then the role of transhydrogenase would be clearly indicated. However, the evidence for this is not definite. Rat heart muscle, kidney and liver, which all have high transhydrogenase activities, contain 3–6 $\mu$g. of $TPN^+$/g. but the concentration of TPNH is 33–205 $\mu$g./g., the $TPN^+$/TPNH ratio being 0·03–0·13 (Glock & McLean, 1955). Rat prostate, seminal vesicle, spleen and testis, which have negligible transhydrogenase activity, contain negligible quantities of $TPN^+$ ($<2\,\mu$g./g.) and only 8–12 $\mu$g. of TPNH/g. It could be argued that transhydrogenase occurs along with the higher concentrations of $TPN^+$ + TPNH; on the other hand, it would be expected that the presence of transhydrogenase would lead to a value for $TPN^+$/TPNH higher than that observed (0·03–0·13), especially since all tissues so far tested contain more $DPN^+$ than DPNH, e.g. Glock & McLean (1955) give $DPN^+$/DPNH for rat heart as 1·6 and Jedeikin & Weinhouse (1955) give 5·5 for the same tissue, the total concentrations given being respectively 483 and 563 $\mu$g./g.

## SUMMARY

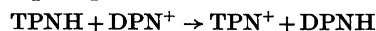
1. Transhydrogenase occurs in animal tissues, particularly in heart muscle and to a smaller extent in skeletal muscle, kidney and liver. Other tissues such as brain, prostate, seminal vesicle, spleen and testis show small or negligible activities.

2. Transhydrogenase is localized in mitochondria.

3. Transhydrogenase can be purified by precipitation with ammonium sulphate in the range 20–35 % saturation.

4. Inert material can be removed from solutions of transhydrogenase and the activity thus increased, by centrifuging for 3 hr. at 160 000 *g*.

5. The pH optimum for each of the reactions

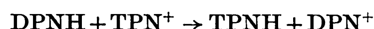


$$TPNH + DPN^+ \rightarrow TPN^+ + DPNH$$

and $$DPNH + TPN^+ \rightarrow DPN^+ + TPNH$$

is 6·3.

6. Michaelis constants for the reaction



$$DPNH + TPN^+ \rightarrow TPNH + DPN^+$$

are $1·4 \times 10^{-5}$ M DPNH and $15·7 \times 10^{-5}$ M $TPN^+$.

7. The action of transhydrogenase is strongly inhibited by sulphydryl reagents.

8. Solutions of transhydrogenase show maximum light absorption at 275 m$\mu$.

9. There is no evidence at present for the presence of any flavine or metal prosthetic group in the enzyme.

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# The Utilization of Inorganic Sulphate by Granulation Tissue

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In a preceding paper (Kodicek & Loewi, 1955), the uptake of $^{35}SO_4^{2-}$ by granulation tissue *in vivo* and *in vitro* was described. It was found that a sulphated mucopolysaccharide akin to chondroitin sulphate was formed, the formation depending on enzymic activity of the tissue.

The present communication is concerned with studies of the relationship between the respiratory activity of granulation tissue at various stages of maturation and the uptake of $^{35}SO_4^{2-}$. The effect of various added substances has also been investigated. We have examined the inhibitory effects on the system of various phenols, some sulphated substances and fluoroacetate. Addition of glucose to respiring sections of cartilage (Boyd & Neuman, 1954; Boström & Jorpes, 1954) resulted in increased incorporation of $^{35}SO_4^{2-}$, and Boström, Roden & Vestermark (1955) reported that glutamine acted as an accelerator of chondroitin sulphate synthesis in cartilage.

Since granulation tissue possesses a system for the synthesis of sulphated mucopolysaccharides, we thought it of interest to determine whether this tissue would also sulphate phenols. Such activity

has been shown in liver slices by De Meio & Arnolt (1944), but Segal (1955) was unable to show this activity in preparations of rat spleen, heart, kidney or hepatoma.

