converted, with fission of the C-O bond of the sulphate ester, into 3:6-anhydro-L-galactose residues and that the enzyme is therefore not a true sulphatase.

3. The biosynthesis and structure of porphyrin are discussed in the light of the characterization of this enzyme.

4. During the normal processes of collecting, drying and extracting the seaweed, the enzyme does not modify porphyrin to any measurable extent.

5. The enzyme shows diminished activity towards the products of partial acidic and enzymic hydrolysis of porphyrin, and its action is not reversible.

I thank Professor Stanley Peat, F.R.S., and Dr J. R. Turvey for their continued interest, and Professor E. L. Hirst, C.B.E., F.R.S., for encouragement. I am also grateful to Dr D. J. Manners and Dr E. E. Percival for loans of equipment and to D.S.I.R. for a Fellowship.

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Biochem. J. (1961) **81**, 352

The Inhibitory Effect of 2:4-Dinitrophenol on Metastatic Tissue Calcification Induced by Calciferol

By R. A. GRANT,* M. HATHORN† AND T. GILLMAN*
Department of Physiology, University of Natal, Durban, South Africa

(Received 8 June 1961)

Whitehead & Weidmann (1959) showed that, in calcifying cartilage in living kittens, the synthesis of adenosine triphosphate (measured by incorporation of ³²P) was inhibited by 2:4-dinitrophenol. It was also found that the uptake of the isotope into bone salt was similarly inhibited only after longer treatment with 2:4-dinitrophenol. Moreover, Gutman & Yui (1949) demonstrated that 2:4-dinitrophenol is capable of inhibiting the calcification of cartilage *in vitro* at a concentration of 0.1 mM.

* Present address: Department of Experimental Pathology, Agricultural Research Council, Institute of Animal Physiology, Babraham, Cambridge, England.

† Present address: Department of Physiology, University of Witwatersrand, Johannesburg, South Africa.

Because calcification of the rat aorta, induced by calciferol, is accompanied by a large increase in this vessel's phosphorus content (Gillman, Grant & Hathorn, 1960), we decided to investigate the effect of 2:4-dinitrophenol on mineralization of rat aorta, lung and stomach produced by acute calciferol intoxication. The results clearly indicate that 2:4-dinitrophenol exerts an inhibitory effect on this process.

METHODS AND MATERIALS

Twenty male Wistar-strain albino rats with a mean weight of 329 ± 7 g. were randomly allocated to each of the following four groups of five rats.

Rats in group 1 were given, twice daily, intraperitoneal injections of 3 ml. of 0.9% sodium chloride solution for

20 days. Group 3 rats were given identical saline injections together with oral calciferol (C. Petersen and Co., Cape Town, South Africa) in daily doses as a solution in arachis oil from day 6 to 10 inclusive, a total of 100 000 units/kg. body wt. being given. Rats in group 2 were given injections, twice daily, of 3 ml. of a saturated solution of 2:4-dinitrophenol in 0.9% sodium chloride for 20 days. Group 4 rats received the same injections as group 2 together with calciferol given orally as in group 3. On day 20 all the animals were killed and the aorta, lungs and stomach dissected out. The tissues were dehydrated with acetone, defatted with ether-alcohol (1:1, v/v) and dried to constant weight at 100°. A second series of animals were treated in the same four groups but were killed and bled from the heart on day 9 of the experiment, i.e. 4 days after starting calciferol treatment; the serum was analysed for calcium and inorganic phosphate.

Total phosphorus. Accurately weighed portions of dry fat-free tissue (about 100 mg.) were digested with 1 ml. of a 1:2 (v/v) mixture of perchloric acid (70%) and nitric acid (sp.gr. 1.52) at 150° till clear. The digest and washings were transferred to a 10 ml. volumetric flask and made to volume. Suitable portions (depending on the amount of P present) were transferred to a 25 ml. volumetric flask. To each flask was added 2 ml. of 2N-hydrochloric acid, followed by 2 ml. of reducing-agent solution (Metol 1%; sodium sulphite 5%) and 2 ml. of ammonium molybdate solution (2.5%). The volume of the solution was adjusted to 25 ml. with water and mixed thoroughly. A phosphate standard (= 1 mg. of P) was treated in the same way. After 10 min. the solutions were transferred to 1 cm. cuvettes and the extinctions determined at 660 m μ .

Calcium determinations. Portions of the digest were titrated with 0.01N-ethylenediaminetetra-acetic acid with the calcium indicator of Diehl & Ellingboe (1956) screened with thymol phthalein (Tucker, 1957) in the presence of 1 ml. of 2.5N-sodium hydroxide. Serum calcium was determined similarly in volumes of 1 ml.

Serum inorganic phosphate. This was determined by a modification of the method of Fiske & Subbarow (1925).

