The possible relation between the uncoupling action of salicylate on oxidative-phosphorylation reactions and other effects of the substance on carbohydrate metabolism in animals has already been discussed by Smith & Jeffrey (1956). Some further examples have been reported. Manchester, Randle & Smith (1958) found that salicylate increased the glucose uptake of rat diaphragm incubated in a bicarbonate medium. This observation is explicable (Randle & Smith, 1958) on the basis that the entry of glucose into the muscle cells is normally restrained by a process dependent on a substance generated during oxidative phosphorylation and that this restraint is diminished by the uncoupling action of salicylate. The incorporation of ^{14}C into the liver glycogen of intact rats after the injection of [2-^{14}C]acetate is inhibited by salicylate (Smith, 1958). This finding is consistent with the view that salicylate impairs carbohydrate synthesis by interfering with oxidative-phosphorylation processes and hence the production of high-energy-phosphate-bond compounds which are necessary at various steps in the major pathway by which acetate carbons are incorporated into liver glycogen.

SUMMARY

1. The effects of salicylate on oxidative-phosphorylation reactions in mitochondria prepared from rat liver have been studied.

2. The phosphorylation associated with the oxidation of succinate and β-hydroxybutyrate was completely uncoupled by 1 mM-salicylate and higher concentrations; 0·1 and 0·5 mM produced smaller but still definite effects.

3. Part of the phosphorylation accompanying the oxidation of α-oxoglutarate appeared to be resistant to salicylate.

4. 5 mM-Salicylate increased the rate of oxygen consumption in an acceptor-deficient system above that in a corresponding control system saturated with glucose and hexokinase.

5. 5 mM-Salicylate completely prevented the swelling of mitochondria occurring in 0·25 M-sucrose solution. Lower salicylate concentrations caused less-marked effects.

6. Some implications of these results are discussed.

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Some Cation-binding Properties of Cartilage

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For some years there has been discussion of the importance of chondroitin sulphate in the calcification of cartilage. It has been suggested (Stetten, 1950; Neuman, 1952) that chondroitin sulphate should behave analogously to ion-exchange resins in the binding of cations.

Boyd & Neuman (1951) have shown that sodium, calcium and barium ions are equally bound to decalcified calf costal cartilage, that the binding capacity is closely related to the chondroitin sulphate content and that the binding of calcium ions is an ion-exchange reaction.

Sobel (1955) has considered the inactivation of the calcification *in vitro* of rachitic-bone sections by cations. The decreasing order of inactivating power was shown to be Be^{2+}, Cu^{2+}, Mg^{2+}, Na^{+}, Sr^{2+}, K^{+}, and this order is in the same direction as the reciprocal of the ionic radii. The mechanism of inactivation is said to involve competition between the ions for the chondroitin sulphate complex present in the organic matrix.

Simkiss & Tyler (1958) have studied the cation-binding properties of the organic matrix of egg shells. An acid mucopolysaccharide has been sug-

gested as the factor responsible for the binding, and the decreasing order of affinity of metal ions for the matrix is Fe^{3+}, Al^{3+}, Be^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, Ca^{2+}, Sr^{2+}, Li^{+}, Na^{+}. It has been proposed that chelate complexes are formed between the cation and the acid mucopolysaccharide, and that those metal ions which have the greater affinity for the matrix form the more stable complexes.

The cation-binding capacity of chondroitin sulphate has been investigated by Farber & Schubert (1957), using a dialysis technique. It has been shown that Na^{+} and K^{+} ions half saturate the available binding sites and that Ca^{2+} ions saturate about four-fifths of these sites. These authors used chondroitin sulphate in solution and its behaviour in this state will differ from that of insoluble ion-exchange material; with the latter the whole of the fixed charge is normally neutralized by excess of counter-ion.

The work of Boyd & Neuman (1951) did not include the cation inactivators considered by Sobel (1955), and magnesium and potassium were not included in the series studied by Simkiss & Tyler (1958). A further study of the ion-exchange processes involved in the cation binding by cartilage was considered desirable to test the applicability of Boyd & Neuman's findings (1951) to the cations that inactivate the calcification process. The cations studied in this work are Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Be^{2+}, Cu^{2+} and NH_4^{+}. Barium was included because it was one of the ions studied by Boyd & Neuman (1951), and ammonium was employed because of its possible use in equilibrium studies of the type described by Kressman & Kitchener (1949). It was considered advisable to use a tissue which contained considerable quantities of chondroitin sulphate. Bovine nasal septum, which contained approximately 25% of chondroitin sulphate, was selected for this purpose, despite the fact that it is not a normally calcifiable tissue.

EXPERIMENTAL

Materials. Solutions of cations were prepared from the chlorides (A.R.) of the metals with the exceptions of copper, where the (cupric) acetate (A.R.) was used, and beryllium, where beryllium carbonate was dissolved in formic acid. In the experiments on the effect of anions the solutions were prepared by dissolving $CaCO_3$ (A.R.) in the calculated quantities of the required acids. Chondroitin sulphate was extracted from bovine nasal septa by the method A 2 of Einbinder & Schubert (1950) and purified by the method recommended by these authors. A second sample, obtained from L. Light and Co., was also used.

