116. THE CITRIC ACID CONTENT OF ANIMAL TISSUES, WITH REFERENCE TO ITS OCCURRENCE IN BONE AND TUMOUR[1]

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THE distribution of citric acid in the animal body has not previously been systematically studied, for although Thunberg and his colleagues have made a careful examination of the citrate content of body fluids, that of the solid tissues has been largely neglected. This is all the more surprising, since Pucher *et al.* [1936] have described a sensitive colorimetric method which, unlike the enzymic one of Thunberg, is readily applicable to such material. Pucher *et al.* [1936] and Sherman *et al.* [1936] analysed only a few tissues by the colorimetric method (liver, kidney, abdominal and heart muscle of the dog), for which very low values were found (0·1–1·9 mg. citric acid/100 g. tissue). It is clear from their paper that they regarded these results as typical of the major tissues of the body, but this view is shown to be incorrect by the present work.

In contrast with these low results on solid tissues, it has long been known that several body fluids contain relatively large amounts of citrate. Thus milk contains enough for the crystallization of Ca citrate from evaporated milk, an occurrence which led to the first identification of citric acid as a normal constituent of the animal organism by Soxhlet & Henkel [1888]. Citric acid was not isolated from urine until many years later [Fasold, 1930], although it was shown to be a normal constituent by Amberg & McClure [1917], and in fact urine may also contain amounts of citric acid of similar order to that present in milk (20–120 mg./100 ml.; for literature see Östberg [1931], Scherstén [1936], Grönvall [1937], Sherman *et al.* [1936]). According to the Scandinavian workers, much less citric acid is present in cerebrospinal fluid, amniotic and follicular fluids, blood serum, aqueous humour, saliva and sweat, which contain in diminishing order about 5–0·1 mg./100 ml. Pucher *et al.* [1936] found similar low figures for blood and saliva. Finally there is the surprising observation of Scherstén [1929, 1936] that a high concentration of citric acid (up to 410 mg./100 ml.) is present in semen, and is particularly associated with the secretion of the seminal vesicles, which may contain as much as 633 mg./100 g. This brief outline of the present knowledge of citric acid distribution may be supplemented by the comprehensive reviews already quoted.

The present investigation is primarily the outcome of analyses of tumour tissue, a material of which the citric acid content has not previously been studied. The results showed that tumour frequently, though not invariably, contains a relatively high concentration of citric acid. However, certain normal organs

[1] The greater part of this work was reported to the Biochemical Society at Sheffield in Feb. 1940 [Dickens, 1940]. Its completion was delayed by difficulty in obtaining necessary replacements for the photometer used.

such as skin, hair and embryonic tissue were found to have much higher amounts than those previously reported for solid tissues. In addition, the hard substance of bone has been shown to be a hitherto unsuspected store of citric acid, which may constitute as much as 1·6% of the dry, fat-extracted bone. It follows that the skeleton is the site of by far the greatest proportion of citric acid in the vertebrate, for it occurs in high concentration in the bones of all species yet investigated.

Experimental

Methods. Freshly excised tissues were used in all experiments except those on materials such as horse hair, ox bone or dried extracted bone. The animals received their last food 18 hr. before the experiment. Immediately after dissection and weighing, the tissue was ground with sand and extracted three times with 10% trichloroacetic acid. When bone was analysed it was first chopped or pulverized, then ground with sand and digested, usually with more concentrated (40%) trichloroacetic acid at about 30°, until the hard substance had dissolved. The solid residue was then re-extracted with 10% trichloroacetic acid as above. After boiling down the combined extracts with H_2SO_4 (final conc. *ca.* *N*) and cooling, the precipitated $CaSO_4$ was removed by centrifuging.

The citric acid was estimated by the colorimetric pentabromoacetone method of Pucher *et al.* [1936], with dioxan [Johnson, 1939] instead of pyridine as the final diluent. This method of analysis is the best available, but there are some sources of error which need special attention. The fresh, filtered solution of Na_2S must be prepared immediately before use. If an aliquot part of the trichloroacetic filtrate after boiling with H_2SO_4 is taken, it is important that after dilution further H_2SO_4 is added to make about *N* concentration before the oxidation. The use of $FeSO_4$ to decolorize the $KMnO_4$ was not found consistently reliable [cf. Thunberg, 1941, 1] owing to the serious effect of even minute traces of residual Fe after washing, and instead the method of Pucher *et al.* [1934] using H_2O_2 at 6° instead of $FeSO_4$ has been found to be preferable. The addition of H_2O_2 must be carried out with all the care of a titration, a slight excess leading to rapid destruction of pentabromoacetone. Some specimens of light petroleum were unsatisfactory, as Pucher *et al.* [1936] state. Instead of using water in the blank for colorimetry, the mixture of Na_2S and dioxan was employed. By this method, accurate estimations are obtained between 0·1 and 1·0 mg. citric acid: the specificity is discussed by Pucher *et al.* [1936] and is referred to in the discussion of the present paper.