Statistical methods. Except for serum calcium and serum inorganic phosphate, where the variances were homogeneous, analysis of variance was performed on the results after logarithmic transformation in order to render the variances homogeneous (Maxwell, 1958). In order to assess the significance of differences between group means, 2-tailed *t* tests were used at the 5% level of significance, the standard error of the difference having been calculated from the residual error variance in the analysis of variance (Maxwell, 1958). In Table 1, significant differences in means are indicated by the sign '>' whereas groups not differing significantly from each other are separated by a comma.

RESULTS

The mean percentage calcium and phosphorus contents of the tissues, together with the mean aortic dry weights and the serum calcium and inorganic phosphorus values, are given in Table 1. This also includes an analysis of variance of the individual results obtained in 20 animals.

The present results confirm our previous observation (Gillman *et al.* 1960) that, with calciferol

alone, treatment resulted in an approximately 2½-fold increase in the mean aortic dry weight; for this reason the results for calcium and phosphorus are expressed in mg./aorta. Table 1 also shows that 2:4-dinitrophenol administered during calciferol treatment significantly reduced the effect of calciferol on total aortic dry weight, although 2:4-dinitrophenol alone had no effect at all on the aortic weight.

Both the calcium and phosphorus contents of the aorta were greatly increased by calciferol treatment; 2:4-dinitrophenol alone had no effect on these constituents, but, when given simultaneously with calciferol, significantly reduced, by approximately one-half, the degree of mineralization induced by calciferol alone. The calcium:phosphorus ratios of the aortae also tended to be reduced in the two groups receiving 2:4-dinitrophenol injections, but, owing to wide variations in individual values, the differences were not significant.

Similar results were obtained for lung and stomach; calciferol produced significant increases in the calcium and phosphorus contents of these organs and 2:4-dinitrophenol given simultaneously reduced this effect.

The results of the serum calcium and inorganic phosphate determinations showed that calciferol markedly increased both these serum constituents. However, despite its marked effects on tissue mineralization, 2:4-dinitrophenol given simultaneously with calciferol did not affect the increases in the serum calcium and inorganic phosphorus. It would thus appear that the inhibitory effect of 2:4-dinitrophenol on calcification is not mediated via any overt action on blood calcium or phosphate levels.

In Table 2 the results for tissue calcium and total phosphorus are expressed in the form of increments over the saline control values. The calcium:phosphorus ratios obtained, whether calciferol was given alone or with 2:4-dinitrophenol, tended to be in the region of 2:1. This indicates that the mineral deposited is calcium phosphate with a calcium and phosphorus composition similar to that of bone salt. Table 2 also shows the extent to which 2:4-dinitrophenol inhibited the calciferol-induced metastatic mineralization. This Table reveals that for the tissues other than aorta, treatment with 2:4-dinitrophenol given together with calciferol resulted in an equal reduction of both calcium and phosphorus as compared with the amounts of these constituents found in rats treated with calciferol only. In the aorta, however, the degree of inhibition of the calciferol-induced calcium increase due to 2:4-dinitrophenol was 20% greater than its effect on phosphorus accumulation. In consequence the Ca:P ratio of the aortae of rats in

Table 1. *Effects of calciferol and 2:4-dinitrophenol, given separately and together, on the calcium and phosphorus contents of rat aorta, lung and stomach*

NS, Not significant.

		Serum			Aorta				
Group	Treatment	Ca	Inorganic phosphate	Ca-P product	Wt. (mg.)	Ca	P	Ca/P	
		(mg./100 ml.)	(mg./100 ml.)			(mg./aorta)	(mg./aorta)		
1	Saline	11.9±0.2*	6.5±0.2	77	16.7±1.2	0.206±0.064	0.073±0.006	2.84	
2	DNP	11.6±0.2	6.6±0.6	77	16.7±1.0	0.108±0.014	0.079±0.011	1.37	
3	Calciferol + saline	14.2±0.3	8.8±0.5	125	43.8±5.5	6.23 ±0.91	2.30 ±0.33	2.71	
4	Calciferol + DNP	15.2±0.1	8.4±0.2	128	28.1±4.0	2.88 ±1.03	1.15 ±0.31	2.51	
Analysis of variance									
Main effects:		<i>F</i> †	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Calciferol		98.0	—	18.86	<0.001	42.57	—	44.16	<0.001
DNP		2.06	—	0.11	NS	4.25	—	4.56	<0.05
Interaction:									
Calciferol - DNP		6.04	<0.05	0.50	NS	4.60	<0.05	1.18	NS
Significant differences between means‡		4 > 3 > 1, 2 3, 4 > 1, 2			3 > 4 > 1, 2		3 > 4 > 1, 2		3 > 4 > 1, 2
		Lung			Stomach				
Group	Treatment	Ca	P	Ca/P	Ca	P	Ca/P		
		(% dry wt.)	(% dry wt.)		(% dry wt.)	(% dry wt.)			
1	Saline	0.17±0.03	0.84±0.01	0.20	0.12±0.01	0.89±0.03	0.13		
2	DNP	0.11±0.01	0.83±0.02	0.13	0.15±0.02	0.85±0.01	0.18		
3	Calciferol + saline	1.10±0.41	1.38±0.20	0.80	0.95±0.33	1.23±0.16	0.77		
4	Calciferol + DNP	0.33±0.09	0.93±0.04	0.35	0.46±0.09	1.06±0.06	0.43		
Analysis of variance									
Main effects:		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>		
Calciferol		31.44	<0.001	17.54	—	44.72	<0.001	13.15	<0.01
DNP		9.53	<0.01	7.50	—	1.04	NS	1.74	NS
Interaction:									
Calciferol - DNP		1.82	NS	6.22	<0.05	3.23	NS	0.43	NS
Significant differences between means‡		3 > 4, 3 > 1, 2 4 > 2		3 > 4, 1, 2		3 > 4, 1, 2		3 > 1 4 > 2	