Preparation of wash water. Deionized water (from the portable Deminrolit plant MK 4; The Permutit Co.) was boiled for 30 min., cooled rapidly and used immediately. The pH of this water varied from 7·5 to 8·5.

Preparation of cartilage. Bovine nasal septum was cleaned of other tissue, minced finely and dried overnight at 95°. The dried material was ground finely and decalcified with a 3% (w/v) solution of the disodium salt of ethylenediaminetetra-acetic acid (previously adjusted to pH 7·3–7·4 with NaOH), until the calcium content was less than 6×10^{-3} m-equiv./g. of dry cartilage.

Cation binding by cartilage (*Method* 1). Approximately 200 mg. samples of decalcified cartilage were shaken with 100 ml. portions of the cation solution for 3 hr. Solutions containing 1 equiv. of cation/l. were employed, except for copper, where a saturated solution of cupric acetate was used. The pH of the cation solutions varied between 5·0 and 6·1, with the exception of solutions of Ca^{2+} and NH_4^{+} ions, where values as high as pH 10·5 and 8·7 respectively were used. The pH of the supernatant solution remained practically constant during the experiments. The supernatant solution was removed by centrifuging and the cartilage was washed five times with 10 ml. portions of boiled-out deionized water, the solid being removed each time by centrifuging. The solid material was then transferred to small beakers with a further 10 ml. portion of water. The water was decanted and the solid dried at 95° overnight. Samples containing NH_4^{+} ions were dried for 48 hr. *in vacuo* over P_2O_5.

The dried samples were then analysed for sulphate and bound cation.

Equilibrium distribution experiments (*Method* 2). Samples (100 mg.) of decalcified cartilage were placed in 50 ml. stoppered bottles and 25 ml. portions of cation solution of known concentration and pH 6·0–7·0 were added quickly. Shaking was carried out manually at intervals during several hours. After equilibrium had been reached portions of solution were removed for analysis, and the cartilage was washed, dried and analysed as described previously.

Analysis of samples. (i) Sodium and potassium were determined by dry ashing at 450–500°, dissolving in 2N-HCl and estimating the cations with a flame photometer (Beckman model DU with flame-photometer attachment, and Lange flame photometer model 5). (ii) Magnesium was determined by dry ashing, dissolving in 2N-HCl and titrating with ethylenediaminetetra-acetic acid (EDTA), with Eriochrome Black T as indicator (Griswold & Pace, 1956). (iii) Calcium was determined by dry ashing, dissolving in 2N-HCl and titrating with EDTA, murexide being used as indicator (Dunstone, 1957). (iv) Barium and strontium were determined by dry ashing, adding 2 drops of 10N-H_2SO_4, evaporating to dryness and weighing the residue (Boyd & Neuman, 1951). (v) Beryllium was determined by dry ashing, dissolving in 9N-H_2SO_4 and then estimating by the method of White, Meyer & Manning (1956). (vi) Copper was determined by dry ashing, dissolving in 2N-HNO_3 and measuring the extinction of the copper–EDTA complex at a wavelength of 280 mμ in a Unicam SP. 500 spectrophotometer. Owing to the fact that the NO_3^{-} ion absorbs strongly at this wavelength (Buck, Singhadeja & Rogers, 1954), it was necessary to construct a calibration curve by measuring the extinctions of the EDTA complexes of known amounts of copper in the presence of the same concentration of NO_3^{-} ion as that used in the analysis of the cartilage. The concentration of copper in the sample was then determined by reference to this calibration curve. By this method a percentage recovery of copper from mixtures of known amounts of

$CuSO_4,5H_2O$ (A.R.) and decalcified cartilage was found to be 101 ± 1 (three analyses). (vii) The NH_4^+ ion was determined by placing the sample in the distillation unit of the apparatus described by Hoskins (1944), adding 10 ml. of a saturated solution of sodium borate, steam-distilling the NH_3 liberated into boric acid-indicator mixture (Conway, 1947) and then titrating with standard acid. (viii) Sulphate was determined by refluxing the sample for 4 hr. with 2N-HCl and then using the method of Fritz, Yamamura & Richard (1957). With cartilage to which Ba^{2+}, Ca^{2+} and Sr^{2+} ions were bound, it was possible to adjust the quantity of 2N-HCl so that all the SO_4^{2-} ion present would remain in solution. (ix) Chloride was estimated by comparison of the opalescence produced by mixing the test solution and a solution of $AgNO_3$, acidified with HNO_3, with that obtained by treating a standard chloride solution in similar fashion. Solutions were prepared in 50 ml. Nessler tubes and comparisons of opalescence were made visually as recommended by The British Drug Houses Ltd., & Hopkins and Williams Ltd. (1949).