Throughout the results, citric acid is always expressed as the anhydrous substance and with the exception of dried bone, tissue weights are wet weights.

The citric acid content of tumour tissue

Table 1 shows the citric acid contents of some typical tumours. It will be seen that the animal tumours used all had large amounts, from 12 to 18 mg./100 g. These tumours, of mouse, rat and rabbit, were fairly large and varying degrees of necrosis were present. In a suitable tumour, which could be dissected into mainly necrotic and mainly non-necrotic portions, the citric acid was estimated separately in the two. The results (Table 1) show that the non-necrotic part of this Crocker mouse sarcoma contains nearly twice the concentration of the necrotic tissue. The high citric acid content is therefore a property of the tumour tissue itself, and not the result of a change, such as calcification, accompanying the necrosis. In one experiment the liver of a rat bearing a Guérin tumour was analysed simultaneously with the tumour; the liver had less than 1/5 of the

Table 1. *The citric acid content of tumour tissue*

Species	Tumour	Citric acid mg./100 g.
Rabbit	Brown-Pearce carcinoma	13·6
Rat	Walker 256 carcinoma	16·6
,,	Walker 256 carcinoma	18·4
,,	Guérin tumour	16·8
,,	Guérin (liver of same rat)	(3·0)
Mouse	Crocker sarcoma 180	14·2
,,	Crocker sarcoma 180	14·3
,,	Crocker sarcoma 180 (mainly non-necrotic)	12·3
,,	Crocker sarcoma 180 (mainly necrotic)	(7·7)
Man	Malignant dysgerminoma (seminoma) of testis	9·9
,,	Fibroadenoma of uterus	5·0
,,	Carcinoma of stomach	2·7
,,	Adjacent gastric mucosa	(2·0)
,,	Carcinoma of vulva	8·8
,,	Carcinoma of vulva	7·0
,,	Leucoplakia of vulva	8·2
,,	(Adjacent normal skin)	(5·3)

Bracketed values refer to same animal or same case.

citrate content of the tumour (Table 1). Among human tumours the results were more variable; although high values were found for the carcinoma of the vulva and seminoma, the citric acid content of the carcinoma of stomach examined was only slightly higher than that of the adjacent gastric mucosa. In one case of carcinoma of the vulva, it was possible to compare the carcinoma with the nearby leucoplakia (which may be considered as precancerous) and adjoining skin which were excised together with the carcinoma; it will be noted that the citric acid content was greater in both the precancerous and malignant tissues than in the normal skin. The fairly high value obtained for the latter may be compared with the still higher values found below for animal skin.

Comparison of the total citric acid of the tumour with that of the remainder of the animal. Since low values have hitherto been reported for the total amounts of citric acid in animal organs, it appeared that analysis might show the greater part of the citric acid of a tumour-bearing animal to be contained in the tumour. This expectation was not, however, realized. A mouse bearing a Crocker sarcoma of weight about equal to that of the animal itself was selected, and the tumour and mouse were separately analysed (Table 2). In spite of the high content of

Table 2. *Analysis of a mouse tumour and of remainder of mouse*

	Total wt. g.	Citric acid mg.	Citric content mg./100 g.
Crocker sarcoma 180	20·8	2·95	14·2
Remainder of mouse	25·2	3·62	14·4

citric acid in the tumour (14·2 mg./100 g.), this contained only 45% of the total citric acid present in mouse and tumour together. It followed that some rich source of citrate was present in the tumour-free mouse, which brought up the citrate content of the whole animal to the high level of 14·4 mg./100 g.

The possibility that the intestinal contents were responsible was excluded by analysis of another mouse receiving the same diet. This animal weighed 32·1 g. and contained a total amount of 4·17 mg. citric acid, towards which the stomach, intestines, bladder and their contents (4·1 g.) contributed only 0·253 mg.

Complete analysis of the normal animal

In an endeavour to clear up this anomaly, the analyses shown in Table 3 were made. It will be noticed that the citric acid contents reported for the rabbit, guinea-pig and mouse are on the whole somewhat higher than those of

Table 3. *Citric acid content of tissues of adult animals*

Species	Tissue	Citric acid content mg./100 g.
Rabbit	Liver	2·8
	Skeletal muscle	2·5
	Kidney	6·0
	Whole brain	4·6
Guinea-pig	Liver	1·6
	Kidney	3·9
	Whole brain	3·8
	Testis	11·5
Mouse	Skin, fur mostly removed	12·2
	Fur	133
	Seminal vesicles	128
Mouse	Fur, total citric acid	114
	Fur, hot water extract	83
Mouse	Fur	93
	Skin, fur mostly removed	9·7
Horse	Hair, unwashed	27·7*
	Hair (commercial 'washed horse hair')	21·1
Man	Sebaceous cyst from hand	11·7
	Sebaceous cyst, mainly cyst wall, with only traces of adhering contents	5·0
Mouse	Skeleton, with eyes and closely adherent connective tissue	46†
	Muscle	2·8
	Skin, fur and tail sheath	8·6
	Seminal vesicles, testes and prostate	119
	Viscera and brain	4·6

* 16·2 mg./100 g. were hot water soluble.

