growth when cobalamin is the growth factor, but not when methionine is supplied. It does not inhibit the growth of strains which do not require cobalamin for growth.

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Chemical Changes in Rabbit Aorta and Ear Cartilage Induced by Papain and Calciferol

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It has been shown both histochemically (Thomas, 1956) and chemically (Tsaltas, 1958) that the intravenous administration of a solution of crude papain decreases the mucopolysaccharide content of rabbit-ear cartilage.

With regard to the action of papain *in vivo*, it has been shown by McCluskey & Thomas (1958) that the injection of crystalline papain results in depletion of cartilage matrix only if the enzyme is inactivated by oxidation or sulphhydryl blocking agents before administration. Cysteine-activated crystalline papain produces little effect on cartilage when injected. The changes produced by injection of inactivated crystalline papain were found to be indistinguishable from those produced by crude papain. These workers suggested that the reason for the failure of activated papain to enter cartilage

after intravenous injection is that it probably reacts with one or more substrates present in blood. Oxidized or inactivated papain, on the other hand, appears to be taken up by cartilage and converted into the active form. In the present study a solution of crude non-activated papain (L. Light and Co. Ltd) in 0.9% sodium chloride solution was used.

It has also been shown that after toxic doses of calciferol in rats, there is an increase in total hexose and hexosamine content of the aorta, which parallels the aortic mineralization which occurs (Gillman, Grant & Hathorn, 1960). Because of the possibility that mucopolysaccharides may play an important role in the process of calcification, it was decided to test the effects of papain on the action of calciferol given simultaneously.

METHODS

Four young hybrid rabbits (three male and one female) with a mean weight of 1960 ± 70 g. (s.e.) were randomly allocated to each of the following treatment groups (a total of 16 rabbits being employed): group I, these were injected intravenously in the left ear with a 1% solution of crude papain (L. Light and Co. Ltd.) in 0.9% NaCl solution (1 ml./kg. body wt.) on the first, fourth, seventh and tenth days of the experiment; group II, as in group I, but injected with 0.9% NaCl solution instead of papain solution; group III, papain injections were given as in group I, together with 0.5 mg. of calciferol (Petersen and Co., Cape Town)/kg. body wt. given orally daily for 10 days as a solution in arachis oil (15 mg./ml.); group IV, these were given injections of 0.9% NaCl solution as in group II together with calciferol as in group III.

The rabbits were killed after 10 days, and the cartilage of the right ear and the entire aorta was removed for chemical analysis. Freshly excised tissues were freed from connective tissue, rinsed in 0.9% NaCl solution, cut into small pieces and placed under dry acetone. After 24 hr. the acetone was renewed and allowed to stand for a further 24 hr. period. The acetone was then replaced with a mixture of ether and ethanol (1:1, v/v) and after standing overnight the solvent was removed and the tissues were dried to constant weight in a hot-air oven at 100° .

Hydrolysis. Portions of dry tissue (50 mg.) were heated with 4 ml. of 0.5 N-HCl for 4 hr. at 100° in sealed tubes. The solutions were then filtered and the volumes adjusted to 10 ml.

Hexoses. These were determined in 1 ml. portions of the hydrolysate with anthrone according to the method of Trevelyan & Harrison (1952), the results being expressed as glucose. Tests with glucose showed that the amount of destruction during the hydrolysis period was insignificant. The yield of hexose as determined by anthrone appeared to be maximal under these conditions.

Hexosamines. These were determined by a modification of the Elson & Morgan method (1933). The acetylacetone reagent was made by dissolving 1 ml. of redistilled acetylacetone in 25 ml. of M- Na_2CO_3 (A.R.) solution. To 1 ml. of hydrolysate 2 ml. of reagent was added and the mixture heated for exactly 15 min. in a boiling-water bath. After cooling, 6 ml. of diluted reagent [consisting of 1.6 g. of *p*-dimethylaminobenzaldehyde (Merck) dissolved in a mixture of 30 ml. of redistilled ethanol and 30 ml. of conc. HCl and diluted with 300 ml. of ethanol] was added. The tube was allowed to stand at room temperature for 10 min. with occasional shaking to remove CO_2 and the colour density of the solution was measured at $530 \text{ m}\mu$ in a Zeiss spectrophotometer, model P.M. QII. Each run included a duplicate standard (50 μg . of glucosamine). Tests with finely divided, acetone-dried and defatted aorta and cartilage and also with chondroitin sulphate showed that the hydrolysis conditions resulted in a maximum yield of hexosamine as determined by the Elson-Morgan reaction. Also, examination of the absorption spectra of the reaction products between 450 and $600 \text{ m}\mu$ showed a single well-defined peak with a maximum at $530 \text{ m}\mu$, closely resembling the curve obtained with pure glucosamine. According to Boas (1953) this indicates that only small amounts of interfering chromogens are present, and it was not therefore