Potentiometric-titration experiments. Potentiometric titrations were carried out by the addition of 3N-NaOH from an Agla micrometer syringe burette, and measurements of pH were made with a Jones model B pH electrometer. Titrations were made on the following materials: (i) 0·5 g. of decalcified cartilage (approx. 0·8 m-equiv. of sulphate/g.) suspended in 50 ml. of 0·02N-HCl. (ii) Same as (i) with the addition of 0·5 m-equiv. of cation. (iii) 0·5 m-equiv. of cation dissolved in 50 ml. of 0·02N-HCl. (iv) 0·1 g. of chondroitin sulphate dissolved in 50 ml. of 0·02N-HCl. (v) Same as (iv) with the addition of 0·5 m-equiv. of cation.

In the titration of cartilage suspensions, periods of up to 1 hr. must be allowed after each addition of alkali for pH equilibrium to be attained.

RESULTS

Preliminary experiments

Measurement of velocity of attainment of equilibrium. Samples (100 mg.) of decalcified cartilage were treated with 25 ml. portions of a solution containing 7·5 m-equiv. of calcium/l. Shaking was carried out manually at 10 min. intervals. Portions (1 ml.) of supernatant solution were taken for assay at times 0, 0·5, 1·0, 2·0, 4·0 and 7·0 hr. It was found that equilibrium had been established after 1·0 hr. In all subsequent measurements periods longer than this were allowed to ensure complete equilibrium.

Validity of method. During the process of cation binding by cartilage it was necessary to shake the finely minced heat-dried cartilage with solutions of cations and subsequently to remove unbound cation by repeatedly washing with aqueous solutions. During these procedures a proportion of the chondroitin sulphate is likely to be extracted from the tissue, and some of the bound cation may exchange with H^+ ions.

In order to investigate the role of chondroitin sulphate in the binding of cations by cartilage, it was necessary to consider only the chondroitin sulphate present in the cartilage after cation binding. The chondroitin sulphate that may have been extracted from the tissue need not be considered, as it would not affect the amount of calcium bound to the remaining cartilage.

Table 1. *Effect of washing cartilage to which cation is bound*

Calcium-binding experiments were carried out as described in Method 1 (Experimental section). Samples of the various washings, and of cartilage which had been washed 4, 5, 6, 7, 8, 9, 16, 21 and 24 times, were collected and analysed. All results are expressed as mean values of figures obtained from triplicate experiments. Analyses on the dried cartilage are shown in (*a*) and those on the washings in (*b*).

(*a*)

Sample	Calcium present (m-equiv./g.)	Sulphate content after binding (m-equiv./g.)
Cartilage washed 4 times	0·91	—
Cartilage washed 5 times	0·88	0·82
Cartilage washed 6 times	0·80	0·81
Cartilage washed 7 times	0·77	—
Cartilage washed 8 times	0·83	—
Cartilage washed 9 times	0·80	—
Cartilage washed 16 times	0·86	0·81
Cartilage washed 21 times	0·79	—
Cartilage washed 24 times	0·79	—

(*b*)

Sample	Calcium present (m-equiv./l.)	Chloride present (m-equiv./l.)
Fifth washing	2·50	—
Sixth washing	0·85	>0·28
Seventh washing	0·15	Approx. 0·28
Eighth washing	0·10	<0·14
Ninth washing	0·10	Not detectable
Fifteenth washing	0·05	Not detectable
Twenty-fifth washing	0·05	Not detectable

In the study of the possible effects of H^+-ion exchange with bound cation, experiments were carried out by shaking 100 ml. portions of a solution of calcium chloride (1 equiv./l., pH 6·0) with 200 mg. samples of decalcified cartilage. The supernatant solution was removed by centrifuging and the cartilage was washed with 10 ml. portions of boiled-out deionized water, the solid being removed each time by centrifuging. The washings were collected and analysed for calcium and chloride. The solid samples were transferred to small beakers and dried at 95°. The dried samples were then analysed for calcium and sulphate. The analyses of the washings and the cartilage are shown in Table 1. These results indicate that: (i) No significant amounts of calcium chloride solution remain after the sixth washing. (ii) No chondroitin sulphate is removed by the washing procedures. (iii) After the sixth washing only very small amounts of bound calcium are removed from the

cartilage. This presumably occurs by exchange with H^+ ions, but the amount exchanged may be regarded as negligible when the variation in the amount of cation bound is considered.

Equilibrium distribution experiments. Experiments were carried out by equilibrating 100 mg. samples of decalcified cartilage with 25 ml. portions of calcium chloride solution (pH 6·0). Portions of solution were removed and analysed for calcium. The amount of calcium bound by the cartilage was thus determined. The concentration of calcium solution was increased until the cartilage would bind no more cation. The results of these experiments are given in Table 2. The maximum binding capacity of the same sample of cartilage determined by Method 1 (Experimental section) is also included in Table 2. Both methods were found to give the same value for the calcium-binding capacity of the cartilage.

Table 2. *Equilibrium distribution experiments*

Portions (25 ml.) of calcium chloride soln. (pH 6·0) were equilibrated with 100 mg. samples of decalcified cartilage. Portions of solution were removed after equilibrium had been reached and analysed for calcium. All results are expressed as mean values of figures obtained from triplicate experiments.