† As it was not possible to remove a good deal of adherent tissue, this value is too low for the skeleton alone.

dog kidney, muscle and liver (0·1–1·9 mg./100 g.) reported by Pucher *et al.* [1936]. In agreement with the results of Scherstén [1936] for other species, a high content of citric acid is present in the seminal vesicles and total male gonads of the mouse, and in the testes of the guinea-pig. Low concentrations are present in skeletal muscle of rabbit and mouse (2·5, 2·8 mg./100 g.) and in liver of rabbit and guinea-pig (2·8, 1·6 mg./100 g.), while the brains of these two species have rather larger amounts (4·6, 3·8 mg./100 g.). Kidney tissue of rabbit and guinea-pig contains considerably more (6·0, 3·9 mg./100 g.) than that of the dog (1·9, 1·2 mg./100 g. [Pucher *et al.* 1936]). The skin and hair are fairly rich sources of citric acid (cf. also human abdominal skin, Table 1). The very high content of horse hair and particularly of mouse fur was very surprising; contamination by urine or semen may be excluded as the source of this citric acid, since it occurred in both male and female mice to the same extent, and care was taken to avoid contamination. It is of interest that a large proportion of the citric acid of hair is extractable by water (200 vol., boiled with hair for 5 min., extracts 58 % of the total citrate from horse hair and 73 % from mouse fur). It seemed possible that the citric acid arose from secretion of glands in the skin, and since mice are stated not to sweat on the furry parts (in any case sweat has

only 0·1–0·2 mg. citrate/100 ml. [Scherstén, 1936]), it was thought that the sebaceous glands might be the source. This hypothesis could hardly be tested in the mouse, but analysis of a sebaceous cyst from the human hand showed the fairly high citric acid content of 11·7 mg./100 g., towards which the cyst wall contributed only a minor part (Table 3). No doubt part of the high citric acid content of the skin may be attributed to a similar origin.

In spite of its high concentration, the citric acid in the fur and skin of a mouse comprises only a small fraction (about 10%) of the total in the animal. Similar considerations apply to the seminal vesicles, which in the adult mouse might add a further 5% to the total. Even excluding these known rich sources of citric acid, there is an overall concentration in the residue of the animal of over 10 mg./100 g.

The source of the greater part of this citric acid was revealed by the complete analysis of a whole mouse after dissection (Table 4). It will be seen that about

Table 4. *Analysis of a dissected mouse*

Male adult, fed 18 hr. before, live wt. 31·2 g.

Tissues	Tissue wt. g.	Citric acid mg.	Citric acid % of total in mouse
Skeleton, with closely adherent connective tissue and eyes	8·2	3·75	69
Muscle, with dissected connective tissue and a little fat	6·8	0·19	3·5
Outer skin, including skin of tail, and fur	4·3	0·37	6·8
Seminal vesicles, testes and prostate	0·6	0·71	13·1
Liver, spleen, brain, heart, lungs, kidneys and other viscera	9·0	0·41	7·5
Total	28·9	5·43	99·9

70% of the total citric acid in the mouse is contained in the skeleton, and this provides the explanation of the remarkably high citric acid content of the whole animal.

Bone, cartilage and marrow

Bone from the foreleg of an ox was obtained from the butcher, and the marrow removed. Great difficulty was experienced in crushing the hard bone, and in consequence its solution in trichloroacetic acid was incomplete; the citric acid content found is therefore probably low. Nevertheless the result showed that while the hard substance contained at least 272 mg./100 g., the red marrow had only 41 mg./100 g. Hence the greater part of the citric acid is contained in the hard bone. A similar experiment with bone and cartilage from a kitten (Table 5) showed that the content of citric acid in the cartilage is less than 10% of that in the bony substance, including marrow. In this and in the following experiments with bone, better solution of the hard material was obtained by taking 1–2 g. quantities of fresh substance, chopped when possible, and grinding with sand, 5 g. trichloroacetic acid and 1 ml. water (or, in the case of dried, extracted bone, 0·3 g. finely powdered material with 5 g. trichloroacetic acid and 10 ml. water) and keeping overnight.

I am indebted to Mrs C. M. Burns for the specimens of bone from specially treated animals, and for kindly supplying the analyses for Ca, P and CO_2, which are included with the citric acid determinations in Table 5. For technical details of preparation and analysis reference may be made to Burns & Henderson [1935].

