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Salicylate-induced inhibition of collagen and mucopolysaccharide biosynthesis by a chick embryo cell-free system

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The ability of a chick embryo cell-free system to synthesize collagen, mucopolysaccharide and non-collagen protein in the presence of sodium salicylate was studied. Added creatine phosphate together with endogenous creatine kinase was the ATP generating system. The incorporation of labelled proline or labelled glucose into collagen or mucopolysaccharide respectively depended on ATP level in the cell-free system used. Salicylate inhibited collagen and mucopolysaccharide synthesis to a greater extent than non-collagen protein synthesis. The ability of the cell-free system to hydroxylate labelled protocollagen was inhibited 50% by storage at $-18°$. Ferrous iron reversed this inhibition. Salicylate prevented the restoration of the enzyme activity by ferrous iron. Incorporation of radioactivity into hyaluronic acid, when labelled UDP-glucuronic acid and UDP-*N*-acetylglucosamine were supplied, was inhibited 17% by salicylate. Under the same conditions 47% inhibition of incorporation into chondroitin sulphate was seen. This suggests that UDP-*N*-acetylglucosamine-UDP-*N*-acetylgalactosamine epimerase is inhibited by salicylate.

Rokosova-Cmuchalova & Bentley (1968) reported the effect of salicylate on the synthesis of collagen, chondroitin sulphate and non-collagen protein by slices of puppy epiphysial cartilage incubated with appropriate precursors of the above compounds. The biosynthesis of collagen and of chondroitin sulphate was markedly inhibited by a salicylate concentration of 10 mM whilst the synthesis of non-collagen protein was minimally, if at all, affected. The various steps in the biosynthesis of chondroitin sulphate are inhibited by salicylate. No information is available about the inhibition of the final polymerization of UDP-glucuronic acid and UDP-*N*-acetylgalactosamine which leads to the formation of the chondroitin sulphate chain. The present communication describes the biosynthesis of collagen, mucopolysaccharides and non-collagen protein by a chick embryo cell-free, mitochondrial-free preparation, and the effect of salicylate on these processes.

Since salicylate is known to uncouple oxidative phosphorylation (Smith & Smith, 1966) it is entirely possible that some of the effects noted were due to lack of ATP. The use of such a cell-free system permits the concentration of ATP to be controlled more readily since the uncoupling effect can be ignored. It also permits studies of the effect of salicylate on the hydroxylation of proline in labelled protocollagen, and of

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the incorporation of UDP monosaccharides into mucopolysaccharides. This latter is not possible in tissue slices due to the impermeability of cell membranes to UDP sugars (Telser, Robinson & Dorfman, 1965).

MATERIALS AND METHODS

The chick embryo cell-free system used was a dialysed 15 000 *g* supernatant (S-15) fraction prepared as described by Peterkofsky & Udenfriend (1963).

Incubation procedure

Incubations were carried out in air in rubber-stoppered 50 ml Erlenmeyer flasks at 37° with shaking. The final volume was 10 ml and contained the following components (mM): KCl, 20; $MgCl_2$, 4; sucrose, 250; ATP, 2; glutamine, 1; NAD, 0·5; creatine phosphate, 20; tris-HCl buffer (pH 7·6), 50; dialysed S-15 fraction equivalent to 100 to 120 mg of protein. [^{3}H]L-proline, [^{14}C]glucose and [^{14}C]UDP-glucuronic acid were added as described in the various experiments.

Isolation of imino-acids from collagen and non-collagen protein

After incubation, trichloroacetic acid (TCA) was added to a final concentration of 5%. After centrifugation the precipitate was heated for 60 min at 90° in 5% TCA and the supernatant solution added to that from the previous TCA treatment. This extraction procedure was repeated three additional times. The final insoluble residue was hydrolysed at 105° in 6 N HCl for 16 h. The hydrolysate was treated with nitrous acid followed by ether extraction to remove amino-acids (Myhill & Jackson, 1963) and the imino-acids separated on columns of AG50X8-H^+ (Eastoe, 1961). No hydroxyproline was recovered from the TCA-insoluble residue which is referred to as non-collagen protein. The supernatants from the hot TCA extraction steps were dialysed against 0·5% proline in 0·01 M acetic acid in an attempt to remove adsorbed labelled proline. Dialysis against several changes of 0·01 M acetic acid was continued for a further four days at 4°. Following this step a measured amount of highly purified calf skin collagen was added as carrier* before reduction of volume and hydrolysis. The hydrolysate was treated as before, leading to the isolation of collagen hydroxyproline and collagen proline.