Concn. of $CaCl_2$ soln. (m-equiv./l.)	Calcium bound (m-equiv./g.)
4·8	0·58
7·8	0·68
12·4	0·73
21·2	0·79
38·5	0·79
49·0	0·80

Calcium bound [determined by Method 1 (Experimental section)] in m-equiv./g. (±S.D.): 0·80±0·03 (7)

Table 3. *Effect of anions on calcium binding by cartilage*

Calcium-binding experiments were carried out as described in Method 1 (Experimental section), with 100 ml. portions of solutions containing 1 equiv. of the following per litre: calcium acetate, calcium chloride, calcium formate, calcium nitrate and calcium perchlorate at pH 6·7–7·0 and pH 10·5–10·6. All results are expressed as mean values of figures obtained from duplicate experiments.

pH	Anion	Calcium bound (m-equiv./g.)
6·9	$CH_3CO_2^-$	0·85
10·6	$CH_3CO_2^-$	0·89
6·8	Cl^-	0·86
10·5	Cl^-	0·87
6·9	HCO_2^-	0·85
10·5	HCO_2^-	0·85
7·0	NO_3^-	0·85
10·5	NO_3^-	0·86
6·7	ClO_4^-	0·85
10·5	ClO_4^-	0·83

Influence of the anion. The degree of cation binding by cartilage might be expected to vary considerably according to the nature of the anion present, particularly during variation of pH. Cation-binding experiments were carried out by treatment of 200 mg. samples of cartilage with solutions containing 1 equiv. of the following per litre: calcium acetate, calcium chloride, calcium formate, calcium nitrate and calcium perchlorate. Two series of experiments were carried out, one at pH 6·7–7·0 and the other at pH 10·5–10·6. The results given in Table 3 show that the cation-binding capacity of cartilage is independent of the anion in both ranges of pH.

Effect of particle size. Experiments were carried out with decalcified cartilage of particle sizes 10–30, 30–60, 60–100 and finer than 100 mesh respectively. The cation-binding capacity was found to be constant, provided that the particle size was smaller than 30 mesh. Particles of 40–100 mesh were used for all subsequent experiments.

Effect of pH *on metal binding.* The calcium-binding capacity of cartilage has been shown to

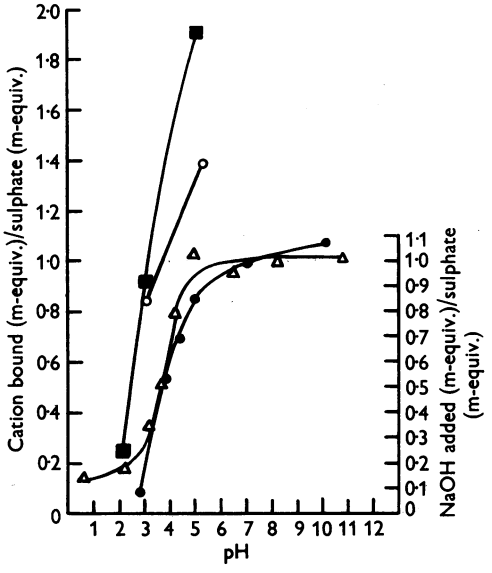

Fig. 1. Effect of pH on the binding of cations and a potentiometric titration of chondroitin sulphate. The cation-binding capacity of decalcified cartilage for Ca^{2+}, Cu^{2+} and Be^{2+} ions was determined at various pH values as previously described (Method 1, Experimental section). The potentiometric titration of a solution of 0·1 g. of chondroitin sulphate dissolved in 50 ml. of 0·02 N-HCl was carried out with 3 N-NaOH as described in the Experimental section. The part of the titration curve associated with the neutralization of the excess of acid is not shown in the Figure. △, Ca^{2+}; ○, Cu^{2+}; ■, Be^{2+}; ●, potentiometric titration of chondroitin sulphate with NaOH.

vary considerably with pH. This effect is illustrated in Fig. 1. It appears that maximum binding occurs above pH 5·0. Similar effects have been shown for Be^{2+} and Cu^{2+} ions, but no data above pH 5·0 are available owing to the difficulty in obtaining a high pH without cation precipitation or the addition of undesirable anions, which may compete with the cartilage for the cation. Constant binding capacities for NH_4^+ ions have been shown over the range pH 5·0–9·0.

Owing to the fact that chondroitin sulphate is likely to be extracted from the tissue, particularly when alkaline pH is used, all analyses were carried out on the dry, washed cartilage after cation binding.

Cation binding by cartilage

Owing to the fact that maximum binding occurs at pH 5·0 and above, all experiments were carried out at, or above, this value. No attempts were made to buffer the cation solutions because of the possible competitive effects that other anions and cations might exert on the degree of cation binding. Where pH adjustments were necessary (experiments at low and high pH values), these were made by suitable additions of hydrochloric acid or base (hydroxide of cation under investigation) so that no interfering anions or cations were present.