## METHODS

*Material.* Granulation tissue was obtained from guinea pigs by tendonectomy as previous described (Kodicek & Loewi, 1955). The tissue was sliced thinly with a razor blade and distributed in flasks so that the tissue from the same animal furnished both test and control. This is essential in view of the great variation among individual guinea pigs. Radioactive sulphate used was carrier-free $Na_2{}^{35}SO_4$, supplied by Radiochemical Centre, Amersham, Bucks.

*Incubation procedures.* (i) Tissue slices were incubated in Krebs–Ringer–phosphate (Krebs, 1950), containing $10^{-2}$M glucose (except in the few cases denoted where a glucose-free medium was used). To each flask, containing 4 ml. of medium, 4 $\mu$c of $Na_2{}^{35}SO_4$ was added. Substances under test were added dissolved in 0·1 ml. of medium and neutralized (pH 7·4) where necessary. Incubation was carried out in Warburg flasks at 37° with $O_2$ as gas phase. At the end of incubation (4 hr.) the tissue slices were washed as previously described (Kodicek & Loewi, 1955), dried and weighed and the radioactivity was assayed. In general, experiments were carried out in duplicate.

(ii) In experiments on phenyl sulphate synthesis, incubation was performed as above, in the presence of phenol or *m*-aminophenol at $10^{-3}$M (final concentration). Two methods were used to detect the formation of sulphated phenols. In the first, the tissue, after incubation for 4 hr., was removed and the medium was treated with 1 ml. of 20% trichloroacetic acid. To the filtrate, 0·6 ml. of 0·05N-$Na_2SO_4$ (non-radioactive) and 0·4 ml. of N-$BaCl_2$ were added and the solution was left at 80° for 0·5 hr. The precipitated $BaSO_4$ was removed by centrifuging and the addition of $Na_2SO_4$ and $BaCl_2$ was twice repeated. The final supernatant was hydrolysed by the addition of 2 ml. of N-HCl and heating in a boiling-water bath for 15 min. A volume (5 ml.) of 0·05N-$Na_2SO_4$ and 3 ml. of N-$BaCl_2$ were added, and the resulting precipitate, after standing at about 80° for 2 hr., was assayed for radioactivity.

*Chromatographic analysis.* In the second method, involving the above incubation procedure, the sulphated products were detected chromatographically. The medium, after incubation, was extracted three times with ether to remove free phenol. The combined aqueous layers were concentrated to dryness under reduced pressure and the residue was taken up in 96% ethanol. This extract was examined by ascending chromatography for 15–16 hr. on Whatman no. 1 paper with *n*-butanol–ethanol–water (4:1:5, by vol.). After drying at room temperature, the papers were suspended in HCl vapour for 1 min. and then heated at 100° for 2 min. to hydrolyse *O*-sulphates. The phenol thus liberated was identified by spraying the paper with a solution of diazotized sulphanilic acid (Evans, Parr & Evans, 1949). When the chromatogram was finally exposed to $NH_3$, coupled phenol was visible as an orange-yellow spot ($R_F$ 0·47). Free phenol moved to the solvent front. Unhydrolysed phenyl sulphate gave no colour. Phenyl sulphate, for use as a marker, was prepared by the method of Feigenbaum & Neuberg (1941).

*Measurement of radioactivity.* (i) Radioactivities of $BaSO_4$ precipitates were counted at infinite thickness with a mica end-window counter tube of diameter 1 in. (General Electric Co., London; type E.H.M.2S). Counts were corrected for decay and expressed in terms of dry wt. of tissue.

(ii) Radioactive components on paper chromatograms were counted by direct application of a Geiger-Müller tube, type E.H.M.2S, with a thin end-window.