Isolation of mucopolysaccharides

After incubation, TCA was added as before to a final concentration of 5%. The precipitate, after adjustment of pH, was papain-digested as described by Antonopoulous, Gardell & Hamnström (1965), and subjected to a second precipitation step with 5% TCA. The supernatant was added to that from the initial TCA precipitation step and both were dialysed against repeated changes of a 3% glucose solution to remove adsorbed labelled glucose and then against repeated changes of distilled water for four days at 4°. Carrier chondroitin-6-sulphate prepared from shark cartilage was added* followed by cetyl pyridinium chloride (CPC) to a final concentration of 1% in order to precipitate charged mucopolysaccharides and leave much of the uncharged glycopeptide material in solution. The precipitate was dissolved in a

* The specific activity data were not corrected for the addition of carrier collagen or chondroitin sulphate which accounts for the relatively low activity of these fractions when compared with non-collagen protein or with hyaluronic acid.

small amount of 60% propanol, precipitated with ethanol at a concentration of 70% together with traces of sodium acetate, and dried in a vacuum. This mixed mucopolysaccharide precipitate was dissolved in 0·4 M NaCl solution, and a 1% solution of CPC dissolved in 0·4 M NaCl was added to precipitate sulphated polysaccharide [the critical electrolyte concentrations (Scott, 1960) of which exceed this concentration of NaCl]. This is referred to as the "chondroitin sulphate fraction." The supernatant was subjected to ethanol precipitation as before and the precipitate referred as "hyaluronic acid fraction".

Hydroxylation system

[^{3}H]Protocollagen was prepared by incubation of the minced 9-day-old chick embryos (31 g) in a modified Krebs-Ringer buffer containing α,α'-dipyridyl (1 mM) and [^{3}H]proline (2 mCi) for 90 min at 37° (Hutton, Tappel & Udenfriend, 1966). The incubation mixture was then centrifuged at 15 000 *g* at 4° for 15 min and the resulting pellet was extracted with 0·5 M acetic acid. The extract was dialysed against distilled water for 3 days. The precipitate appearing on dialysis was dissolved in 0·1 M acetic acid and NaCl was added to a final concentration of 10%. The resulting precipitate was dialysed against 0·1 M acetic acid for 3 days and centrifuged. Following adjustment to pH 7·6, the supernatant was used as [^{3}H] protocollagen substrate. It contained $3{\cdot}8 \times 10^5$ d/min ml^{-1}, 2·36 mg protein/ml. Incubations and isolation of imino-acids were carried out as above with the addition of 0·3 ml [^{3}H]protocollagen substrate per flask.

Analytical procedures

Proline was determined on the Technicon autoanalyser by a modification of the method of Troll & Lindsley (1955). Hydroxyproline was determined by an automated modification of the Stegemann procedure (Stegemann, 1958). Uronic acid was determined by the procedure of Balazs, Bernsten & others (1965). ATP was determined (Kornberg, 1955) with a Biochemica Test combination (Boehringer, Mannheim). Radioactivity was measured by liquid scintillation spectrometry with adequate correction for quenching.

RESULTS AND DISCUSSION

Under the conditions used, the cell-free system was capable of incorporating labelled proline into collagen hydroxyproline, into non-collagen protein proline and of incorporating labelled glucose into the "chondroitin sulphate fraction". The synthetic activities continued for between 1 and 2 h after which time no further incorporation occurred. This cessation of synthetic activity can in part be accounted for by the rapid utilization and degradation of ATP by the cell-free system. It was found by ATP assay that the half life of ATP in this system was approximately 18 min, and that the conversion of added creatine phosphate into ATP by endogenous creatine kinase had largely ceased after 45 min. It is also probable that most other enzyme systems had become inactive after this time. It has previously been demonstrated that salicylate inhibits the production of ATP from creatine phosphate by creatine kinase (Dawkins, Gould & Smith, 1966).

