding experiments were carried out side by side with those described above.

Table I.

Additions to basal scorbutic diet	No. of guinea-pig	Curative method			Preventive method		
		Clinical	Post mortem findings		No. of guinea-pig	Histological condition	
			Ribs	Joints		Teeth	Ribs
5 cc. extract of fresh sheep-liver	1 a	Healthy, but inactive	Tr.	+	A ₃	—	—
	1 b	Mild scurvy, died from pneumonia	—	Tr.	A ₄	Tr.	—
	1 c	Very mild scurvy, died from pneumonia	Tr.	—			
5 cc. extract of sheep-liver stored at -19° for 6 days	2 a	Severe	+	+	D ₁	+	—
	2 b	Severe	++	+	D ₂	++	+
	2 c	Severe, died 16 days from onset of scurvy	+++	++			
5 cc. extract of sheep-liver stored at -19° for 6 months	3 a	Very severe, died 21 days from onset	+++	+++	E ₁	+++	?
	3 b	Death from pneumonia 12 days from onset	.	.	E ₂	++	—
	3 c	Death from scurvy 16 days from onset	++	++			
10 cc. extract of fresh sheep-liver	4 a	Completely cured; animal active	—	—	A ₁	—	—
	4 b	Normal, death from pneumonia	Tr.	—	A ₂	—	—
	4 c	Slight stiffness (death from gastro-intestinal infection)	—	—			
10 cc. extract of sheep-liver stored at -19° for 6 days	5 a	Death from infection after 11 days	—	—	B ₁	+	—
	5 b	Death from infection after 12 days	Tr.	Tr.	B ₂	++	—
	5 c	Death from infection after 9 days	—	—			
10 cc. extract of sheep-liver stored at -19° for 6 months	6 a	Very severe	+++	+++	C ₁	++	Tr.
	6 b	Very severe	++	+++	C ₂	++	Tr.
	6 c	Very severe	++	++			
Positive control 5 g. cabbage	7 a	Normal	—	—	F	—	—
	7 b	Normal	—	—			
5 cc. autoclaved extract +1 g. cabbage	8 a	Mild scurvy	—	+	J	—	—
	8 b	Healthy but inactive	Tr.	+			
5 cc. autoclaved extract +0.5 g. cabbage	9 a	Severe scurvy, death 8 days from onset (pneumonia)	++	++	I	+	—
	9 b	Severe scurvy, death 12 days from onset	++	++			
Negative control	10 a	Very severe scurvy, death 10 days from onset	+++	+++	H	.	++
	10 b	Very severe scurvy, death 11 days from onset	+++	+++			

Degrees of scurvy: normal (—), trace (Tr.), mild (+), severe (++), very severe (+++).

The results of this comparison are shown in Table I and Figs. 1 and 2. It will be observed that there is a definite loss of antiscorbutic activity from sheep-liver which has been stored at -19° for 6 days and then thawed, for while the minimum protective dose (Höjer's method) lies between 5 and 10 cc. of the fresh liver extract, 10 cc. of the extract from frozen liver are not sufficient to prevent histological scorbutic changes from occurring in the teeth.

The loss after freezing for 6 days is much less than that observed in the extract of liver stored at -19° for 6 months, 5 cc. of the aqueous extract of the former being approximately equal in effect to 10 cc. of the latter.

Fig. 1. Growth curves of guinea-pigs, showing the comparative curative power of 5 cc. aqueous extract of (I) fresh sheep-liver, (II) sheep-liver stored at average temperature of -19° for 6 days, (III) sheep-liver stored at average temperature of -19° for 6 months, when dosed to scorbutic animals receiving a vitamin C-free basal diet. (Arrow denotes onset of scurvy and commencement of administration of the sheep-liver extracts.)

Fig. 2. Growth curves of guinea-pigs, showing the comparative curative effects of 10 cc. (*per diem*) of aqueous extracts of (I) fresh sheep-liver, (II) sheep-liver stored for 6 months at temperature of -19° . (Arrow denotes onset of scurvy and commencement of administration of sheep-liver extracts.)