## RESULTS

*Relationship between $Q_{O_2}$, sulphate uptake and age of tissue.* Fig. 1 (*a*) shows nineteen values for $Q_{O_2}$ ($\mu$l. of $O_2$/mg. dry wt. of tissue/hr.) of granulation-tissue slices obtained at varying intervals of time

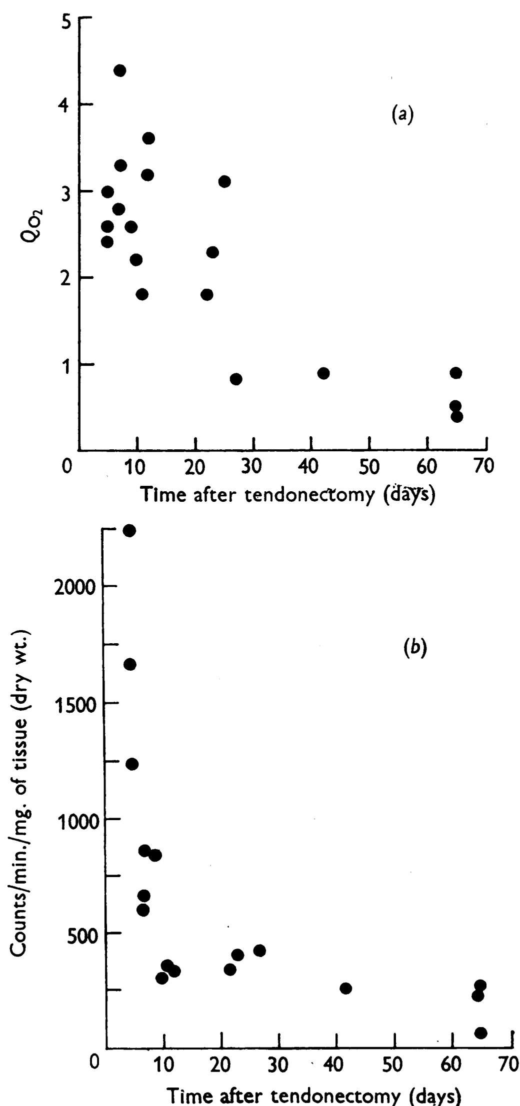


Fig. 1. Relationship of (*a*) $Q_{O_2}$ and (*b*) ${}^{35}SO_4{}^{2-}$ uptake of granulation-tissue slices to maturation of the tissue.

after tendonectomy in nineteen animals. Though there is a wide scattering of points, in general a decrease with maturation of the tissue can be observed. Fig. 1 (*b*) shows the corresponding values (with two pairs of coincident points) for $^{35}SO_4^{2-}$ uptake, and though it exhibits also a wide scattering of results, it similarly suggests a decrease with maturation of the tissue.

*Effect on $Q_{O_2}$ and sulphate uptake of various added substrates.* It is clear from the data in Table 1 that although addition of glucose at varying concentrations did not appreciably affect the $Q_{O_2}$, the uptake of $^{35}SO_4^{2-}$ was increased considerably. Pyruvate similarly caused a rise in the uptake of sulphate, but in this case the $Q_{O_2}$ was increased at the same time. Addition of L-glutamine did not have any effect on $Q_{O_2}$ or sulphate uptake of granulation tissue. Increasing concentrations of fluoroacetate caused a progressive fall in both $Q_{O_2}$ and uptake of sulphate.

The inhibition of sulphate uptake produced by salicylate (Kodicek & Loewi, 1955) and our observation that phenol was sulphated by granulation tissue, led to an investigation of the effects of various phenolic substances. The results are given in Table 2. Phenol, at concentrations of $10^{-3}$ M and $10^{-2}$ M, caused no appreciable fall of $Q_{O_2}$ and only a slight decrease of sulphate uptake. At $10^{-1}$ M, the $Q_{O_2}$ was abolished and the uptake of sulphate almost completely inhibited. It appears therefore that phenol, at concentrations which do not interfere with oxygen uptake, has only a small effect on sulphate uptake. Aminophenol at $10^{-3}$ M caused a similar slight effect. Inhibition by salicylate, as already reported, was 63% at $10^{-4}$ M. On the other hand, sodium gentisate at $10^{-3}$ M did not inhibit sulphate uptake and the $Q_{O_2}$ did not appear to be affected, even at $10^{-1}$ M.

