INHIBITOR STUDIES ON THE BIOSYNTHESIS OF CHONDROMUCOPROTEIN BY CHICKEN EMBRYONIC CARTILAGE

By P. L. JEFFREY*† and K. G. RIENITS*

[Manuscript received 18 August, 1971]

Abstract

Using chick embryonic cartilage, the effects of puromycin, ribonuclease, and chloramphenicol on the incorporation into chondromucoprotein of radioactively labelled leucine, glucose, and inorganic sulphate have been studied.

In cell-free extracts, puromycin inhibited the incorporation of both glucose and leucine but did not affect sulphate incorporation. Ribonuclease had no effect on glucose incorporation, and chloramphenicol inhibited the incorporation of leucine but not that of glucose.

Puromycin and ribonuclease inhibit to a greater extent than leucine the incorporation of glucose into chondroitin sulphate isolated from chondromucoprotein; this suggests that both inhibit chondromucoprotein synthesis at some second point in addition to inhibiting protein synthesis. The action of chloramphenicol could be due to an effect on protein synthesis alone.

I. INTRODUCTION

The paths of synthesis of the components of chondromucoprotein are moderately well understood but at present the pattern of synthesis of the whole molecule is not clear. This, in part, stems from the complex nature of these substances. Chondromucoproteins exist in cartilage as high-molecular-weight compounds which can comprise some 40–50% of its dry weight (Malawista and Schubert 1958).

The recognition that chondromucoprotein could be separated into a light and heavy fraction (Gerber, Franklin, and Schubert 1960) did not affect the conclusion that in cartilage most, if not all, of the chondroitin sulphate was covalently linked to protein. Each protein molecule carried a number of chondroitin sulphate chains linked through the hydroxyl of the serine residues by a specific linkage region consisting of Gal-Gal-Xyl-Ser (Lindahl and Roden 1965; Roden and Smith 1966). Keratan sulphate is found in the light fraction of chondromucoprotein (Franek and Dunstone 1967). However, keratan sulphate is absent from embryonic cartilage (Mathews 1965) and thus from chondromucoprotein isolated from embryonic cartilage.

Metabolic studies have indicated that chondromucoprotein is metabolized as a unit, i.e. that the protein and polysaccharide moieties of the chondromucoprotein are synthesized and broken down in an integral fashion. This conclusion is based on studies using tissue preparations in which cellular integrity has been maintained (Gross, Mathews, and Dorfman 1960; Campo and Dziewiatkowski 1962; Telser, Robinson, and Dorfman 1965). The experiments of Telser *et al.* further indicate that, in cell-free extracts of cartilage, protein synthesis and chondroitin sulphate synthesis need not be linked as they appear to be in intact cells.

* School of Biochemistry, University of New South Wales, P.O. Box 1, Kensington, N.S.W. 2033.

† Present address: Department of Biochemistry, Monash University, Clayton, Vic. 3168. (Address for reprints.)

The investigations reported here show that three inhibitors of protein synthesis, i.e. puromycin, ribonuclease, and chloramphenicol, affect the synthesis of chondromucoprotein, but in different ways.

II. Materials and Methods

(*a*) *Epiphyseal Crushes*

Epiphyses were obtained from the tibias and femurs of 14- to 16-day-old chick embryos. Epiphyseal crushes were prepared by squeezing epiphyses between roughened microscope slides moistened with cold 0·9% NaCl (w/v). Microscopical examination indicated that this type of preparation consisted almost entirely of whole cells. The preparation gave more reproducible results than cartilage slices and was also thought to allow a larger surface area of the cells to be exposed to the media. Cell-free extracts were prepared as previously described (Jeffrey and Rienits 1970).