3. EFFECTS OF SLOW AND RAPID FREEZING WITHOUT STORAGE.

The foregoing experiment was designed to distinguish between the separate effects of freezing and of storage in the frozen state, and the results indicated that a short period of freezing, followed by thawing, brings about a loss of vitamin C, while storage for a prolonged period in the frozen state causes further destruction. Further experiments were carried out to confirm the inactivating effect of freezing without prolonged storage and at the same time a comparison was also made of the effects of slow and rapid freezing.

Preparation of material. Fresh whole sheep-livers were procured daily, and from each of the two lobes a portion weighing 150–200 g. was cut to fit exactly into a rectangular tin of cross section 1×3 inches. The remainder was sealed in greaseproof paper to prevent evaporation, and stored in a chamber at 0° .

Slow freezing in air. One tin containing the sample of liver was packed in granulated cork to a depth of $1\frac{1}{2}$ inches in each direction, the exposed surface of the liver being covered by $1\frac{1}{2}$ inches of cotton-wool and the tin sealed with cork. Under these conditions a period of 21 hours was required for the sample to cool from $+10^{\circ}$ to -19° (measured by a thermocouple placed at the centre of the sample of liver). A further storage period of 24 hours at this temperature was allowed to ensure complete thermal equilibrium.

Rapid freezing in brine. The tin containing the second sample was suspended in well-stirred brine at -19° (time of cooling from $+10^{\circ}$ to -19° 32 mins.) and after allowing a period of 80 minutes in the tank, was transferred to the -19° chamber.

At the end of 48 hours the brine- and air-frozen samples were removed from the cold chamber and were thawed in air at $+10^{\circ}$ for 22 hours¹. Aqueous extracts of these, and of the unfrozen sample which had been stored for the same length of time at 0° , were then prepared and tested on guinea-pigs as in the previous experiment.

Feeding experiments. These consisted of an examination of the extracts by

(a) a measurement of their curative power when administered to scorbutic guinea-pigs in graded daily doses;

(b) a comparison of their power to prevent histological scorbutic changes in the teeth of normal guinea-pigs, the extract being administered in graded daily doses, for 14 days, in addition to the scorbutic basal diet.

The selection, grouping and treatment of the animals were exactly the same as in the previous experiment. The scorbutic basal diet contained, in addition to the aforementioned constituents, 1% mineral salt mixture.

The results of these investigations are shown in Table II and Fig. 3, and it will be seen that the growth changes, the *post mortem* observations, and the histological condition of teeth and ribs combine to prove that a definite loss of antiscorbutic activity occurs in sheep-liver which has undergone the pro-

¹ This is slow thawing. Experiment has not yet been made of the effects of rapid freezing followed by rapid thawing.

Table II.

Addition to basal scorbutic diet	Curative method				Preventive method			
	No. of guinea-pig	Post mortem condition			No. of guinea-pig	Post mortem examination	Histological condition	
		Ribs	Joints				Teeth	Ribs
5 cc. extract of sheep-liver stored at 0° (3 days)	IV 32	+	-		IV 10	Tr.	+	-
	33	-	+		11	-	+	-
	34	+	+		12	-	+	-
5 cc. extract of sheep-liver frozen slowly in air at -19°	V 35	+	++		V 13	Tr.	++	-
	36	+++	+++		14	Tr.	++	-
	37	-	+		15	Tr.	++	-
5 cc. extract of sheep-liver frozen rapidly in brine at -19°	VI 38	++	+++		VI 16	+	++	-
	39	++	+++		17	-	+	-
	40	++	-		18	-	+	-
10 cc. extract of sheep-liver stored at 0° (3 days)	I 23	-	-		I 1	-	-	-
	24	-	-		2	-	-	-
	25	-	-		3	-	-	-
10 cc. extract of sheep-liver frozen slowly in air at -19°	II 26	+	-		II 4	-	-	-
	27	-	-		5	-	-	-
	28	+	-		6	-	-	-
10 cc. extract of sheep-liver frozen rapidly in brine at -19°	III 29	Tr.	-		III 7	-	-	-
	30	Tr.	-		8	-	-	-
	31	-	-		9	-	-	-
Negative control	VII 43	+++	++		VII 19	+++	+	++
	44	+++	++		20	++	+	+
Positive control, 5 g. cabbage	VIII 41	-	-		VIII 21	-	-	-
	42	-	-		22	-	-	-

Degrees of scurvy: normal (-), trace (Tr.), mild (+), severe (++), very severe (+++).