The cations Na^+, K^+, Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+} (group A) appeared to be bound to cartilage in a similar fashion, whereas the cations Be^{2+}, Cu^{2+} and NH_4^+ (group B) behaved differently. In discussing the experimental results, the group A cations will be considered collectively and the group B cations will be considered individually. The results, which show maximum binding capacity for all ions, in terms of the ratio cation bound:sulphate content are shown in Table 4.

In group A the sulphate content of the cartilage shows a definite correlation with the amount of cation bound. Although some variation in the amount of cation bound was observed, the ratio cation bound (in m-equiv./g. of dry cartilage):sulphate content (in m-equiv./g. of dry cartilage) may be considered as unity.

With Be^{2+} ions some difficulty was encountered in maintaining solutions at approximately pH 5·0, owing to hydrolysis of the Be^{2+} ion, which may be represented by the following equation:

$$Be^{2+} + H \cdot OH \rightleftharpoons BeOH^+ + H^+.$$

This difficulty was overcome by dissolving beryllium carbonate in the calculated quantity of formic acid. A buffer solution of pH 5·0 was thus obtained. The pH of this solution remained constant over long periods of time. This is attributed to the fact that the Be^{2+} ion is only very slowly hydrolysed in the presence of the formate ion (Sidgwick, 1950). At this pH the Be^{2+} ion was bound by the cartilage to a greater extent than the

Table 4. *Cation-binding capacity of decalcified cartilage*

Cation-binding experiments were carried out as described in Method 1 (Experimental section). All results are expressed as the mean values of figures obtained from the replicate experiments.

Cation used	Anion present	pH	No. of expts.	Cation bound (m-equiv./g.)	Sulphate content after binding (m-equiv./g.)	Cation bound (m-equiv.) / Sulphate present (m-equiv.)
Na^+	Cl^-	5·7	4	0·78	0·77	1·01
			2	0·99	1·01	0·98
K^+	Cl^-	5·6	3	0·90	0·89	1·01
			4	0·75	0·77	0·97
Mg^{2+}	Cl^-	6·1	2	0·95	0·87	1·08
			2	0·81	0·78	1·04
			4	0·75	0·78	0·96
Ca^{2+}	Cl^-	5·0–10·4	4	0·83	0·81	1·02
			3	0·92	0·94	0·98
			2	0·86	0·89	0·97
Sr^{2+}	Cl^-	5·2	3	0·87	0·86	1·01
			2	0·77	0·80	0·96
Ba^{2+}	Cl^-	5·9	2	0·91	0·86	1·06
			2	0·84	0·80	1·04
			4	0·73	0·75	0·97
Be^{2+}	HCO_2^-	5·0	2	1·40	0·83	1·69
			2	1·57	0·77	2·04
			3	1·55	0·75	2·07
Cu^{2+}	$CH_3CO_2^-$	5·3	4	1·33	0·91	1·46
			2	1·23	0·98	1·26
			4	1·07	0·75	1·43
NH_4^+	Cl^-	5·6–8·7	7	0·75	0·95	0·79
			3	0·69	0·85	0·81

Table 5. *Effect of drying conditions on bound ammonium ions*

Known amounts of decalcified cartilage were shaken with 100 ml. portions of NH_4Cl solutions (1 equiv./l.; pH 5·6–8·7); the solution was decanted and the cartilage washed with boiled-out deionized water as previously described (Method 1, Experimental section). The cartilage was either: (*a*) dried at 95° and analysed; or (*b*) dried *in vacuo* over P_2O_5 and analysed; or (*c*) analysed without drying. All results are expressed as the mean values of figures obtained from the triplicate experiments.

No. of expts.	Drying conditions	Ammonium ion bound (m-equiv.) / Sulphate present (m-equiv.) After 16 hr.	After 40 hr.	After 72 hr.	After 29 days
3	95°	0·60	0·53	0·48	0·33
3	*In vacuo* over P_2O_5	0·79	—	0·78	0·76
3	No drying	0·81	—	—	—

Table 6. *Dependence of cation-binding capacity on the chondroitin sulphate content of cartilage*

Chondroitin sulphate was successively removed from cartilage by method A 2 of Einbinder & Schubert (1950) and cation-binding experiments were carried out as described in Method 1 (Experimental section). All results are expressed as the mean values of figures obtained from the replicate experiments.

No. of expts.	Calcium bound (m-equiv./g.)	Sulphate content after binding (m-equiv./g.)
3	0·93	0·94
2	0·85	0·88
2	0·82	0·80
2	0·69	0·68
3	0·64	0·60
4	0·39	0·35

group A cations. Measurements at lower pH were carried out with beryllium chloride solutions to which hydrochloric acid had been added. At these lower pH values the cartilage was also found to bind Be^{2+} ions to a greater extent than Ca^{2+} ions at corresponding pH values (see Fig. 1).