Table 5. *Bone analyses*

	Citric acid mg./100 g.
Fresh bone, marrow and cartilage	
Ox	
Bone of foreleg	272
Red marrow from this bone	41
Kitten (3 days old)	
Bone, including marrow, from ribs and legs	373
Cartilage, from ribs and leg joints	33
'Steamed bone meal' (ox and sheep)	1420
Dried, alcohol-extracted, powdered bone	
Pup A. Normal	1310
Pup B. After prolonged administration of parathyroid hormone	1660
Kitten C. Normal	644
Kitten D. Rachitic; low Ca diet	345

Ca and P analyses

	Blood Ca mg./100 ml.	Bone analysis (%) Ca	P	CO_2
Pup A	11·2	21·4	10·8	2·42
Pup B	13·8	20·6	10·3	2·59
Kitten C	N.D.	22·7	11·3	2·78
Kitten D	N.D.	20·0	9·59	2·43

Bone cortex was taken from the middle of the shaft, and samples are thus directly comparable in all experiments; after drying and extraction the bone was reduced to a fine powder and was dried to constant weight. The two pups, A and B (Table 5), were fed on identical diets which consisted largely of milk and porridge; B received massive and prolonged dosage with parathyroid extract. The effect of diet was studied in kittens C and D; C was fed on the usual mixed domestic diet while D received a similar diet but without milk or bones. This diet was clearly deficient in Ca, for whereas the bones of animal C although thin were not rachitic, those of D were definitely rachitic, being typically bent and having large epiphyses. The result of this experiment is discussed later in this paper, but attention may here be drawn to the increase in citrate in the bone of pup B which received parathyroid extract, and the fall in citric acid in kitten D which was on a diet deficient in Ca, and was rachitic. The great variation between the two species is also noteworthy.

The composition of steamed bone meal

The great hardness of fresh bone made it necessary, for the purpose of isolating citric acid, to find a more tractable material, which was readily available in 'steamed bone meal'. This is prepared commercially from coarsely pulverized bone, mainly of ox and sheep, which after mechanical separation from skin, hair, etc. is extracted with light petroleum and with steam, and is finally dried and reduced to a fine powder. The specimen used contained 4·20% moisture (6 hr. at 105°), 2·0% ether-extractable (mainly fat), 0·82% protein-N (*ca.* 5% protein); of the total meal 7·8% was insoluble in dilute HCl. The acid-soluble part (88·0% of the original meal, dry wt.) contained 30·3% Ca, 14·0% inorganic P and 16·05% total P, all calc. on the original wt. of meal.

The citric acid content (Table 5) was 1·42% and the CO_2 content 2·06%, both calc. on the original meal (i.e. 1·61 and 2·34% respectively, calc. on dry, fat- and protein-free, acid-soluble meal). Comparison with a typical analysis of

the 'inorganic' phase of human bone (Table 6) shows that the 'inorganic' part of the sample of bone meal used in these experiments has sustained some loss of CO_2 and Ca, but on the whole closely resembles the typical bone analysis in its mineral composition. This is regarded as evidence of the suitability of this material for comparison with natural bone.

Table 6. *Comparison of the compositions of normal bone ash and of 'steamed bone meal'*

	Bone meal, % of dry, fat- and protein-free wt.	Inorganic residue of bone (human) [Gabriel, 1894] %
CaO	48·2	51·31
P_2O_5 (inorganic)	36·5	—
(total)	41·8	36·65
CO_2	2·34	5·86
Citric acid	1·61	—
Minor constituents	—	5·92*
	93·95	99·74

* Water, 3·78; MgO, 0·77; K_2O, 0·32; Na_2O, 1·04; Cl, 0·01%.

The solubility of citric acid present in bone meal. 1 g. bone meal was shaken for 2 hr. with 100 ml. distilled water at room temperature and filtered. The clear filtrate (95 ml.) contained 0·629 mg. citric acid, or 5% of that present. Boiling with 200 ml. water extracted from the residue only a further 1·24 mg. (10% of the total). Since the solubility of $Ca_3Cit._2,4H_2O$ (Beilstein, III, 564) is 85 mg./100 ml. at 18°, equivalent to 57 mg. citric acid, it is evident that the citric acid in bone meal, if present as the Ca salt, must be in some form not readily extractable by water. The presence of appreciable amounts of free citric acid, which is in any case improbable, or of the freely soluble alkali salts seems to be excluded by this experiment.

The isolation of citric acid from bone meal

On account of the great preponderance of $Ca_3(PO_4)_2$ the isolation of a pure specimen of citric acid was accompanied by much loss; but the isolation in good yield of pentabromoacetone, as described later, appears to make further work on these extractions of citric acid hardly necessary. Two methods were used, of which the first is more laborious and more wasteful, but yields a product which is readily obtained quite pure.