(*b*) *Incubation Conditions*

Epiphyseal crushes (approximately 500 mg wet weight from three embryos) were incubated in 3·0 ml of Krebs–Ringer–bicarbonate, pH 7·4, with magnesium chloride instead of magnesium sulphate, in double-side-arm Warburg flasks with shaking. The incubation temperature was 37°C and the gas phase was oxygen containing 5% carbon dioxide. After a 15-min pre-incubation, the reaction was commenced by tipping in radioactive substrate contained in 0·2 ml 0·9% (w/v) NaCl from a side-arm. Inhibitors, when used, were added as neutralized aqueous solutions (0·2 ml) from a side-arm during the pre-incubation period. The incubation was terminated by the addition of 100 μmoles of either *p*-chloromercuribenzoate or potassium cyanide (Wolfe and Vickery 1964). When using cell-free extracts, the reaction mixtures contained, in a total volume of 3·0 ml and final pH 8·0, the following: 200 μmoles Tris (pH 8·4); 30 μmoles ATP; 10 μmoles magnesium chloride; 20 μmoles cysteine; 10 μmoles glutathione; 1·0 ml cell-free extract. Inhibitors, when used, were added in 0·1 ml of neutralized solution. The temperature of incubation was 37°C, the gas phase was air, and the vessels were shaken. The reaction was terminated in the same manner as for the crush preparations.

The time from the addition of radioactive substrate to termination of the reaction was 2 hr, except for the experiment in Figure 1.

The cell-free extract was usually diluted so that three embryos were contained in 1 ml of extract and yielded 8–10 mg of chondromucoprotein. However, 500 mg of epiphyseal crushes (also the approximate yield from three embryos) gave 12–17 mg of chondromucoprotein.

The preparation of the cell-free extract involved a centrifugation, at 750 *g* for 20 min, of the initial homogenate, and there would have been some chondromucoprotein contained in the pellet which was discarded.

(*c*) *Isolation of Reaction Products*

Chondromucoprotein was isolated from the reaction mixtures essentially by the procedure of Malawista and Schubert (1958), modified for experiments with radioactive tracers. The reaction mixtures were made up to 30 ml with cold distilled water and homogenized in a Virtis 45 homogenizer at 0°C. A sequence of 30 min at top speed, 30 min at one-quarter speed, and 30 min at top speed was used. Two volumes of 95% ethanol were added and the precipitate of collagen was centrifuged down at 8000 *g* for 20 min, and washed with 30 ml of 95% ethanol. To the combined supernatant and washing 1 g of potassium acetate was added to precipitate the chondromucoprotein overnight at 0°C. A second extraction of the collagen was carried out by the above procedure, using homogenization times of 3 min and 10 ml of water, and a second precipitate of chondromucoprotein obtained. The two precipitates were collected by centrifugation at 8000 *g* for 20 min, and washed with 75% ethanol containing 1·0% (w/v) potassium acetate. The chondromucoprotein was then dissolved in 3 ml of water and 6 ml of 95% ethanol added. To the clear supernatant remaining after a short centrifugation, 3 ml of 95% ethanol were added, followed by 300 mg of potassium acetate to precipitate the chondromucoprotein. The precipitate was collected by centrifugation and washed five times with 75% ethanol containing 1% (w/v)

potassium acetate. It was suspended in a small volume of fresh wash fluid, collected by filtration on tared filter paper disks (Whatman No. 42), and weighed after drying.

Chondroitin sulphate was isolated by subjecting the chondromucoprotein to papain digestion and precipitating the chondroitin sulphate as the cetyl trimethylammonium complex in the presence of 0·2M sodium sulphate (Muir 1958). "Total mucopolysaccharide fraction" was isolated, as described by Perlman, Telser, and Dorfman (1964), from a papain digest of the entire cell-free extract reaction mixture inactivated as described above.

Chondromucoprotein isolated as described above contained glucuronic acid, hexosamine, and sulphate in molar ratios of 1·0, 1·06, and 0·89, and 61·7% protein on a dry-weight basis. Chondroitin sulphate isolated after papain digestion of the chondromucoprotein contained glucuronic acid, hexosamine, and sulphate in molar ratios of 1·0, 1·13, and 0·47, and 14% protein on a dry-weight basis.

(*d*) *Radioactivity Measurements*

Radioactivity of the plated-out precipitates was measured in a windowless Nuclear Chicago model 181B gas flow counter which had a counting efficiency of 13·4% for ^{14}C at infinite thinness. Specific activities are expressed as counts/min/mg at infinite thickness of the compounds isolated, and duplicates agreed to within 6·5%.

(*e*) *Source of Inhibitors and other Materials*

Puromycin dihydrochloride was obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio. Pancreatic ribonuclease, A grade, 50 enzyme units/mg, protease-free, was obtained from Boehringer and Soehne. Chloramphenicol came from Parke, Davis and Co., Detroit, Michigan. [$^{14}C_6$]glucose (76·3 mCi/mmole), L-[^{14}C]leucine (8·0 mCi/mmole), and carrier-free [^{35}S]sulphate were obtained from the Radiochemical Centre, Amersham, England.