The effect of phenyl sulphate was considerably greater than that produced by the same concentra-

Table 1. *Effect of various substances on the $Q_{O_2}$ and [$^{35}$S]sulphate uptake of granulation tissue*

For conditions see incubation procedure (i) under Methods.

| | | Experiment | | Control | | |
|---|---|---|---|---|---|---|
| Substance | Concn. (M) | $Q_{O_2}$ | Counts/min./100 mg. dry wt. | $Q_{O_2}$ | Counts/min./100 mg. dry wt. | Change in $^{35}SO_4^{2-}$ uptake (%) |
| D-Glucose | $2\times10^{-2}$ | −1·8 | 27 200 | −1·8 | 14 400 | +89 |
| | $10^{-1}$ | −1·8 | 40 800 | −1·9 | 17 000 | +140 |
| | $2\times10^{-1}$ | −1·7 | 32 850 | −1·6 | 14 500 | +127 |
| Sodium pyruvate | $2\times10^{-2}$ | −2·9 | 42 600 | −1·5 | 17 600 | +142 |
| L-Glutamine | $10^{-3}$ | −2·1 | 92 500 | −1·7 | 88 000 | +5 |
| | $10^{-2}$ | −2·0 | 95 250 | −2·3 | 89 500 | +6 |
| Sodium fluoroacetate | $10^{-3}$ | −1·2 | 43 000 | −2·9 | 88 400 | −51 |
| | $10^{-2}$ | −0·7 | 25 200 | −2·5 | 80 000 | −68 |
| | $2\times10^{-2}$ | 0 | 2 918 | −2·3 | 79 000 | −98 |

Table 2. *Effect of phenolic substances and some sulphated substances on [$^{35}$S]sulphate and $Q_{O_2}$ of granulation tissue*

For conditions see incubation procedure (i) under Methods.

| | | Experiment | | Control | | |
|---|---|---|---|---|---|---|
| Substance | Concn. (M) | $Q_{O_2}$ | Counts/min./100 mg. dry wt. | $Q_{O_2}$ | Counts/min./100 mg. dry wt. | Change in $^{35}SO_4^{2-}$ uptake (%) |
| Phenol | $10^{-3}$ | −3·0 | 43 375 | −3·2 | 58 050 | −15 |
| | $10^{-2}$ | −2·2 | 30 700 | −2·4 | 38 200 | −20 |
| | $10^{-1}$ | 0 | 3 030 | −3·0 | 75 000 | −96 |
| Sodium gentisate | $10^{-3}$ | −2·7 | 27 200 | −2·2 | 20 900 | +30 |
| | $10^{-2}$ | −3·1 | 43 600 | −4·4 | 79 600 | −45 |
| | $10^{-1}$ | −3·2 | 4 560 | −2·8 | 85 200 | −95 |
| Phenyl sulphate | $10^{-4}$ | −2·8 | 23 800 | −2·2 | 28 900 | −20 |
| | $10^{-3}$ | −2·1 | 28 900 | −2·6 | 54 500 | −47 |
| | $10^{-2}$ | −3·5 | 10 500 | −2·7 | 28 800 | −64 |
| Potassium methanesulphonate | $2\times10^{-2}$ | −1·9 | 24 400 | −2·2 | 55 650 | −56 |
| | $4\times10^{-2}$ | −1·0 | 13 250 | −1·9 | 59 500 | −76 |
| Potassium ethyl sulphate | $2\times10^{-2}$ | −3·4 | 30 500 | −3·3 | 28 400 | +7 |

tion of phenol. This suggested that the effect was not due to phenol liberated by hydrolysis during the incubation.

The inhibition of $^{35}SO_4^{2-}$ uptake shown in the presence of phenyl sulphate suggested the trial of other sulphur-containing compounds. Though potassium methanesulphonate produced inhibition, potassium ethyl sulphate, even at $2 \times 10^{-2}$ M, showed no such effect.