Isolation by fractional precipitation. 1 kg. bone meal was extracted three times at 100° with 2·5 l. of 2*N* HCl. The filtered extract was made alkaline with NH_3 and the precipitate collected. The precipitate contained 93% and the filtrate only 5% of the citric acid initially present. The moist precipitate was thoroughly stirred with 1 l. water and 0·67 l. 18*N* H_2SO_4. After adding 1·7 l. alcohol and filtering, the filtrate was evaporated at 30° to remove alcohol; magnesium acetate (0·5 kg.) was added followed by ammonia to make alkaline. The precipitate of phosphate was removed, and to the Ca- and PO_4-free solution were added 100 g. Ba acetate and 1·9 l. alcohol. The precipitate of Ba salts was collected and washed with 70% alcohol. Three extractions of the precipitate with a mixture of 90 ml. alcohol and 10 ml. conc. HCl were combined and concentrated almost to dryness (with considerable accidental loss at this stage). The residual syrup was treated with 50 ml. 10% $CaCl_2$ and the boiling solution made alkaline with NH_3. The Ca salt, after washing and drying, weighed 2·15 g.; it

was freed from Ca by the calculated amount of N H_2SO_4 in 50% alcohol. The syrup remaining after evaporation on the water bath was dried; yield 0·55 g. It was recrystallized, with a trace of animal charcoal, from ether by refluxing with 60 ml. for 6 hr., evaporating the greater part of the ether, and keeping in a stoppered flask. The sparkling white crystals were washed twice with ether. Yield 0·325 g., M.P. (slow heating) 153·0° corr., mixed M.P. 153·5° corr., M.P. of authentic anhydrous citric acid 153·5° corr.

Direct extraction after acidification. 95 g. bone meal were extracted with ether for 3 hr. in a Soxhlet apparatus to remove fat. The dried residue was cooled in ice and well stirred with 95 ml. 7N H_2SO_4. Three ether extractions of this paste, each of 24 hr., were combined. The yields of citric acid in each, by analysis, were 31, 26 and 3·0% of the total present. The extract was shaken with $NaHCO_3$ and the alkaline extract acidified with HCl; CO_2 and dissolved ether were removed on the water bath, and after boiling with animal charcoal the citrate was precipitated by addition to the boiling solution of 35 ml. 25% $CaCl_2$ and NH_3 to make alkaline. The precipitate of Ca salt was collected, washed twice with boiling water and dried. Yield 1·778 g. Ca salt containing by analysis 0·749 g. citric acid, or 60·2% of that present in the original meal. The Ca salt was ground with H_2SO_4 (16·6 ml. N) in 50% alcohol, filtered, and the precipitate re-extracted with 20 ml. 50% alcohol. The combined extracts were evaporated; the residue was dried in the oven, dissolved in a little alcohol (later experience showed this to be undesirable) and refluxed with ether. After removal of some insoluble material, the evaporated ether solution largely solidified on long keeping in a vacuum desiccator to a yellow crystalline residue (0·743 g.) which, however, still contained some alcohol. It was redissolved, with a trace of animal charcoal, in 20 ml. ether, filtered, evaporated and dried. Yield of nearly pure citric acid 0·502 g. = 40% of that calculated from analysis of original bone meal. The pale yellow crystals after one recrystallization from anhydrous ether yielded pure white citric acid, M.P. 152–153° corr.

Preparation of pentabromoacetone from bone meal

The following is based upon the preparative methods described by Wöhlk [1902] and Grönvall [1937]. 50 g. bone meal, containing by analysis 0·620 g. citric acid, were ground with a solution of 170 g. trichloroacetic acid in 100 ml. warm water. Rapid solution of all but a few coarse bony particles occurred, and after 30 min. the liquid was separated by filtration and centrifuging. To the filtrate cooled in ice 110 ml. 18N H_2SO_4 were added (temp. $<15°$). The precipitate of $CaSO_4$ was well washed with water and the combined filtrate and washings (700 ml.) were evaporated over a flame to 500 ml.; two lots of 750 ml. water were added, boiling down to 500 ml. again after each addition, to destroy trichloroacetic acid. After keeping overnight in the ice-box, the clear solution was filtered from $CaSO_4$ and some fatty material.

5 g. KBr were added, and the solution warmed to 30° while 50 ml. 5% $KMnO_4$ were added. During 30 min. at 30° three lots of 10 ml. 5% $KMnO_4$ were added at intervals; the mixture was then decolorized with 30% $FeSO_4$, when a white crystalline precipitate appeared immediately, and more separated overnight at 0°. Yield 1·165 g. of nearly pure pentabromoacetone, M.P. 68–70° (equivalent to 0·495 g. citric acid or 80% of that found by analysis of the original bone meal). After one recrystallization from a little 50% alcohol, brilliant white crystals, M.P. 73°, mixed M.P. 73°, M.P. of authentic pentabromoacetone 73°, were obtained.