Copper (Cu^{2+}) ions were also found to be bound to a greater extent than the group A cations, but to a lesser extent than Be^{2+} ions. At lower pH values Cu^{2+} ions (cupric chloride solutions to which hydrochloric acid had been added) were bound to a greater extent than Ca^{2+} ions at corresponding pH values (see Fig. 1).

When cartilage to which NH_4^+ ions had been bound was dried overnight at 95°, a yellow–brown material was produced. The NH_4^+-ion content of this material was approximately one-half of that found for similar experiments with the cations in group A. On longer periods of drying at this temperature, the material became darker and the NH_4^+-ion content less. When samples were dried *in vacuo* over P_2O_5 unvarying results were obtained over a wide range of drying times. Other samples were not dried but assayed wet, and results comparable with those found by drying *in vacuo* over P_2O_5 were obtained (Table 5). The maximum binding of NH_4^+ ions was found to be less than that observed for the cations of group A.

Dependence of cation binding on chondroitin sulphate content of cartilage

Chondroitin sulphate was extracted from the decalcified cartilage by the method A 2 of Einbinder & Schubert (1950), and cation-binding experiments were then carried out on the cartilage as described in Method 1 of the Experimental section, with preparations which contained differing amounts of chondroitin sulphate. The results of these experiments, given in Table 6, show that the amount of cation bound depends to a large extent on the chondroitin sulphate content of the cartilage.

Potentiometric-titration experiments

It has been shown (Albert, 1950; Martell & Calvin, 1952) that potentiometric-titration methods may be used in the detection of chelation. When a compound is titrated in the presence and the absence of cations, shifts in the titration curves indicate chelate formation.

To investigate the nature of the binding of cations by cartilage, titrations of decalcified-cartilage suspensions and chondroitin sulphate solutions were carried out in the presence and the absence of the cations K^+, Be^{2+}, Mg^{2+}, Ca^{2+} and Cu^{2+}.

When cartilage or chondroitin sulphate was titrated in the presence of Be^{2+}, Mg^{2+} and Cu^{2+} ions small deviations of the titration curves away from those of the cartilage or chondroitin sulphate alone were observed. No deviations were observed when titrations of these materials were carried out in the presence of K^+ and Ca^{2+} ions. The titration curves obtained by titration of Be^{2+}, Mg^{2+} and Cu^{2+} ions showed steps associated with the precipitation of the metal hydroxides (or basic salts). These steps occurred at pH 4·7 and 6·0 for Be^{2+}, pH 10·5 for Mg^{2+} and pH 4·9 for Cu^{2+} ions. In the presence of either cartilage or chondroitin sulphate the titration curves showed similar steps but at somewhat higher pH values. With chondroitin sulphate the steps occurred at pH 4·9 and 6·8 for Be^{2+}, pH 10·8 for Mg^{2+} and pH 5·5–6·1 for Cu^{2+} ions. At all stages of these titrations no precipitation of metal hydroxide (or basic salt) was observed,

although precipitates were noticed when chondroitin sulphate was absent. With cartilage considerable time (up to 1 hr.) had to be allowed after each addition of 3N-NaOH for equilibrium to be established. The titration curves obtained showed steps similar to those with chondroitin sulphate. The titration curves obtained for the potentiometric titrations of (i) 0·5 m-equiv. of copper as $CuCl_2,2H_2O$ in 50 ml. of 0·02N-HCl, (ii) 0·5 m-equiv. of copper as $CuCl_2,2H_2O$ and 0·1 g. of chondroitin sulphate in 50 ml. of 0·02N-HCl, and (iii) 0·1 g. of chondroitin sulphate in 50 ml. of 0·02N-HCl, are given in Fig. 2.

DISCUSSION

The equivalent binding capacity of the cartilage for sodium, potassium, magnesium, calcium, strontium and barium indicates that a similar mechanism of binding is involved in each case. The close correlation between the binding capacity and the sulphate content of the cartilage indicates that chondroitin sulphate is primarily responsible for the binding. Each disaccharide-repeating unit of chondroitin sulphate has one carboxyl and one ester sulphate group (Davidson & Meyer, 1955), and it can be seen that both of these groups would account for the observed binding capacity. It is thought that the bivalent metals of group A would combine with the chondroitin sulphate moiety through the sulphate ester and carboxyl groups to form chelate compounds.

A mechanism for the binding of Be^{2+} and Cu^{2+} ions is difficult to devise. It is peculiar that these two ions, which are bound to the greater extent, are the most powerful inhibitors of calcification *in vitro* (Sobel, 1955).

