DISCUSSION

Dr. George R. Nichols (Boston): Dr. Schubert, you described two fractions which you extract from cartilage, one heavy and the other light. I didn't understand what you thought caused one to be heavy. I had the idea that this might be a fraction which contained a good deal of crystalline mineral, as for instance, in calcified collagen.

Dr. Schubert: They were called heavy and light depending on the ease of sedimentation in the centrifugal field. The heavy fraction settles very readily. The difficulty involved here is that you have to repeat this centrifugation a number of times. You can't do it just once and separate them. That is why I stressed the idea of entanglement.

Dr. Nichols: Did you analyze them for salts contained in either fraction?

Dr. Schubert: Oh, yes, they have been analyzed for inorganic ions. The cation content of either one of them corresponds to the calculated counterion content.

Dr. Nichols: Dr. Dorfman, you spoke of trypsin-isolated cartilage cells and pointed out that trypsin damages cells. Could you tell us more about the metabolism of these cells, the oxygen consumption, and so forth, and also whether you tried other means of separation? Other enzymes and agents have been used for such purposes; some, for instance, have isolated renal tubular cells with collagenase, others have used chelating agents.

Dr. Dorfman: To the first question, the answer is that we have no data. We have not studied these cells for other metabolic parameters. They take up oxygen in a few experiments.

As for the second question, in some earlier studies by Dr. Gross in our laboratory, we thought, originally, we could approach this problem by using a combination of hyaluronidase and collagenase. I would say we were somewhat less than successful.

I would add that this was not studied extensively. We did not have the best preparations of collagenase available. I would not say it is impossible to do it in this manner, but we have found the trypsin method the simplest one to work with.

Dr. H. Holtzer (Philadelphia): I would like to ask Dr. Dorfman about this extracellular chondroitin sulfate. It sounded very intriguing. You stressed the use of trypsinized cells and suggested that such cells allow the entry of molecules which are normally excluded. Is it not then possible that molecules which normally do not leak out do so under the conditions used, particularly as many of the cells are

lysing? Second, is there any evidence that unpolymerized chondroitin sulfate "pours" into the medium, rather than first being polymerized on the cell surface? In the latter case the chondroitin sulfate detected in the medium might first have been deposited in or on the cell membrane.

Dr. Dorfman: I'm not sure I understand your question. I might just restate the circumstances and the limitations. We simply made the suspensions with trypsin, washed until they showed no further uronic acid in the washings, and then incubated the suspensions. The chondroitin sulfate was isolated from the medium. This has the composition that was shown both with respect to chondroitin A and C and sulfate content, as far as amino acid content goes. I think I stated that we can't say how much extrusion occurs because some cell breakdown may occur during the period of incubation.

There are other limitations I should have indicated but did not because of lack of time. We are very concerned about the fact that this does not have as high a protein content as the complex that Dr. Schubert talked about, and sooner or later we must come to grips with what is actually made in the cell. Is there further polymerization or linkage to protein after the original particle is made?

There are two limitations in our experiment. One is concerned with whether the trypsin attached to cells has degraded to original particle. We tried to control this by immediate washing and adding the trypsin inhibitor. A second difficulty which we can't control is the possibility that some of the cells may be breaking down and releasing lysosomal enzymes of the kind that Dr. Thomas is going to discuss this afternoon. I don't know of any experimental way to stop this or to prove it, and so the particle we have may have undergone some proteolysis after synthesis.

I think what we have studied reflects the amino acids present, but whether a longer peptide was present and was lost or whether this is the real particle that is made cannot be ascertained.

Question: I wondered whether Dr. Dorfman had repeated those experiments on chondroitin sulfate synthesis, using homogenates of cells in any system?

Dr. Dorfman: Well, I think it is obvious that we are quite conscious of the importance of working with cell-free systems. I am put in a difficult spot to say that these experiments are under way. Shall I say, they are promising?

Dr. David J. Hamerman (New York): I would like to make a comment on Dr. Dorfman's intriguing suggestion about the genesis of Hurler's syndrome. In listening to him and thinking about it, it occurred to me that possibly we are moving into a new stage in the field of protein polysaccharides. For years we were concerned with the difficulties of getting macromolecules out of tissues, and then with characterizing the polysaccharides. I think increasingly we are going to have to come to reckon with the proteins that appear bound to the polysaccharides, and of great importance to the physical behavior of the polysaccharides, as we heard

this morning from Dr. Schubert, and as we will hear later this afternoon from Dr. Thomas. The nature of these proteins is still relatively unknown. They are not collagen, certainly, for they don't contain hydroxyproline, and they may not be plasma proteins.

The intriguing thing about these proteins in the chemistry of both abnormal connective tissue and normal connective tissue is that the composition of these proteins is under genetic control. Even slight variations may affect the properties of the protein polysaccharides, whereas the polysaccharides seem to be fairly similar in composition from condition to condition.

Dr. Meyer: Do you mean to say that the polysaccharides are not under genetic control? Assuming this is a polysaccharide, it is not under genetic control?

Dr. Hamerman: I presume it is, but surely the protein is, and this is known from data we now have. It may be that variations in the amount, or in the amino acid composition of the proteins, have a profound effect on the polysaccharide behavior.