Citric acid in the embryo

The embryo is of particular interest in connexion with citric acid, because the mainly carbohydrate metabolism of the young embryo [Dickens & Šimer, 1930] and the later extensive calcification are both processes in which citric acid may be expected to play a part. Table 7 gives some preliminary results on this

Table 7. *Citric acid content of the embryo*

Species	Age	Average wt. of one embryo g.	No. of embryos for analysis	Citric acid: mg. per embryo	Citric acid: mg./100 g. embryo
Mouse	—	0·9	9	0·112	12·4
Mouse	New-born—a few hr. old	1·2	7	0·194	16·2
Chick	3½ days	0·037	6	(0·012)*	(31·0)*
	5 ,,	0·217	6	0·059	27·4
	8 ,,	1·263	2	0·168	13·3
	12 ,,	5·60	1	0·740	13·2
	21 ,,	48·60	1	4·56	9·4

* At lower limit of accuracy of the method.

material, from which it appears that an unusually high content of citric acid is indeed a feature of embryonic tissue. This is particularly marked in the earliest stage which can be readily investigated with a method of the sensitivity of the present one (3½- to 5-day chick); thereafter up to the 12th day the citric acid falls to a level comparable with that of the more advanced mouse embryo, the new-born mouse, and indeed the adult mouse (cf. Table 4). In comparing these results with the adult values, however, it must be remembered that 70% of the citric acid of the adult mouse is contained in the skeleton. The determination of the distribution of citric acid in the organs of the embryo should therefore be particularly informative. This is also a necessary step before any useful comparison with the citric acid content of tumours, that is of normal and pathological growth, can be made.

Discussion

The main result of this investigation is the discovery of citric acid as a major constituent of normal bone. The preliminary estimations [Dickens, 1940] have now been confirmed for the bones of several animals by Thunberg [1941, 1], who found by the colorimetric method that the bone substance of all species studied (horse, ox, sheep, pig and also the hen and 5 species of fishes) contained citric acid amounting to 'I bis einige Prozent der Trockensubstanz'. He also found about 0·5% of citric acid in the teeth of the cat, ox and pig. These results, together with those on the bones of the mouse, ox, kitten and puppy and on mixed bone meal which are contained in the present paper, show that the presence of a high concentration of citric acid is a very widespread, probably general, property of bone.

That the substance estimated is really citric acid, and not some unknown constituent giving a similar colorimetric reaction, is shown first by the isolation of pure citric acid from bone meal and secondly by the isolation of pure pentabromoacetone in 80% of the yield calculated from colorimetric analysis. This latter proof may reasonably be taken to mean that practically the whole of the substance is citric acid, for the isolation would not be expected to be entirely quantitative, and the formation of pentabromoacetone is such an unusual reaction, depending as it does on the intermediate formation of the enolic form of $\alpha\alpha'$-acetonedicarboxylic acid, that it is very highly specific for citric acid [cp.

Grönvall, 1937, pp. 62 *seq.* for literature]. Tartaric, succinic, malic, lactic, oxalic, pyruvic, camphoglycuronic, uric, tricarballylic, *trans*aconitic, itaconic, citraconic, and mesaconic acids are stated not to interfere, nor do glycerol, sucrose, glucose, $CaSO_4$ or $Ca_3(PO_4)_2$. Pucher *et al.* [1936] add to this list acetone, glycogen, acid haematin, creatine, creatinine, cholesterol, amytal, urea, taurine, acid digest of casein, allantoin; hippuric, maleic and fumaric acids: they found, however, that β-hydroxybutyric gave about 1/1000–1/200 and the ethyl ester of acetone-dicarboxylic acid about 1/110 of the colour given by citric acid. *cis*Aconitic acid gives no colour [Johnson, 1939]. From these data it must be concluded that there is no known physiologically occurring substance other than citric acid which can be held responsible for the pentabromoacetone prepared from bone.

The fact that so large a quantity of a familiar substance has hitherto escaped detection by bone analysis is surprising, but it is upon bone ash that the most accurate and complete analyses have been made, and in these the citrate would presumably appear as an addition to the carbonate fraction. It has long been recognized that the nature of the organic combination of bone cations is an almost unknown field, and a systematic study of bone for the presence of other physiologically important organic acids would now appear profitable.

Since the citric acid of bone meal is insoluble in alcohol and ether and is only slightly extracted by water, it is assumed that it may be present in combination with calcium, though this combination may be in the form of a complex ion, such as citrates are known to form with Ca [cf. Hastings *et al.* 1934]. The nature of its distribution throughout the bone, and its possible entry into the mixed crystal structure of bone, are questions for future investigations.