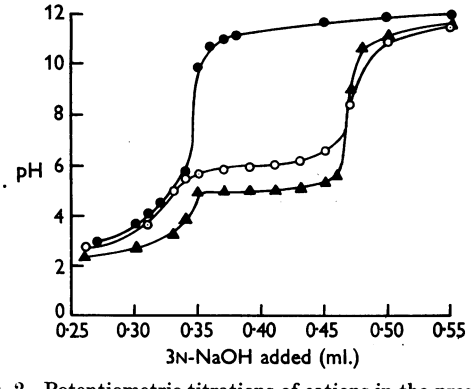

Fig. 2. Potentiometric titrations of cations in the presence and the absence of chondroitin sulphate. ●, 0·1 g. of chondroitin sulphate in 50 ml. of 0·02N-HCl; ○, 0·1 g. of chondroitin sulphate and 0·5 m-equiv. of copper (as $CuCl_2,2H_2O$), i.e. 5 m-equiv. of cation/g. of chondroitin sulphate, in 50 ml. of 0·02N-HCl. ▲, 0·5 m-equiv. of copper (as $CuCl_2,2H_2O$) in 50 ml. of 0·02N-HCl.

Bower & Truog (1940) have observed that certain clay fractions bind Be^{2+} and Cu^{2+} ions to a greater extent than the group A ions considered in this work. They have reasoned that the greater binding of these ions is due to the presence in the cation solution of monohydroxide ions (MOH^+, where M^{2+} represents the cation), which may attach themselves to the clay particle, the degree of binding being dependent on the relative concentrations of the MOH^+ and M^{2+} ions. They have suggested that MOH^+ ions combine with a weak clay acid in the following manner:

$$Clay^- + MOH^+ \rightarrow clay\ |\text{—}M\text{—}OH$$

A similar, though not identical, situation may prevail in the binding of Be^{2+} and Cu^{2+} ions by cartilage. Feldman & Havill (1952), Gurd & Wilcox (1956) and Mattock (1954) have reported that monohydroxide ions of Be^{2+} and Cu^{2+} are formed in aqueous solution by the hydrolysis of the metal ions and that these monohydroxide ions tend to associate to form multinuclear complexes. The formation of these multinuclear complexes occurs in the region of pH somewhat below the point at which the insoluble metal hydroxides (or basic salts) begin to precipitate.

Ionic species such as Be^{2+}, $BeOH^+$, Be_2OH^{3+}, $Be_3(OH)_2^{4+}$ and a second series of ions $Be_n(OH)_n^{n+}$ have been postulated as being present in solution by Mattock (1954). Rollinson (1956) reports that the most abundant ionic species in beryllium nitrate solutions is $[Be(H_2O)NO_3]^+$, and that, on ageing, the nitrate group is replaced by an hydroxyl group and the resulting hydroxo compound is thought to be capable of dimerizing:

$$2\,[Be(H_2O)OH]^+ \rightarrow [(H_2O)Be\text{—}O\text{—}Be(H_2O)]^{2+} + H_2O.$$

In a study of copper (Cu^{2+}), Gurd & Wilcox (1956) report that Pedersen (1943) has found evidence for the presence of dimers, which he has formulated as

$$[Cu_2OH]^{3+} \quad \text{or} \quad [(H_2O)_7Cu_2OH]^{3+},$$

and $$[Cu_2O]^{2+} \quad \text{or} \quad [(H_2O)_6Cu_2(OH)_2]^{2+}.$$

The conditions under which these multinuclear ions of Be^{2+} and Cu^{2+} are formed are similar to those used for the cation-binding experiments reported here. If such ions combine with cartilage through the chondroitin sulphate, then the greater binding capacities for these ions can be explained. The degree of such binding would be dependent on the relative concentrations of the various ionic species present in solution. The binding of these multinuclear ions by cartilage may occur by combination with both the sulphate ester and carboxyl groups of the chondroitin sulphate moiety.

The binding of such multinuclear ions to cartilage would thus involve the formation of ring systems, and this would explain the stabilization of the cation against precipitation during titration with alkali.

The potentiometric-titration experiments carried out in this study give results which are similar to those obtained by Albert (1950) for the titration of a mixture of two substances which do not mutually form a complex. Under these circumstances the precipitation of metal hydroxide (or basic salt) takes place at the same pH as it does when the metal ion is titrated alone. In the titrations with alkali of Be^{2+}, Mg^{2+} and Cu^{2+} ions, in the presence of chondroitin sulphate no such metal hydroxide (or basic salt) is precipitated. Although each titration curve shows a similar step to the precipitation step, the pH at which this occurs is somewhat higher than the pH at which the metal hydroxide (or basic salt) is precipitated in the absence of chondroitin sulphate. These facts lead to the conclusion that some complex between the cation and chondroitin sulphate is formed and that the complex is sufficiently stable to prevent the precipitation of the metal hydroxide (or basic salt). Therefore the steps observed in the potentiometric-titration curves cannot be associated with precipitation, but might be associated with the combination of OH^- ions to cation which is already bound to chondroitin sulphate. It would be possible for OH^- ions to combine with such a cation–chondroitin sulphate complex by occupying positions in the co-ordination sphere of the particular cation not occupied by the binding groups of the chondroitin sulphate. This presumably could occur by replacement of co-ordinated water molecules with hydroxyl groups.

In these potentiometric-titration experiments, the cations showed almost identical behaviour towards chondroitin sulphate and cartilage suspensions. This fact further implicates chondroitin sulphate as a factor involved in the binding of cations by cartilage.