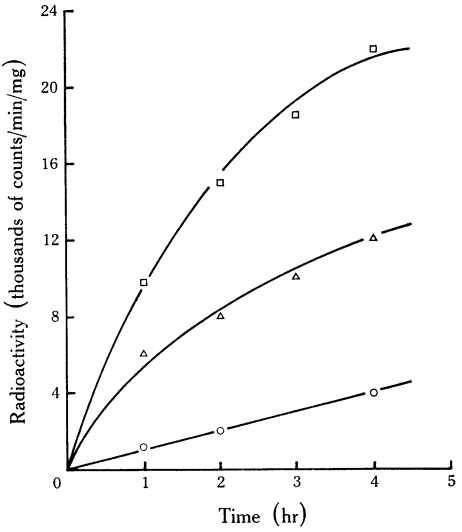


Fig. 1.—Incorporation of radioactivity into chondromucoprotein by epiphyseal crushes from [^{14}C]glucose ($2·2 \times 10^{-5}$M) (○), [^{14}C]leucine ($2·1 \times 10^{-4}$M) (△), and [^{35}S]sulphate (carrier-free) (□). For experimental conditions see Section II(*b*).

III. Results

(*a*) *Preparations without Inhibitor*

Figure 1 illustrates the kinetics of incorporation of the radioactively labelled substrates into chondromucoprotein by epiphyseal crush preparations. The percentages of incorporation of added radioactivity after 2 hr of incubation were 2·5%

for [^{14}C]glucose, 5·3% for [^{14}C]leucine, and 10% for [^{35}S]sulphate. No analyses were made to determine endogenous levels, and hence calculations of absolute rates of incorporation could not be made.

Cell-free extracts, supplemented with ATP, magnesium chloride, cysteine, and glutathione were able to transform [^{14}C]glucose, [^{14}C]leucine, and [^{35}S]sulphate into chondromucoprotein and chondroitin sulphate.

TABLE 1

COMPARISON OF SPECIFIC ACTIVITIES OF CHONDROMUCOPROTEIN AND CHONDROITIN SULPHATE ISOLATED FROM CRUSH PREPARATIONS AND CELL-FREE EXTRACTS

Specific activities were normalized to correspond to the addition of 2·5 μCi of each radioactive substrate. Incubation conditions are described in Section II(*b*). Results of individual experiments are given

Substrate	Specific activity (counts/min/mg): Chondromucoprotein			Chondroitin sulphate		
			Crush preparations			
[^{14}C]glucose	10500	8200	14250	12000	5720	12900
[^{14}C]leucine	4100	5350	8760			
[^{35}S]sulphate	7500	7070	11000			
			Cell-free extracts			
[^{14}C]glucose	2120	1030	1660	1650	1400	1860
[^{14}C]leucine	345	199	291			
[^{35}S]sulphate	192					

Table 1 allows a comparison to be made between results obtained from crush and cell-free preparations. Even though roughly equivalent, in terms of amount of epiphyseal material used to make the crush and cell-free preparations, it can be seen that the former achieved a much greater specific activity in both chondromucoprotein and chondroitin sulphate from the three radioactive substrates. More of note, however, was the marked difference in the pattern of incorporation of the three substrates into chondromucoprotein by the two preparations. The incorporation of sulphate was very much less in comparison to glucose in the cell-free extract than in the crush preparations.

(*b*) *Effects of Puromycin*

Figures 2 and 3 show that puromycin inhibited the incorporation of radioactivity into chondroitin sulphate and chondromucoprotein. There were differences in behaviour between the crush preparation and the cell-free extracts. In the crush preparation the inhibitory effect of puromycin was very similar, although not quantitatively identical, with each of the labelled substrates. These findings are similar to those of Telser, Robinson, and Dorfman (1965) using cartilage mince and a single high concentration of puromycin.