Fig. 1 shows that the response of collagen synthesis to increasing creatine phosphate concentration is similar to that seen for the two mucopolysaccharide fractions up to

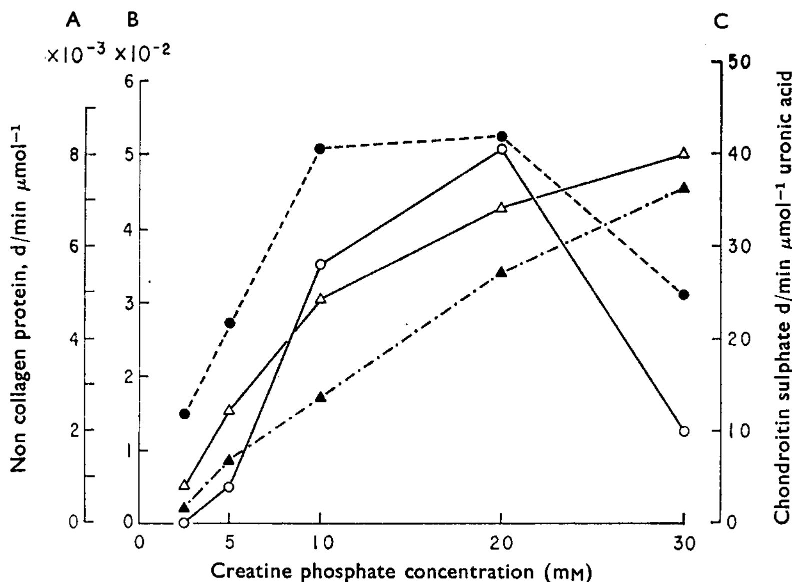

FIG. 1. The effect of creatine phosphate concentration on the incorporation of [^{14}C]glucose into chondroitin sulphate (closed triangles) and into hyaluronic acid (open triangles) and on the incorporation of [^{3}H]proline into collagen hydroxyproline (open circles) and into non-collagen protein proline (closed circles). Ordinate: A = non collagen proline, d/min μmol^{-1}. B = collagen hydroxyproline, d/min μ mol^{-1} and hyaluronate d/min μmol^{-1} uronic acid. C = chondroitin sulphate, d/min μ mol^{-1} uronic acid.

a concentration of 20 mM in that a continuous rise is seen. The specific activity rises by approximately 40% between 10 and 20 mM creatine phosphate. The non-collagen protein curve, however, follows a different course in that a mere 2% stimulation of synthesis is noted between these two creatine phosphate concentrations. Creatine phosphate, 30 mM, further stimulates mucopolysaccharide synthesis whereas it is inhibitory to both collagen and non-collagen protein synthesis. Such differences at very high concentrations of creatine phosphate are not, however, surprising since both the collagen and non-collagen protein are being synthesized on such structures as endoplasmic reticulum and polysomes, whereas the carbohydrate of the mucopolysaccharide is probably being produced by addition of monosaccharide units to preformed protein acceptors. In the range of creatine phosphate concentration between 10 and 20 mM the increased rate of collagen and mucopolysaccharide synthesis noted may be due to the increased availability of ATP, but the increased rate of collagen synthesis may equally be due to the concomitant increase in mucopolysaccharide synthesis.

As shown in Fig. 2, salicylate inhibited the incorporation of labelled proline or labelled glucose into collagen hydroxyproline or chondroitin sulphate respectively to a greater extent than the incorporation of labelled proline into non-collagen protein. The result may be accounted for by the decreased availability of ATP, since salicylate inhibits the production of ATP from creatine phosphate by creatine kinase, and collagen and mucopolysaccharide syntheses, as shown in Fig. 1, depend on the ATP level more than does non-collagen synthesis in the system used.

The biosynthesis of collagen is distinguished from that of other proteins by the hydroxylation of prolyl and lysyl residues previously incorporated into a large polypeptide chain, protocollagen (Lukens, 1965; Prockop & Juva, 1965; Kivirikko

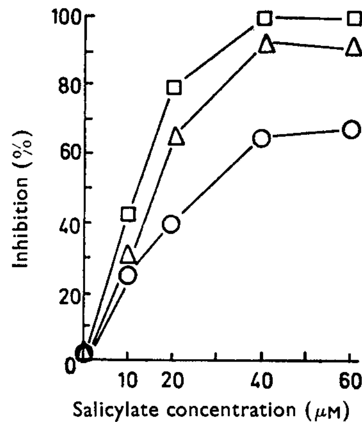

FIG. 2. The effect of salicylate on the incorporation of [^{14}C]glucose into "chondroitin sulphate fraction" (triangles) and on the incorporation of [^{3}H]proline into collagen hydroxyproline (squares) and into non-collagen protein proline (circles).