Dr. Dorfman: I agree. This is the point I was trying to make this morning. I think that the polysaccharides are under genetic control in that the enzymes that are concerned in all these steps of biosynthesis are under genetic control. But I think there is some merit to this point of view, in that I believe the direct polymerization, once the enzyme is there, is probably not dependent on RNA for information supplied in that way.

On the other hand, the protein could be very slightly changed, as is well known, in so many situations. Dr. Matthews has recently been studying the comparative biochemistry of these polysaccharides and already it would appear that, actually, the basic pattern of polysaccharide is a rather early kind of constant genetic information, which does not vary a great deal in different species. It seems that the extracellular material appears very early in phylogenetic development.

I have been fond of saying sometimes that the capsule of the streptococcus is the connective tissue between different microorganisms, and this pattern is laid down early, and, in the specific kinds of polysaccharides, it is fairly early phylogenetic development, but there may be great variations in the protein polymerization.

Dr. Meyer: There must be genetic control of the polysaccharides produced since, in different types of connective tissue, one finds different polysaccharides, whether 4 sulfate or 6 sulfate, whether B or keratosulfate, and so on. There obviously must be some genetic control which determines that, not only for the different tissues, but at different times in differentiating tissues.

Dr. Dorfman: But I think this control may be of a different kind; this may be a second level of control, not informational but, rather, enzyme induction or inhibitor or all the various other things which are now popular with all of us who read the *Scientific American.*

Dr. Meyer: I would also like to ask a question of Dr. Dorfman; namely, he showed us his oligosaccharide which contained galactose, attached, presumably, to the protein and polysaccharides. I don't know whether the galactose forms the glycosidic bond to the serine or where the galactose is. I assume your data are based on the anthrone reaction and/or chromatography. However, in embryonic cartilage, at least there is no non-dialyzing material, after digestion with testicular hyaluronidase which gives hexose reaction.

Now, this is one point. The other point of disagreement which I have with what Dr. Dorfman said concerns chondroitin sulfate B. This is unpublished work which Miss Anderson of our laboratory has been doing, in particular. Using the same procedure, which avoids alkali, and using proteolytic digestion (we use panprotease as a rule) followed by fractionation and digestion with bacterial proteolytic enzymes, we find that the chondroitin sulfate A and chondroitin sulfate C fractions isolated from any tissue have amino acids attached to them, unless you treat them with fairly strong alkali. In contrast, chondroitin sulfate B of skin (mainly from pigskin) is free of amino acid after hydrolysis with 6 normal acid.

Now, the evidence, of course, is overwhelming that chondroitin sulfate B occurs as a protein complex. We have tried to get this protein complex into solution, to extract it by various procedures, by water, by salt solution, by urea, by calcium chloride, and what not, and we have never succeeded. It is insoluble.

I was very much surprised when Dr. Dorfman said that his chondroitin sulfate B, which was also derived from skin, but human skin, as I remember, had amino acid in it.

Dr. Dorfman: Let me take these questions one at a time. As far as the galactose is concerned, it is important, Karl, to note that these preparations are different from yours. Using the kind of material that Dr. Gregory and Dr. Rodén have prepared, when a large amount of chondroitin sulfuric acid chains has been removed and you are down to something like a tri- or disaccharide, the analytical situation as to the amount of galactose is very different from the case in which you hydrolyze chondroitin sulfuric acid. If there were one residue—and I'm not claiming there is one residue—for a 50,000 molecular chain, it would be a very hard thing to find, but when it gets down to one galactose with only two uronic acids and one hexosamine, it is quite a large amount, and this shows up by anthrone, by phenol, and by paper chromatography, as you surmise.

As for the other question about the chondroitin sulfuric acid B, again, I would have to compare notes with you on methodology. As you saw from the nitrogens that I showed, the amount of excess nitrogen here is very small. Don't forget, the slides I showed were in terms of amino acid residues per 100 galactosamine residues, so this is not very many of the amino acids. Nevertheless, they become very important.

Now, it is not only in the human skin preparation that I showed. A very pure

preparation that Dr. Schiller has prepared from rabbit skin shows an amino acid pattern similar to that shown; and in a preparation purified from the material that was furnished to all of us by Dr. Winterstein, there were also amino acids. I would say, therefore, that our experience is different from yours, but I wonder if it isn't a methodological difference we are talking about.

Dr. Meyer: These data are not based on nitrogen analysis, but on hydrolysis and two-dimensional paper chromatography, in which we can very well distinguish, of course, the amino acid pattern in A and C.

What we have usually considered pure C contains some amino acids. After the work of Schubert and others we probably can no longer consider these fractions "pure" mucopolysaccharides. This discrepancy will remain, I'm afraid, because it is not based on nitrogen.

Dr. Dorfman: We would both agree that it occurs as a complex, but we can find the amino acids and you cannot.

Dr. Schubert: Can I get into this fight, too? Dr. Rosenberg in our laboratory has been doing some work on extraction of cartilage, not from embryos, as you have, Dr. Meyer, but from very young children. He finds considerable amounts of glucosamine in the preparations that he gets. The amount of glucosamine was of the order of 20 per cent of the total hexosamine.