During these experiments it was observed that puromycin caused an inhibition of oxygen uptake by epiphyseal crushes. In one experiment inhibitions of 64 and 82%

in oxygen uptake were obtained at concentrations of 10 and 40 μg/ml of puromycin (2×10^{-5}M and 8×10^{-5}M respectively). At these concentrations [^{14}C]leucine incorporation into chondromucoprotein was inhibited 52 and 87% respectively. Puromycin has not been previously recorded as inhibiting respiration and it has been tentatively assumed that the effect observed here reflects an indirect respiratory control. Protein synthesis may well be a major consumer of energy in the cells of embryonic cartilage. Inhibition of protein synthesis could diminish the requirement

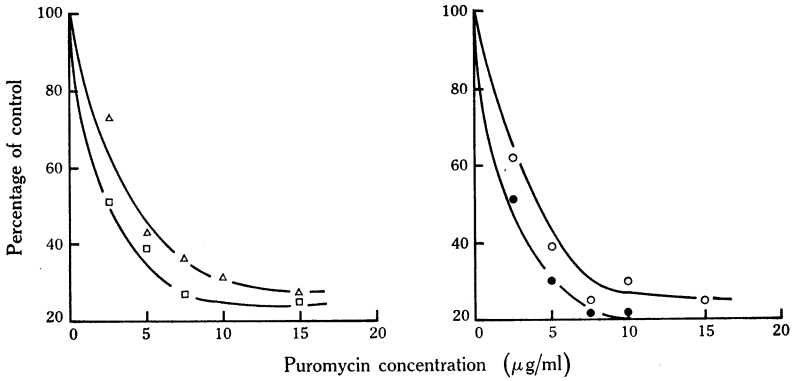


Fig. 2.—Inhibition by puromycin of uptake of [^{14}C]leucine (4×10^{-5}M) (△), [^{35}S]sulphate (carrier-free) (□), and [^{14}C]glucose ($1\cdot1\times10^{-5}$M) (○) into chondromucoprotein, and [^{14}C]glucose ($1\cdot1\times10^{-5}$M) (●) into chondroitin sulphate, by epiphyseal crushes. For experimental conditions see Section II(*b*).

for ATP, allowing it to build up at the expense of ADP. This would bring into operation the respiratory control mechanism originally proposed by Chance and Williams (1956). This interpretation is supported by the observation that the inhibition of respiration brought about by puromycin is released on the addition of dinitrophenol (unpublished observations of Williams and Rienits).

It is notable that in the cell-free system (Fig. 3) puromycin caused a very slight stimulation of [^{35}S]sulphate incorporation into chondromucoprotein, but varying degrees of inhibition of [^{14}C]glucose and [^{14}C]leucine incorporations into chondroitin sulphate and chondromucoprotein.

A comparison of Figures 2 and 3 indicates that in cell crushes puromycin inhibits [^{14}C]leucine incorporation into chondromucoprotein slightly more than it does in the cell-free system. The incoporation of [^{14}C]glucose into chondromucoprotein was much less inhibited by puromycin in the cell-free system than in the crush preparation. On the other hand, [^{14}C]glucose incorporation into chondroitin sulphate was more severely inhibited by puromycin in the cell-free system than in the crush preparation. The lack of inhibitory effect of puromycin upon [^{35}S]sulphate incorporation in the cell-free system was in marked contrast to the inhibition seen in the crush preparation.

In the above experiments the chondroitin sulphate was isolated by degrading chondromucoprotein isolated from the incubation mixture. This may have represented a selected fraction of the total chondroitin sulphate synthesized in the extract,

especially in the presence of puromycin which had undoubtedly inhibited protein synthesis. A comparison was therefore made of the effect of puromycin on [^{14}C]glucose incorporation into chondroitin sulphate isolated via chondromucoprotein and into the total mucopolysaccharide fraction of the extract (i.e. the initial isolation of

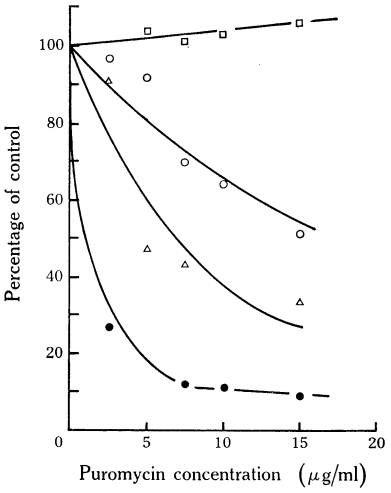
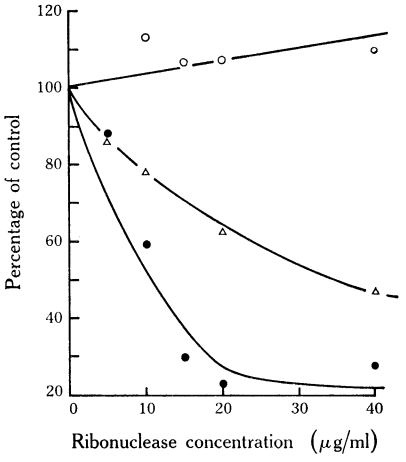