* Mean ± s.e. [s.e. calculated as S.D./(*n* - 1)].

† Ratio of the variance due to the effect considered to the error variance (tested with 1 and 16 degrees of freedom).

‡ Standard error of difference of means calculated from error variance; 2-tailed *t* test with 5% level of significance.Table 2. *Increases in tissue concentrations of calcium and phosphorus induced by calciferol given alone and with 2:4-dinitrophenol*

	Calciferol alone Increments*			Calciferol + 2:4-dinitrophenol Increments			Inhibition of calciferol effect (%)	
	Ca	P	Ca:P	Ca	P	Ca:P	Ca	P
Aorta	12.8	4.8	2.7:1	7.3	3.2	2.3:1	43	33
Lung	0.93	0.54	1.7:1	0.16	0.09	1.8:1	83	83
Stomach	0.83	0.34	2.5:1	0.34	0.15	2.3:1	59	56

* Experimental level - saline control level (expressed as percentage of dry fat-free tissue).

group 3 was significantly greater than of those in group 4. Although the reasons underlying this differential effect of 2:4-dinitrophenol on the calcium and phosphorus levels in the aortae of calciferol-intoxicated rats remains obscure, nevertheless this finding indicates differences in the responses of the various tissues to the action of 2:4-dinitrophenol.

DISCUSSION

In earlier studies by Trout (1958) and by Gillman *et al.* (1960) it was shown that mineral continued to accumulate in the aortae of calciferol-intoxicated rats long after administration of this compound was stopped and after the serum calcium level had returned to normal. It therefore appears that, once initiated, calcification of the aorta, under these conditions, follows an irreversible course, since even 160 days after calciferol intoxication there was no evidence of a reduction in the mineral content of such injured aortae. Aorta appears to differ in this respect from other tissues, since in the heart, for instance, the calcium disappeared within a relatively short time. The mechanism underlying these differences in reactivity is at present obscure.

Since the increases in blood calcium and inorganic phosphorus, induced by calciferol, were not affected by simultaneous administration of 2:4-dinitrophenol, it would appear that 2:4-dinitrophenol exerts its action directly on the tissues. In this respect our results may perhaps be better understood on the basis of the finding of Whitehead & Weidmann (1959) for kitten cartilage in which 2:4-dinitrophenol was found to inhibit synthesis of adenosine triphosphate and reduce the uptake of ^{32}P into the bone salt. Cartier & Picard (1955) suggested that adenosine triphosphate is responsible for the initiation of calcification by the formation of seed crystals, and the results of Whitehead & Weidmann (1959) are in agreement with this concept. Our own finding, that 2:4-dinitrophenol depresses the degree of soft-tissue mineralization induced by calciferol, is also in conformity with those of the above authors. This similarity in response to 2:4-dinitrophenol of calcification processes in cartilage, lung, stomach and aorta may indicate that all these tissues calcify by a common mechanism involving in part perhaps adenosine triphosphate and adenosine triphosphatase balance. A further indication of this possibility emerges from the similarity in Ca:P ratio of the mineral salts deposited in all the above tissues (see Table 2).

A reduction in the adenosine triphosphate content of the tissues could also result from a stimulation of adenosine-triphosphatase activity,

especially as Chappell & Perry (1954) showed that skeletal muscle adenosine triphosphatase is stimulated by 2:4-dinitrophenol. Stimulation of chick-embryo mitochondrial adenosine triphosphatase by 2:4-dinitrophenol has also been reported by Carey & Greville (1959) and the activation of three rat-liver adenosine triphosphatases by 2:4-dinitrophenol was shown by Myers & Slater (1957). Further evidence for this action of 2:4-dinitrophenol has recently been given by Penniall (1960). Wakid (1960) has also shown that in the absence of Mg^{2+} ions the adenosine-triphosphatase activity of rat myometrial mitochondria was increased about four times by 0.1 mM-2:4-dinitrophenol. Mg^{2+} ions alone produced a tenfold to 20-fold increase in the adenosine-triphosphatase activity. This last observation is of interest in view of Selye's (1958) finding that administration of magnesium chloride prevented calcification of the aortae of rats intoxicated with both dihydrotachysterol and sodium phosphate. This effect of Mg^{2+} ions on the action of drugs studied by Selye may perhaps have been due to the increased adenosine-triphosphatase activity induced by Mg^{2+} ions.