& Prockop, 1967). The enzyme responsible for the hydroxylation of proline in protocollagen has been referred to as protocollagen proline hydroxylase. Ferrous iron is a required cofactor of the enzyme which can be inhibited by such metal chelators as α,α'-dipyridyl, 1,10-phenanthroline or EDTA (Hurych & Chvapil, 1965; Nordwig, Kobrle & Pfab, 1967; Chvapil, Hurych & others, 1967). Salicylate is capable of acting as a metal chelator (Perrin, 1958), although the stability constant for Fe^{2+}-salicylate complex is markedly less than that of similar complex with α,α'-dipyridyl (Baxendale & George, 1948; Krumholz, 1949). It is thus possible that the results noted above could be due to inhibition of the hydroxylation step. This question was studied by incubating the S-15 fraction with previously prepared chick embryo protocollagen, the proline residues of which were labelled with tritium. The subsequent appearance of [^{3}H]hydroxyproline was taken as evidence of hydroxylation. As shown in Table 1, the S-15 fraction was capable of hydroxylating 17% of the proline residues of the protocollagen, but storage at −18° for 12 days reduced this value by half. Addition of 0·5 or 1·0 mM ferrous sulphate restored the activity of the frozen enzyme system to levels equal to that of the fresh material. The addition of 5 or 10 mM salicylate to the frozen stored enzyme system produced no effect but these concentrations were capable of preventing progressively the restoration of

Table 1. *Effect of ferrous iron and salicylate on the hydroxylation of protocollagen.* Incubation conditions: protocollagen 1·14 × 10^5 d/min; Dialysed S-15 fraction, 80 mg protein; final volume, 8 ml; incubation at 37° for 2 h.

Incubation temp. (°C)	Salicylate concn (mM)	FeSO₄, 0 mM d/min Hyp	Pro	Hydroxylation (%)	FeSO₄, 0·5 mM d/min Hyp	Pro	Hydroxylation (%)	FeSO₄, 1·0 mM d/min Hyp	Pro	Hydroxylation (%)
37°	0α	5 610	26 556	17·4						
1°	0*	401	32 240	1·2						
37°	0*	3 104	29 538	9·5	7 083	29 445	19·4	6930	31 513	18·0
37°	5*	2 978	32 706	8·3	5 207	28 202	15·6	4 179	29 041	12·6
37°	10*	2 731	32 022	7·9	3 665	32 194	10·2	3 285	31 573	9·4

* S-15 fraction stored 12 days at −18°.
α Fresh dialysed S-15 fraction was used.

activity by ferrous iron. It would seem that the enzyme contains more than one iron atom and that these are bound with different degrees of stability. Some of the ferrous iron may be removed by storage or by a chelator such as salicylate with a relatively low affinity for ferrous iron. This removal only partially inactivates the enzyme, though the use of chelators with higher stability constants for ferrous iron complexes leads to complete loss of activity.

Characterization of the mucopolysaccharide fraction

In an attempt to further characterize the polysaccharides produced, samples of "hyaluronic acid fraction" and "chondroitin sulphate fraction" were subjected to the cellulose column fractionation procedure of Antonopoulous & others (1965).The cetyl pyridinium complexes of the polysaccharides were eluted with increasing salt concentrations and the analysis of the material eluted is presented in Table 2. It is

Table 2. *Cellulose column fractionation of cetyl pyridinium (CPC) complexes of the hyaluronic acid (HA) and chondroitin sulphate (CSA) fractions.*

	Eluant				
	1% CPC	0·3 M NaCl	0·3 M $MgCl_2$	0·75 M $MgCl_2$ in acetic acid	0·75 M $MgCl_2$
HA Fraction					
Glucosamine (μmol)	0·12	1·28	—	—	—
Galactosamine (μmol)	0·02	0·04	—	—	—
Glucuronic acid (μmol)	0·25	1·30	0	0	0
Radioactivity (d/min)	—	228	0	0	0
CSA Fraction					
Glucosamine (μmol)	—	—	—	0·02	—
Galactosamine (μmol)	—	—	—	2·20	—
Glucuronic acid (μmol)	0	0	0·2	2·21	0
Radioactivity (d/min)	—	0	25	94	10