*Synthesis of phenyl sulphate by granulation tissue.* The data shown in Table 3 suggested that granulation-tissue slices, like liver slices, could sulphate aminophenol, since radioactive sulphate was held in the medium after precipitation of remaining ionic sulphate. Similar results were obtained with phenol, the formation of phenyl sulphate by granulation tissue being shown chromatographically. A coincident coloured and radioactive spot ($R_F$ 0·47) corresponding to phenyl sulphate was obtained. When non-radioactive phenyl sulphate was added to the extract of the medium, the same coincident radioactive and coloured component ($R_F$ 0·47) was observed. In the solvent system used, inorganic sulphate remained almost stationary at the origin. In the presence of liver slices, the results were similar. Approximately 0·2 $\mu$mole of phenyl sulphate was formed/100 mg. of granulation tissue in 4 hr. Control experiments performed with boiled-tissue slices and with tissue without added phenol produced no evidence of the formation of phenyl sulphate. In the same way it was found that phenyl sulphate was formed in comparable amounts when slices of calf costal cartilage were incubated with phenol.

Experiments to determine whether salicylate was sulphated by granulation tissue have so far shown no evidence of the formation of a sulphated derivative.

## DISCUSSION

Although the $Q_{O_2}$ of granulation tissue is relatively low at all times and the scatter of values is great, a general decline in values with age of the tissue is apparent. The cellularity of granulation tissue shows a marked decrease with age, as the tissue becomes more collagenous. The decline in $Q_{O_2}$, together with sulphate uptake, serves to emphasize further the dependence of the sulphation processes on cellular activity. The highest value obtained for $Q_{O_2}$ was $-4·4$, a low value when compared with values from tissues such as liver, brain and kidney (Krebs, 1950). Neuhaus (1929) studied granulation tissue from tuberculous lymph nodes. The average $Q_{O_2}$ in ten experiments with such tissue from guinea pigs was $-6·7$. Injection of kieselguhr produced a more cellular granuloma, slices of which had a $Q_{O_2}$ of $-12·9$. Since sections of the granulation tissue studied by ourselves showed large numbers of collagen fibres rather than many cells, the low values for $Q_{O_2}$ are explicable.

The effect of glucose is difficult to interpret. In the presence of this sugar a very considerable increase in sulphate uptake was achieved. Since, however, no values for specific activities are available (the total amounts of sulphate were too small for estimation) it is not possible to decide whether this increase represents a rise in net synthesis of sulphated polysaccharide or only an increase of the degree of labelling. There was no apparent effect on $Q_{O_2}$, but a small change in these low values might not have been perceptible. It is possible therefore that glucose acted by virtue of its availability as a source of substrate for polysaccharide formation, but it is also possible that it acted by increasing the amount of available energy for the formation of sulphated polysaccharide or sulphate transport into the cell. The present results confirm the findings of Boyd & Neuman (1954) and Boström & Jorpes (1954) in so far as sulphate uptake of cartilage is increased in the presence of glucose.

Addition of pyruvate caused a considerable rise in sulphate uptake and an appreciable increase in $Q_{O_2}$. It is possible therefore, but not established, that an increased supply of energy caused the raised sulphate uptake. That the uptake of sulphate is an energy-requiring process has already been shown in an earlier paper (Kodicek & Loewi, 1955). Boström *et al.* (1955) have found that L-glutamine acts as an accelerator of chondroitin sulphate synthesis in cartilage. We were unable to detect such an effect with granulation tissue.

The effect of fluoroacetate suggests the importance of the citric acid cycle for the provision of energy necessary for sulphate uptake. It serves, further, to demonstrate that the cycle plays a role in the metabolic pathways of fibroblasts. The effect of salicylate in inhibiting sulphate uptake was also exhibited by other phenolic substances; however, phenol caused a marked inhibition only at $10^{-1}$ M, a concentration at which no $Q_{O_2}$ was observed. The addition of a dihydric phenol, sodium gentisate, also caused less inhibition than salicylate. The hypothesis of competition for available sulphate could not be substantiated, since addition of excess of carrier sulphate to the medium did not overcome the