However, the level of tissue adenosine triphosphate does not appear to be the only factor involved in calcification under the action of calciferol since skeletal muscle, which is relatively rich in adenosine triphosphate, calcifies only to a small extent compared with aorta.

SUMMARY

1. 2:4-Dinitrophenol has been found to exert an inhibitory effect on soft-tissue calcification induced by calciferol.

2. 2:4-Dinitrophenol did not affect the increases in blood calcium and inorganic phosphate induced by calciferol and appears to act directly on the tissues.

3. It is suggested that the above effect of 2:4-dinitrophenol on calcification may be in part at least due to its known effects on adenosine triphosphate synthesis and adenosine-triphosphatase activity.

This study was aided by grants from the Wellcome Trust, the Nuffield Foundation and the C.S.I.R.

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Biochem. J. (1961) **81**, 356

Structural Changes in Collagen

2. ENHANCED SOLUBILITY OF BOVINE COLLAGEN: REACTIONS WITH HYDROGEN PEROXIDE AND SOME PROPERTIES OF SOLUBLE EUCOLLAGEN*

By A. COURTS

The British Gelatine and Glue Research Association, 2a Dalmeny Avenue, London, N. 7

(Received 23 November 1960)

Evidence by Fitton-Jackson (1953) and others shows that the generation of collagen *in vivo* takes place intercellularly to the stage of a soluble precursor and becomes insoluble extracellularly by some type of aging process likely to involve cross-linking. A substantial fraction of embryonic bovine collagen is soluble in citrate buffer (Gebhardt, 1960) and accounts for more than 13% of the total collagen at birth. The small fraction of adult human or bovine collagen which is solubilized in citrate buffer generally represents less than 1% of the total (Plotnikova, 1947, 1949) or about 4% in calf skin (Bowes, Elliott & Moss, 1955). The fraction similarly dispersed from the tail tendon of the rat or from teleostean collagen is considerably greater. Dumitru & Garrett (1957) obtained nearly quantitative solution of rat-tail tendon in distilled water at pH 6 after treatment with 0.5M-sodium dihydrogen phosphate, whereas bovine tendon did not dissolve in this way. Clearly, these highly dispersible collagens are different from bovine or human collagen. One common feature of acid-soluble collagens from a wide variety of connective tissues and species appears to be the retention of the highly ordered collagen structure as indicated by well-orientated X-ray-diffraction patterns and typical collagen 640Å spacings, confirmed by electron microscopy (Cowan, North & Randall, 1955; Randall, Booth, Burge, Jackson & Kelly, 1955; Schmitt, Gross & Highberger, 1955) and by high intrinsic viscosity in dilute solution (Boedtker & Doty, 1956; Gallop, 1955; Doty & Nishihara, 1958). Methods that lead to extensive solubiliza-

tion of bovine collagen, such as autoclaving in water above 100°, heating in ethylenediamine (Solomons, 1956) or potassium phosphate (Bate-Smith, 1938), also result in loss of collagen structure.

Courts (1960) has described the conversion of collagen into eucollagen in which substantial disorganization of covalently bonded structure is likely, but regions of hydrogen bonding maintain a stable collagen-type structure. The present study suggests that, during the extensive dispersion of eucollagen into acid buffer solutions, the retention of the collagen fold is more likely than conversion into gelatin; the material thus obtained has therefore been termed 'soluble eucollagen'. It seems probable that the conversion of collagen into eucollagen is a chemical reversal of the aging process. The reactions described in the present work have made possible a large-scale study of a solubilized collagen from a single batch of bovine connective tissue. The method has an added advantage of freeing the tissue from much of the non-collagen impurity. The present account of the fibre-forming properties is a more detailed study of this new form of collagen than that published by Courts & Stainsby (1959). The X-ray diffraction and electron-microscopy work on the fibres reconstituted from soluble eucollagen is reported elsewhere (Little, Kelly & Courts, 1961).

The alternative available method of non-thermal solubilization of native bovine collagen is by the action of lyotropic agents (Gustavson, 1955). Because of the extensive breaking of hydrogen bonds this type of reaction invariably gives gelatin (Courts, 1958), and the rigid rod-like molecular structure associated with native collagen (Boedtker

* Part 1: Courts (1960).