Fig. 3. Growth curves of guinea-pigs, showing the antiscorbutic activities of 5 cc. aqueous extracts of sheep-liver (I) kept at zero without freezing for 3 days, (II) frozen rapidly to -19° , stored for 48 hours at temperature of -19° , (III) frozen slowly to -19° , stored for 48 hours at temperature of -19° . (Arrow denotes onset of scurvy and commencement of administration of sheep-liver extracts.)

cesses of freezing and subsequent thawing. Further, in regard to the method of freezing, this loss is as great after rapid freezing in brine at -19° as after slow freezing in air at the same temperature.

DISCUSSION.

These experiments demonstrate the high antiscorbutic activity of sheep-liver, of which the minimum protective dose lies between 5 and 10 cc. of the extract (*i.e.* between 2.88 and 5.77 g. of the liver, on the assumption made above). This is in agreement with the results of work on the vitamin C content of the livers of other animals.

It has been rendered probable that the rat, the chick, the calf (see references on p. 704) and also that puppies [Harris and Innes, 1931] can synthesise vitamin C and that the livers of these animals have a comparatively high antiscorbutic potency. It is possible, though yet to be proved, that sheep may also have this synthetic power.

The marked loss of antiscorbutic activity caused by the process of freezing and thawing is probably associated with the structural breakdown of the liver tissue brought about through the formation of ice crystals which may act directly by causing mechanical damage or indirectly by concentrating the aqueous phase in frozen liver; it is the latter which is responsible for the changes in living muscle when it is frozen. This disintegration may well lead to the liberation into the free fluid of enzymes or other substances capable of inactivating the vitamin.

The mere lowering of temperature must retard any chemical changes in the vitamin and thus assist in its preservation.

As rapid freezing leads to the formation of smaller ice crystals in the tissue than does slow freezing it should cause a less degree of structural breakdown. No appreciable difference could, however, be observed between the vitamin C contents of slowly and of rapidly frozen liver.

During storage for 6 months in the frozen state a further loss takes place. This may be due to further structural breakdown in the tissue and consequent liberation of substances capable of inactivating the vitamin on thawing. Attention may here be drawn to the work of Bracewell *et al.* [1931] on the effect of cold storage on the vitamin C content of apples. It was found by these workers that prolonged storage at low temperatures had no effect on the antiscorbutic potency of apples. However, the stability of vitamin C has been shown to vary in different media; among other factors an increase in the hydrogen ion concentration of the medium is associated with increased stability of the active constituent [Holst and Frölich, 1912]. It is possible that vitamin C is more stable in apples than in sheep-liver during cold storage on account of the greater acidity of the former material.

SUMMARY.

1. Sheep-liver contains an appreciable amount of vitamin C.
2. The mere act of bringing the liver into the frozen condition followed by thawing brings about a considerable decrease in its vitamin C activity.
3. Still further loss occurs from liver which is stored at -19° for 6 months.
4. It is suggested that the structural breakdown caused by freezing liberates, on thawing, substances from the liver tissue which have a destructive action on the vitamin.
5. No appreciable difference was observed between the effect of rapid freezing and slow freezing on the vitamin C content of sheep-liver.

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REFERENCES.

- Bracewell, Kidd, West and Zilva (1931). *Biochem. J.* **25**, 138.
Harris and Innes (1931). Private communication.
Hart, Steenbock, Lepkovsky and Halpin (1925). *J. Biol. Chem.* **66**, 813.
Höjer (1926). *Brit. J. Exp. Path.* **7**, 356.
Holst and Frölich (1912). *Z. Hyg. Infek.* **72**, 1.
McKillop (1930). Food values. (London.)
Parsons (1920). *J. Biol. Chem.* **44**, 587.
Thurston, Eckles and Palmer (1929). *J. Dairy Sci.* **12**, 394.