Results expressed as μmol/5 ml of 15 000 *g* supernatant.

clear from these results that the "hyaluronic acid fraction" is virtually free of galactosamine and has a glucosamine to glucuronic acid ratio of 1. The analytical figures of the "chondroitin sulphate fraction" agree well with its identity as chondroitin 4 or 6 sulphate. In addition, Table 2 shows that all the radioactivity incorporated into the "hyaluronic acid fraction" can be eluted from the column with 0·3 M NaCl and that most of the activity incorporated into the "chondroitin sulphate fraction" can be eluted with 0·75 M magnesium chloride in acetic acid. These salt concentrations are in agreement with the critical electrolyte concentrations of hyaluronic acid and chondroitin sulphate respectively. Because of the minute amounts available, no attempt was made to characterize further the fractions eluted with 0·3 M $MgCl_2$ and with 0·75 M $MgCl_2$ at neutral pH.

Effect of salicylate on the epimerase and polymerase steps

As previously pointed out (Rokosova-Cmuchalova & Bentley, 1968), salicylate is capable of blocking several reactions in the biosynthesis of mucopolysaccharides. No information is available however regarding the final UDP-*N*-acetylglucosamine

(UDP-GlcNHAc)–UDP-*N*-acetylgalactosamine (UDP-GalNHAc) epimerase reactions or of the UDP-glucuronic acid (UDP-GlcUA)–UDP-GalNHAc or UDP-GlcNHAc polymerase steps. Experiments were made to determine whether salicylate produced different effects on mucopolysaccharide synthesis when labelled glucose only was provided as a precursor and when the direct precursors of hyaluronic acid, UDP-GlcUA and UDP-GlcNHAc were supplied. After incubation, the "hyaluronic acid fraction" and "chondroitin sulphate fraction" were further purified by the cellulose column fractionation procedure described above and their specific radioactivities determined. The results of this experiment are seen in Table 3. Salicylate (20 mM) inhibited the incorporation of [^{14}C]glucose into chondroitin sulphate by 55% and into hyaluronic acid by 43%. A similar degree of inhibition (47%) was noted for the incorporation of [^{14}C]UDP-GlcUA into chondroitin sulphate when UDP-GlcNHAc was supplied, but a much lower degree (17%) of inhibition was seen for the incorporation of [^{14}C]UDP-GlcUA into hyaluronic acid under the same conditions.

Table 3. *Effect of salicylate on mucopolysaccharide synthesis by chick embryo 15 000 g supernatant fraction.*

Labelled precursor	[^{14}C]Glucose (30 μCi)	[^{14}C]Glucose (30 μCi)	[^{14}C]Glucose (30 μCi)	[^{14}C]UDP-GlcUA* (3·3 μCi)	[^{14}C]UDP-GlcUA* (3·3 μCi)	[^{14}C]UDP-GlcUA* (3·3 μCi)
Sodium salicylate	None	None	20 mM	None	None	20 mM
Incubation temp. (°C)	1°	37°	37°	1°	37°	37°
Hyaluronic acid, d/min μ mol^{-1}	5·3	79·8	44·8 (43% inhibition)	7·8	139·7	116·5 (17% inhibition)
Chondroitin sulphate, d/min μ mol^{-1}	2·6	21·6	9·6 (55% inhibition)	1·7	45·4	23·8 (47% inhibition)

Incubation was carried out for 2 h under the conditions as in the text except as follows:
* Contained UDP-glucuronic acid, 0·3 mM; UDP-NHAc glucosamine, 0·3 mM.

It is felt that this 17% inhibition represents the effect of salicylate upon the enzyme system polymerizing UDP-GlcUA and UDP-GlcNHAc. The additional inhibition noted in the chondroitin sulphate synthesis is probably due to the effect of salicylate upon the epimerase responsible for the conversion of UDP-GlcNHAc to -UDP-GalNHAc.

This report serves to point out the complexity of the effect of salicylate on the biosynthesis of connective tissue components.

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