considered necessary to separate the amino sugars by means of ion-exchange resin before analysis.

Calcium. Portions of hydrolysate (1 ml.) were diluted with 2 ml. of water and 1 ml. of 2.5 N-NaOH solution. The calcium was then titrated with 0.01 N-ethylenediaminetetra-acetic acid with the calcium indicator of Diehl & Ellingboe (1956), screened with thymolphthalein (Tucker, 1957).

Uronic acid. This was determined in 1 ml. portions of the hydrolysate by the method of Dische (1947). Glucuronolactone (L. Light and Co. Ltd.) was used for standardizing.

All chemical estimations were carried out in duplicate. No differences were found in the results from the male and female rabbits in each group.

RESULTS

The mean percentage composition of the ear cartilage is shown in Table 1, together with an analysis of variance of the individual results obtained in the 16 animals. It will be seen that papain significantly lowered the hexosamine and uronic acid content of the ear cartilage, irrespective of whether calciferol was administered simultaneously or not. In addition, calciferol resulted in a significant decrease in hexose, irrespective of whether papain was administered simultaneously. There was also an increase in calcium content of ear cartilage in the calciferol-treated rabbits, but the difference did not reach significant levels. Finally, the analysis shows that there was no significant interaction between papain and calciferol, i.e. they neither potentiated nor inhibited each other.

The weight of the fat-free aorta is expressed as dry wt./kg. body wt. As will be seen in Table 2, this weight was almost doubled in the calciferol-treated rabbits. Consequently, if the aortic constituents are expressed in terms of concentration, changes in their absolute values would be obscured. These values are therefore expressed as mg./aorta for a standard body weight (kg. body wt.).

Table 2 shows that calciferol resulted in significant increases in hexosamine and uronic acid (to just less than double) and an approximately 25-fold increase in the calcium content. These changes, induced by calciferol, were so marked that they tended, in the analysis of variance, to obscure changes induced by papain alone without simultaneous calciferol administration. A subsidiary analysis of the original data from groups I and II showed that papain significantly lowered the hexose, hexosamine and uronic acid content [Mann-Whitney *U* test (Siegal, 1956), $P < 0.05$].

Because of the marked differences in standard error (and hence variance) of the calcium results in Table 2, doubt may be cast on the applicability of an analysis of variance in this case. The original data for each group were therefore subjected to a non-parametric test. Similar conclusions were

Table 1. *Chemical composition of rabbit-ear cartilage*

NS, Not significant.

	Percentage of dry, fat-free tissue (mean $\pm$ s.e.*)							
	Hexose		Hexosamine		Uronic acid		Calcium	
Papain	2.75 $\pm$ 0.37		1.64 $\pm$ 0.15		1.45 $\pm$ 0.13		0.39 $\pm$ 0.03	
0.9% Sodium chloride	3.06 $\pm$ 0.04		1.99 $\pm$ 0.08		1.80 $\pm$ 0.08		0.42 $\pm$ 0.04	
Papain + calciferol	2.15 $\pm$ 0.03		1.46 $\pm$ 0.19		1.43 $\pm$ 0.13		0.53 $\pm$ 0.11	
Saline + calciferol	2.22 $\pm$ 0.18		2.04 $\pm$ 0.13		1.80 $\pm$ 0.16		0.63 $\pm$ 0.12	
	Analysis of variance							
	<i>F</i> †	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Main effects								
Papain	0.86	NS	8.72	<0.025	7.30	<0.025	0.56	NS
Calciferol	15.00	<0.01	0.10	NS	0.04	NS	4.52	> 0.05
Interaction								
Papain + calciferol	0.29	NS	0.62	NS	0.04	NS	0.15	NS

* Standard error calculated as S.D./ $\sqrt{(n-1)}$.