The mere presence of the highest concentration of citrate in that tissue which is richest in calcium, however, seems to provide by far the most striking evidence for a participation of citric acid in calcium metabolism. The idea of a citrate-like substance acting as a solvent for bone salts is a very old one [cf. Klinke, 1928], and Greenwald [1926] in his studies of parathyroid activity went so far as to postulate the formation of such a substance directly or indirectly by the parathyroid gland. He actually tested for citrate, presumably in the blood, but with negative results [Greenwald, 1926]. Thunberg and his associates have continually referred to the association of citric acid with calcium in the body, a striking example being the high content of citrate in semen, which has some 50 mg./100 ml. of Ca, about 5 times the concentration in plasma, and also has a high concentration of inorganic phosphate, without the precipitation of Ca occurring [Scherstén, 1936]. The high citric acid contents of skin and embryo, reported in the present paper, may be considered as strengthening this connexion, for skin has on a dry weight basis 'considerably more Ca and less Mg and P than in most other soft tissues' [Schmidt & Greenberg, 1935]; the case of embryo is discussed below. Recently, Thunberg [1941, 1, 2] has demonstrated the presence of 0·08–0·35 % of citric acid in the shell of eggs from many species of birds.

Since it is now well recognized that bone substance is continually in a state of vigorous metabolic exchange with body fluids [cp. Hevesy *et al.* 1940] it may be expected that the citrate of bone will accompany the other bone salts during their mobilization and deposition. Thus the stores of citrate in the bone will probably be available for the metabolic requirements of body tissues in general, whether catalytically in carbohydrate oxidation [Krebs & Johnson, 1937] or as solvent for Ca salts in Ca metabolism. In particular, it seems probable that the presence of citrate in the immediate vicinity of calcifying or decalcifying bone might, by its well-known solvent action, play an important part in preventing premature precipitation of bone salts in this region.

The present evidence is certainly far too meagre to decide if this mechanism is really in operation or not. Some tentative support for a participation of bone citrate in calcification may, however, be claimed from the analyses of Table 5. On the basis of these individual results, it appears that the bone citrate is considerably affected by both hormonal and dietary conditions. In fact, it is much more affected than the other salts of bone, as is clearly shown by the recalculation of the data of Table 5 in terms of the molecular proportions of the three main constituents (Table 8). On the basis of these figures, parathyroid administration,

Table 8. *Molar ratios in dried extracted bone*

	Ratio of atoms (or mol.) of				Estimated molecular proportions* of		
	Ca	P	CO_2	Citric acid	$Ca_3(PO_4)_2$	$CaCO_3$	$Ca_3Cit._2$
Pup A, normal	78·3	50·8	8·04	1	100	33	2·1
Pup B, parathyroid	59·5	38·4	6·80	1	100	38	2·7
Kitten C, normal	198·5	108·2	18·8	1	100	32	0·9
Kitten D, rickets	278·2	172	30·8	1	100	37	0·6
(Dried bone meal	116	69·4	7·06	1	100	20	1·4)

* Approximate only, based on the assumption that all the P is present as $Ca_3(PO_4)_2$, all CO_2 as $CaCO_3$ and all citric acid as $Ca_3Cit._2$.

tending to hypercalcaemia, increases the relative molar proportions of citrate to $Ca_3(PO_4)_2$ and, to a less extent, to $CaCO_3$. On the other hand, a diet deficient in calcium has the opposite effect: the citrate is diminished by some 30%. The weakness of this evidence needs no emphasis; the normal variation has not been taken into account, and in the second experiment the diet deficient in Ca, from which milk and bones were omitted, was almost certainly also low in citric acid content. It shows, however, that the variations in citrate do greatly exceed those of the other salts; further experiments are being undertaken by Mrs Burns and it is hoped that these may provide more definite evidence. In this connexion, attention may be drawn to the calcifying action of citric acid and of citrates when added to a rachitic diet [cf. Hathaway & Meyer, 1939; Day, 1940].

The embryo. It is instructive to compare the data of Table 7 for the citric acid content of the chick embryo with those of Mankin [1929] on the Ca content (Table 9). Calculation of the ratio of equivalents of Ca to those of citric acid

Table 9. *Relationship of Ca to citric acid in the embryo chick*

Age in days	Average wt. g.	Ca, mg./100 g. embryo wet wt. [Mankin, 1929]	Citric acid mg./100 g.	Equiv. Ca / Equiv. Cit.
$3\frac{1}{2}$	0·037	—	31·0	3·3 : 1
4	0·144	32	—	
5	0·217	—	27·4	3·9 : 1
5	0·183	33	—	
8	1·263	—	13·3	5·8 : 1
8	1·010	24	—	
12	5·6	—	13·2	18·8 : 1
12	5·82	77·5	—	
21	—	381	9·4	130 : 1

(column 5) reveals the remarkable fact that in the earliest stages capable of investigation by the present method (4–5 days), the citrate present in the whole embryo is sufficient to combine with almost 1/3 of the total Ca. Thereafter, the total Ca increases much more rapidly than the citric acid.