Table 3. *Residual radioactive counts in hydrolysed, protein-free incubation medium, after precipitation of free ionic sulphate*

For conditions see incubation procedure (ii) under Methods.

| | Counts/min./flask |
|---|---|
| Granulation tissue and *m*-aminophenol | 2827 |
| Granulation tissue alone | 83 |
| Liver slices and *m*-aminophenol | 2601 |
| Liver alone | 58 |

inhibition caused by phenol or aminophenol. Inhibition in the presence of phenyl sulphate was considerably greater than that produced by similar concentrations of phenol. The effect could therefore not be attributed to hydrolysis of phenyl sulphate during incubation. Addition of carrier sulphate to the medium did not overcome inhibition of $^{35}SO_4^{2-}$ uptake by phenyl sulphate. It is possible that phenyl sulphate may be inhibitory by means of a competitive action on enzymes involved in sulphate metabolism. Of other sulphur-containing substances investigated, potassium methanesulphonate inhibited, whereas potassium ethyl sulphate failed to do so. The further elucidation of this problem and that of the mode of action of other inhibitors must await the availability of a satisfactory cell-free system.

The sulphation of phenol by granulation tissue was a somewhat surprising finding, since Segal (1955) found no such activity in a variety of other tissues. The only reported association of chondrosulphatase and aryl sulphatase activities has been found in the large periwinkle (*Littorea littorina*) by Dodgson & Spencer (1954). Our reasons for investigating the sulphation of phenol were, first, to study the specificity of the sulphating system and, secondly, to have a readily available substrate for future experiments on the problem of sulphation in granulation tissue. Hilz & Lipmann (1955) have shown the presence of a sulphate-activating and a transferring system in liver. It seems likely that a similar activating system occurs in granulation tissue and that a transfer to eithei carbohydrate or phenol may then take place.


## SUMMARY

1. The $Q_{O_2}$ and $SO_4^{2-}$ uptake of guinea-pig granulation-tissue slices decreased with the maturation of the tissue after tendonectomy.

2. The $SO_4^{2-}$ uptake by granulation tissue was stimulated by addition of glucose ($2\times10^{-2}$M) or pyruvate ($2\times10^{-2}$M), and was completely inhibited, together with $Q_{O_2}$, by fluoroacetate ($2\times10^{-2}$M).

3. Sulphate uptake by the tissue was also inhibited by gentisate and phenol.

4. Phenol acted as an alternative substrate and was shown by chromatography to be converted into phenyl sulphate, which itself was an inhibitor of $^{35}SO_4^{2-}$ uptake by granulation tissue.


The authors thank Professor H. A. Krebs for his nterest.

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# The Metabolism of Sorbitol in the Human Subject


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Sorbitol has been used as a sweetening agent for diabetics since it was shown that moderate amounts of sorbitol taken by mouth by normal or diabetic subjects cause only a small and insignificant increase in the blood-sugar concentration (Thannhauser & Meyer, 1929). While this small increase was considered to be due partly to a slow rate of absorption from the intestine, it was also widely believed that, in man, orally ingested sorbitol is metabolized by a pathway not involving glucose (Payne, Lawrence & McCance, 1933).

Animal experiments have shown that as much as 64 % of injected sorbitol may be excreted unchanged in the urine (Todd, Meyers & West, 1939; Smith, Finkelstein & Smith, 1940; Ellis & Krantz, 1941, 1943; Stetten & Stetten, 1951), whereas only about 3 % of orally ingested material is lost in this way. Recently Stetten & Stetten (1951), Wick, Almen & Joseph (1951) and Wick, Morita & Barnet (1955) have injected uniformly labelled D-[$^{14}$C]sorbitol (D-[U-$^{14}$C]sorbitol) into normal and alloxan diabetic rats and recovered most of the radioactivity in the respiratory carbon dioxide. Hepatectomized rats were unable to metabolize sorbitol in this way. Embden & Griesbach (1914) and later Anschel (1930) suggested that sorbitol was metabolized via fruc-