Further evidence suggesting that chondroitin sulphate is the main factor responsible for the binding can be obtained by considering the variation in calcium binding with pH. The curve obtained by plotting the ratio calcium bound:sulphate content against pH is roughly similar to the pH titration curve of chondroitin sulphate (Fig. 1). This indicates that the state of ionization of the binding sites of chondroitin sulphate may be a regulating factor which determines the percentage of cation bound.

In the binding of NH_4^+ ion close correlation with the sulphate content was expected. An explanation of the low result, in which only about four-fifths of the available binding sites were saturated, is not known at present.

SUMMARY

1. Decalcified bovine nasal septum showed equal binding capacities for Na^+, K^+, Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+} ions. This capacity was closely correlated with the sulphate content, indicating that chondroitin sulphate was responsible for the major portion of the binding.

2. Cu^{2+} and Be^{2+} ions were found to be bound to a greater extent, and NH_4^+ ions were found to be bound to a less extent, than the ions mentioned above. It is possible that the greater binding of Cu^{2+} and Be^{2+} ions may be related to the powerful inhibition of calcification of rachitic-bone sections (Sobel, 1955) exhibited by these ions *in vitro*. This greater binding is attributed to the binding by chondroitin sulphate of multinuclear ions of Cu^{2+} and Be^{2+}.

3. The binding capacity for calcium has been shown to vary considerably with pH, and the reasons for the variation have been discussed.

4. The possibility that the cartilage may act as a complexing agent in the binding of metals has been studied by potentiometric-titration methods. It has been concluded that complex compounds are formed and the cations Be^{2+}, Mg^{2+} and Cu^{2+} are bound sufficiently strongly to prevent the precipitation of the metal hydroxides (or basic salts).

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The Acute Toxic Action of Dimethylnitrosamine on Liver Cells

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Dimethylnitrosamine has been shown to be selectively hepatotoxic for several species of mammals, producing demonstrable necrosis within 24 hr. (Barnes & Magee, 1954) and, on prolonged administration, to produce malignant liver tumours (Magee & Barnes, 1956). It is rapidly metabolized (Dutton & Heath, 1956) and Magee (1956) demonstrated that the liver is the main, and probably the only, site of its metabolism. Magee (1957) also found that the incorporation of labelled amino acids into liver protein is impaired as early as 6 hr. after treatment.

The rapid necrotic effect on liver cells suggests that dimethylnitrosamine, or a metabolite, might interfere with respiratory metabolism of the liver cells, and this paper reports an investigation of this.

MATERIALS AND METHODS

Animals

The animals belonged to a strain of Sprague–Dawley rats inbred by brother–sister matings to the stage of successful homologous skin grafting. The diet consisted of Poultry Growers' Pellets (Barastoc Products, Melbourne), fresh green vegetables and water. Rats were used irrespective of sex when they weighed between 150 and 250 g. As far as possible, litter mates were used for each series of experiments. Dimethylnitrosamine was injected intraperitoneally in aqueous solution.

Reagents

Inorganic reagents were of analytical grade; glass-distilled water was used throughout. Cytochrome *c* was prepared by the method of Keilin & Hartree (1937) and dialysed against water. Diphosphopyridine nucleotide (DPN), adenosine 5′-phosphoric acid, adenosine 5′-triphosphoric acid (ATP), coenzyme A, L-malic acid and sodium pyruvate were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Thiamine pyrophosphate, flavinadenine dinucleotide (FAD), thioctic acid, α-oxoglutaric acid, β-hydroxybutyric acid and choline chloride were products of L. Light and Co. Ltd. Liver concentrate 202–20 was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Vitamin B_{12} was a product of The Distillers Co. (Biochemicals) Ltd. Sodium citrate (A.R.), sucrose (A.R.), sodium succinate, octanoic acid, nicotinamide and the disodium salt of ethylenediaminetetraacetic acid (EDTA) were obtained from British Drug Houses Ltd. Octanoic acid was purified by redistillation *in vacuo*.

Dimethylnitrosamine was prepared by the method of Hatt (1946).

The quantity of DPN required for each experiment was weighed shortly before use and dissolved in 0·4 M-nicotinamide to a concentration of 5 mg. of DPN/ml. of solution. This precaution aimed at preventing non-enzymic hydrolysis of DPN, which is otherwise rapid at neutral or acid pH, before the beginning of the incubation. A volume of 0·2 ml. of the mixture was added to each flask immediately before addition of the enzyme. In experiments in which four runs of twelve flasks had to be made in rapid succession, volumetric addition of the DPN was the most practical method.

Tissue preparations

Animals were killed by stunning and exsanguination, and the whole liver was rapidly excised and placed immediately in aqueous 0·25 M-sucrose solution at 0°. After chilling, it was removed, blotted dry, weighed on a torsion balance and samples were taken for biochemical and histological studies.

Homogenates. Rat-liver homogenates (10%, w/v) were prepared in a cold room at 0–1° by means of a Potter–Elvehjem all-glass homogenizer with 0·25 M-sucrose solution as the suspension medium.