The presence of so high a concentration of citrate relative to Ca, may, if it proves to be a general feature of embryonic metabolism, be of great importance in securing an adequate supply of readily mobilizable Ca for the use of the very young embryo.

It seemed probable that the shell and contents of the hen's egg would contain enough citric acid to provide all that contained in the embryo, for originally the shell of a 50 g. egg has 7·5 mg., the yolk 2·3 mg. and the white 0·3 mg. [Thunberg, 1941, 2]. In fact a newly hatched chick (Table 7) from an egg, which before incubation weighed 64·2 g., contained 4·56 mg. citric acid, or 3·55 mg. calculated for a 50 g. egg. Unincubated eggs of the same strain (R. I. R) contained, per 50 g. egg, 3·45 mg., and 21-day incubated, non-fertile, eggs 3·87 mg. citric acid (means of 3 each); while the shells of these eggs had only 2·18 and 1·95 mg., a much lower proportion than in Thunberg's experiments. The shell at hatching had 1·55 mg. in the example quoted in Table 7.

Equally important for the question of the origin of citric acid in the body, is a reconsideration of the work of Sherman *et al.* [1936] in the light of the present results. These authors conclude that 'consideration of the large amounts of citrate which are excreted by dogs on low citrate diet during prolonged alkalosis, and the absence of stores of preformed citrate in blood, liver, muscle and kidney, lead to the conclusion that the dog can synthesize citric acid'. Examination of the results of Sherman *et al.* [1936] shows that the three dogs studied by them excreted, over periods of from 18 to 45 days, 392, 335 and 608 mg. citric acid per kg. of dog. Such large amounts could not arise from preformed stores in tissues other than bone, but it seems possible that they might originate in the skeleton. Data for the dry weight of the total bones of the dog have not been found, but in a well fed rabbit the bones constitute 8·7% of the body wt. [Aron, 1909, p. 196]. Assuming a similar value for the dog, this animal would have per kg. some 80–90 g. dry bone. If we take the value from Table 5 for the normal pup, as applying to the whole bone of the dog, and make an allowance for the fat content, these bones might have about 1% citric acid. If these figures prove to be correct, the skeleton alone of dogs would contain about 800–900 mg. citric acid per kg. of dog, more than enough to cover the whole excretion of citrate observed by Sherman *et al.* [1936], provided that the loss of citric acid from the bones could reach 60% of the total amount originally present. From this admittedly very rough calculation it appears doubtful if the evidence of these authors, unsupported by bone analyses, may be interpreted to prove the endogenous synthesis of citric acid in the dog. This important question could now be decided by further experiments in which the citrate of the bones is taken into account. The same authors' preliminary experiments on the effect of alkalosis on citrate excretion in the rat, however, suggest that this point could be more easily proved by the use of this species [cf. Sober *et al.* 1940]. This would be very desirable in order to establish that the synthesis of citrate which Krebs & Eggleston [1940] observed *in vitro* also occurs in the living animal.

Summary

1. The citric acid content of the solid tissues of several species of animals has been determined.

2. It has been found that the hard substance of bone contains a hitherto unsuspected store of citric acid, which may constitute some 70% of that contained in the whole body. The marrow and cartilage contain much smaller amounts. Variations in the citrate content of bone, believed to be due to hor-

monal and dietary conditions, are reported. The variation of citrate greatly exceeds that of the other bone constituents, suggesting that the citrate is in a readily available form *in vivo*. *In vitro* it is not readily extracted from dried powdered bone by water, alcohol or ether.

3. Pure citric acid has been isolated from dried bone meal, and pure pentabromoacetone has been prepared from a trichloroacetic acid filtrate of bone meal in 80% of the yield calculated from analysis. It is concluded that the substance estimated in bone is in fact citric acid.

4. The skin and hair have high contents of citric acid, which may arise, at least in part, from the sebaceous secretion: a high content was found in a human sebaceous cyst. The citrate present in hair is to a large extent extractable by water.

5. The very young (3- to 5-day) chick embryo is rich in citric acid, and the amount present is sufficient to combine with one-third of the total embryonic calcium. As development proceeds, Ca increases more rapidly than citrate.

6. Tumour tissue usually, though not invariably, contains much citric acid.

7. The need for revision of certain experiments on the citric acid balance in animals, caused by the present findings, is pointed out.

8. It is concluded that the existing hypotheses of 'citrate-like substances' being concerned in calcium metabolism receive their main experimental support from the present detection of citrate itself as a major constituent of bone.

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