Fig. 3.—Effects of puromycin on the incorporation into chondromucoprotein of 2·5 μCi [^{14}C]glucose ($1\cdot1\times10^{-5}$M) (○) 1 μCi [^{14}C]leucine (4×10^{-5}M) (△), and 2 μCi [^{35}S]sulphate (carrier-free) (□), and on the incorporation into chondroitin sulphate of 2·5 μCi [^{14}C]glucose ($1\cdot1\times10^{-5}$M) (●), by a cell-free extract of epiphyses. For experimental conditions see Section II(*b*).

chondromucoprotein omitted and all mucopolysaccharide present in the extract included). At two levels of puromycin (10 and 20 μg/ml) no difference was observed in the effect of puromycin on the incorporation into the two products.

Fig. 4.—Influence of ribonuclease on the incorporation into chondromucoprotein of 2·5 μCi [^{14}C]glucose ($1\cdot1\times10^{-5}$M) (○) and 2·5 μCi [^{14}C]leucine (4×10^{-5}M) (△); the incorporation into chondroitin sulphate of 2·5 μCi [^{14}C]glucose ($1\cdot1\times10^{-5}$M) (●), by a cell-free extract of epiphyses. For experimental conditions see Section II(*b*).



(*c*) *Effects of Chloramphenicol and Ribonuclease*

The effects of two other inhibitors of protein synthesis, viz. ribonuclease and chloramphenicol, were examined. In Figure 4 it can be seen that ribonuclease markedly inhibits the incorporation of [^{14}C]leucine into chondromucoprotein and [^{14}C]glucose into chondroitin sulphate. However, the incorporation of [^{14}C]glucose into chondromucoprotein was unaffected.

Chloramphenicol had another pattern of effects (Table 2). The incorporation of [^{14}C]leucine into chondromucoprotein was inhibited, whereas the uptake of [^{14}C]glucose into either chondromucoprotein or chondroitin sulphate was unaffected. The concentration of chloramphenicol used was considerably higher than that necessary to affect bacterial systems.

TABLE 2

EFFECT OF CHLORAMPHENICOL ON THE INCORPORATION OF [^{14}C]GLUCOSE AND [^{14}C]LEUCINE INTO CHONDROMUCOPROTEIN AND CHONDROITIN SULPHATE BY A CELL-FREE EXTRACT OF EPIPHYSEAL CARTILAGE

Reaction conditions and isolation procedures are described in Section II(*b*)

Substrate	Chloramphenicol concn. (μg/ml)	Specific activity (as % of control)	
		Chondromucoprotein	Chondroitin sulphate
[6-^{14}C]glucose	0	100	100
(10 μCi; $4{\cdot}4\times10^{-5}$M)	100	104	88
	1000	98	98
[^{14}C]leucine	0	100	
(2·5 μCi; 4×10^{-5}M)	100	36	
	1000	21	

IV. DISCUSSION

The synthesis of chondromucoprotein in the epiphyseal crush preparations gives the impression of being a highly integrated process. The similar time course of incorporation of various radioactive precursors (Fig. 1) and the quantitatively similar effects of puromycin (Fig. 2) indicate an organized and controlled series of events leading to chondromucoprotein synthesis.

It is accepted that chondromucoprotein synthesis follows a sequence in which a protein core is first formed, and to this are added linkage regions of two galactose and one xylose residues, through the hydroxyls of serine residues in the protein core. Alternate glucuronic acid and *N*-acetylgalactosamine residues are then added to the linkage regions from their uridine diphosphate derivatives to build up polysaccharide chains. Sulphation proceeds simultaneously with the growth of the polysaccharide chains or lags a little behind it. The whole process takes place on the endoplasmic reticulum (Telser, Robinson, and Dorfman 1965; Horwitz and Dorfman 1966; Robinson, Telser, and Dorfman 1966).

Chondromucoprotein is taken as a discrete chemical entity for the purposes of discussion, although it is realized that relatively mild procedures can separate it into a number of fractions, e.g. heavy and light fractions.