† Ratio of the variance due to the effect considered to the error variance. Tested with 1 and 12 degrees of freedom.

Table 2. *Chemical composition of rabbit aorta*

NS, Not significant.

	Aortic wt. (mg./kg. body wt.)	Weights [mg./aorta/kg. body wt. (mean $\pm$ s.e.*)]								
		Hexose		Hexosamine		Uronic acid		Calcium		
Papain	30.2 $\pm$ 3.8	0.30 $\pm$ 0.02		0.15 $\pm$ 0.01		0.11 $\pm$ 0.01		0.29 $\pm$ 0.09		
0.9% Sodium chloride	37.3 $\pm$ 3.9	0.41 $\pm$ 0.04		0.20 $\pm$ 0.02		0.19 $\pm$ 0.02		0.20 $\pm$ 0.04		
Papain + calciferol	70.2 $\pm$ 9.7	0.46 $\pm$ 0.08		0.32 $\pm$ 0.04		0.25 $\pm$ 0.04		5.94 $\pm$ 1.05		
0.9% Sodium chloride + calciferol	65.2 $\pm$ 4.6	0.44 $\pm$ 0.04		0.32 $\pm$ 0.02		0.25 $\pm$ 0.02		6.48 $\pm$ 1.53		
Analysis of variance										
	<i>F</i> †	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Main effects										
Papain	0.01	NS	0.98	NS	0.91	NS	2.60	NS	0.06	NS
Calciferol	29.41	<0.001	4.03	NS	31.52	<0.001	15.65	<0.01	41.23	<0.001
Interaction										
Papain + calciferol	0.97	NS	2.05	NS	1.30	NS	2.02	NS	0.12	NS

* Standard error calculated as S.D./ $\sqrt{(n-1)}$.

† Ratio of the variance due to the effect considered to the error variance. Tested with 1 and 12 degrees of freedom.

reached, namely that papain had no significant effect on aortic calcification whereas the differences induced by calciferol were highly significant (Mann-Whitney *U* test: $P < 0.001$).

DISCUSSION

The present findings in ear cartilage are consistent with previous reports (Thomas, 1956; Tsaltas, 1958; Spicer & Bryant, 1958) that papain decreases the mucopolysaccharide content of rabbit-ear cartilage. It should be noted that papain induced a similar decrease in the hexose, hexosamine and uronic acid contents of the rabbit aorta.

On the other hand, the effects of calciferol were different in the two tissues analysed here. Whereas calciferol induced a massive mineralization of the

aorta, the calcium content of the ear cartilage was only slightly increased. Calciferol decreased the hexose in ear cartilage and did not affect hexosamine and uronic acid contents, whereas in the aorta it resulted in an increase in both hexosamine and uronic acid, unassociated with any significant change in aortic hexose.

In the rat, calciferol administration results in increases in aortic hexose and hexosamine (Gillman *et al.* 1960) whereas the present results show no significant change in the hexose content of the calciferol-treated rabbit aorta. These two experiments were performed under different conditions, however, and a species difference cannot therefore be inferred from our data.

We wish to draw attention to the remarkable difference in sensitivity of the rabbit and the rat to calciferol: on a body-weight basis rats tolerated a

dose approximately 12 times as great as that tolerated by the rabbit.

In conclusion, from these results it would appear that although papain decreases the hexosamine and uronic acid content of the rabbit aorta, it has no effect on the degree of mineralization induced by calciferol administered simultaneously; nor does papain interfere with the ability of calciferol to raise the content of these mucopolysaccharides in the calcifying aorta.

SUMMARY

1. Chemical changes induced by papain and calciferol in rabbit-ear cartilage and aorta have been determined.

2. Papain significantly lowered the hexosamine and uronic acid contents of ear cartilage whether calciferol was given simultaneously or not. Calciferol lowered the hexose content when it was given alone or with papain.

3. The weight of dry aortic tissue was almost doubled in the calciferol-treated rabbits owing to heavy mineralization. Aortic hexosamine, uronic acid and calcium were increased in the calciferol-treated animals, whereas the hexose content was unchanged.

4. In the papain-treated animals there was a decrease in aortic hexosamine and uronic acid. However, when given simultaneously with calciferol, papain had no effect on the extent of mineralization.

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