Compared with the crush preparation, chondromucoprotein synthesis in the cell-free extracts is greatly reduced. There is also a changed pattern of incorporation of radioactive precursors. The data of Table 1 indicate that in the cell-free preparations [^{14}C]glucose incorporation into chondromucoprotein was quantitatively much more important compared to sulphate and leucine incorporation than it was in the crush preparations. A number of factors are possibly concerned in this changed pattern of incorporation; one of them could be that disruption of cell organization

brings enzymes, substrates, and intermediates of chondromucoprotein synthesis into new spatial relationships with one another, giving rise to the possibility of artifacts.

In addition there is the possibility of active uptake of added substrates by the intact cell system, leading to higher effective concentrations of the radioactive precursors than in the cell-free system.

Cell disruption allows large amounts of extracellular chondromucoprotein to become accessible to the sulphotransferases present in both the microsomal fraction (Horwitz and Dorfman 1966) and in the 105,000 *g* supernatant (Meezan and Davidson 1967*a*) of embryonic cartilage. Under these conditions sulphation is unaffected by puromycin, as seen in Figure 3 (also see Meezan and Davidson 1967*a*, 1967*b*). In contrast, in the crush preparation, which consists predominantly of whole cells and extracellular matrix, sulphation is probably confined to the intracellular acceptor. If this acceptor pool is small, sulphate incorporation into chondromucoprotein would become indirectly dependent on factors controlling acceptor synthesis, e.g. puromycin. Sulphate incorporation in the cell-free extracts represents an abnormal situation not obtained *in vivo* (cf. Robinson 1969).

The polymerase responsible for the formation of the glycosaminoglycan of chondroitin sulphate from UDP-glucuronic acid and UDP-*N*-acetylgalactosamine has been located in the microsomal fraction (Perlman, Telser, and Dorfman 1964; Silbert 1964) and the suggestion has been made that polysaccharide chain initiation occurred in the rough, and chain elongation in the smooth, reticulum (Horwitz and Dorfman 1966). The surprising feature of Figures 3 and 4 was that chondroitin sulphate formation from [$^{14}C_6$]glucose was much more sensitive to puromycin and ribonuclease than was [^{14}C]leucine incorporation into chondromucoprotein. An inhibition of chondroitin sulphate synthesis consequent upon the inhibition of synthesis of the protein core of chondromucoprotein would, at the most, be equal to the inhibition of protein synthesis, and in the case where there was present a pool of partially completed chondromucoprotein, e.g. the protein core, the inhibition would be less. Chloramphenicol (Table 2) inhibits protein synthesis without affecting chondroitin sulphate synthesis. These findings imply that puromycin and ribonuclease each inhibited two steps in the overall synthesis of chondromucoprotein. It is well established that puromycin brings about the production of incomplete and non-functional peptides (Davidson 1969).

Adamson, Langeluttig, and Anast (1966) have shown, using embryonic chick bone, that puromycin inhibits amino acid incorporation immediately, though after 1–2 hr an inhibition of transport is expressed. The reduced transport was explained on the basis of an inhibition of synthesis of protein or proteins necessary for amino acid transport. Whether this effect is applicable here is debatable.

Ribonuclease probably inhibits protein synthesis by degrading *m*-RNA and perhaps ribosomal RNA. This may be the basis of its action in chondroitin sulphate synthesis if this takes place on the rough endoplasmic reticulum. Our results with puromycin indicate that all chondroitin sulphate synthesis is part of chondromucoprotein synthesis and does not proceed independently of it. This conclusion follows from the observation that [^{14}C]glucose incorporation into chondroitin sulphate isolated from chondromucoprotein and into total acid mucopolysaccharide are equally sensitive to puromycin.

Inhibition by chloramphenicol of cytoplasmic ribosomal protein synthesis in animal systems does not appear to have been observed previously. Chloramphenicol affects protein synthesis in mitochondria, inhibits respiration at high concentrations (100–200 μg/ml), and causes extensive dilation of the cisternae of endoplasmic reticulum (Firkin and Linnane 1968, 1969). The effect seen in Table 2 appears to be due to an action of chloramphenicol on either cytoplasmic ribosomes as site of protein synthesis or on the endoplasmic reticulum. Any effect on mitochondrial respiration was likely to be unimportant, as the system was adequately supplemented with ATP.

The relationship between [^{14}C]leucine incorporation into chondromucoprotein and [^{14}C]glucose into chondromucoprotein and chondroitin sulphate, shown in Table 2, could follow if the extract contained a pool of incomplete chondromucoprotein to which glucose and its derivatives could be added by the extract, to give chondromucoprotein. The experiments of Cole and Lowther (1969) appear to have shown the presence in cartilage slices of such a pool, which enabled chondroitin sulphate synthesis to proceed for over 2 hr after protein synthesis was stopped by cycloheximide.

Enzyme activity incorporating [^{14}C]xylose into trichloroacetic acid-precipitable material is found both in soluble and particulate fraction of chick embryonic cartilage (Robinson, Telser, and Dorfman 1966) and the incorporation of *N*-acetylgalactosamine and glucuronic acid from corresponding uridine diphosphate compounds can take place onto a variety of polysaccharide acceptors (Telser, Robinson, and Dorfman 1966). In cell-free extracts, this could lead to the same type of abnormal situation that appears to hold for sulphation, and hence not present an accurate picture of the events in intact cells.

V. Acknowledgments

We are grateful for support for this work by the University of Sydney Post-Graduate Medical Foundation and the National Health and Medical Research Council of Australia.

VI. References

Adamson, L. F., Langeluttig, S. G., and Anast, C. S. (1966).—*Biochim. biophys. Acta* **115**, 355.

Campo, R. D., and Dziewiatkowski, D. D. (1962).—*J. biol. Chem.* **237**, 2729.

Chance, B., and Williams, G. R. (1956).—*Adv. Enzymol.* **17**, 65.

Cole, N. N., and Lowther, D. A. (1969).—*FEBS Lett.* **2**, 351.

Davidson, J. N. (1969).—"The Biochemistry of the Nucleic Acids." 6th Ed. p. 284. (Methuen: London.)

Firkin, F. F., and Linnane, A. W. (1968).—*Biochem. biophys. Res. Commun.* **32**, 398.

Firkin, F. F., and Linnane, A. W. (1969).—*Expl Cell Res.* **55**, 68.

Franek, M. D., and Dunstone, J. R. (1967).—*J. biol. Chem.* **242**, 3460.

Gerber, B. R., Franklin, E. C., and Schubert, M. (1960).—*J. biol. Chem.* **235**, 2870.

Gross, J. I., Mathews, M. B., and Dorfman, A. (1960).—*J. biol. Chem.* **235**, 2889.

Horwitz, A. L., and Dorfman, A. (1966).—*J. Cell Biol.* **38**, 358.

Jeffrey, P. L., and Rienits, K. G. (1970).—*Biochim. biophys. Acta* 208, 147.

Lindahl, U., and Roden, L. (1965).—*J. biol. Chem.* **240**, 2821.

Malawista, I., and Schubert, M. (1958).—*J. biol. Chem.* **230**, 535.

Mathews, M. B. (1965).—"The Structure and Function of Connective and Skeletal Tissues." N.A.T.O. Study Institute. p. 181. (Butterworths: London.)

MEEZAN, E., and DAVIDSON, E. A. (1967*a*).—*J. biol. Chem.* **242**, 1685.
MEEZAN, E., and DAVIDSON, E. A. (1967*b*).—*J. biol. Chem.* **242**, 4956.
MUIR, H. (1958).—*Biochem. J.* **69**, 195.
PERLMAN, R. L., TELSER, A., and DORFMAN, A. (1964).—*J. biol. Chem.* **239**, 3623.
ROBINSON, H. C. (1969).—*Biochem. J.* **113**, 543.
ROBINSON, H. C., TELSER, A., and DORFMAN, A. (1966).—*Proc. natn. Acad. Sci. U.S.A.* **56**, 1859.
RODEN, L., and SMITH, R. J. (1966).—*J. biol. Chem.* **241**, 5949.
SILBERT, J. E. (1964).—*J. biol. Chem.* **239**, 1310.
TELSER, A., ROBINSON, H. C., and DORFMAN, A. (1965).—*Proc. natn. Acad. Sci. U.S.A.* **54**, 912.
TELSER, A., ROBINSON, H. C., and DORFMAN, A. (1966).—*Archs Biochem. Biophys.* **116**, 458.
WOLFE, H. J., and VICKERY, A. L. (1964).—*Lab. Invest.* **13**, 743.