DISCUSSION

Dr. David J. Hamerman (New York), *Chairman:* Dr. Dziewiatkowski presented some data on the turnover of hyaluronate and chondroitin sulfate in skin, and Dr. Sara Schiller from the University of Chicago has kindly consented to discuss some of these aspects.

Dr. Sara Schiller (Chicago): We don't feel that the decay curves tell us the entire story about turnover of mucopolysaccharides under the influence of hormones. Recently we determined the concentration of mucopolysaccharides under various hormonal conditions.

In the alloxan diabetic animal we find lower than normal concentrations of hyaluronic acid and chondroitin sulfate. Concentrations of hyaluronic acid and chondroitin sulfate have not been determined, as yet, in animals treated with adrenal steroids but the turnover of hyaluronic acid and chondroitin sulfate is slower than normal, and we suspect that the concentration of both substances is also less than normal.

In the hypothyroid animal the concentration of hyaluronic acid is increased whereas that of chondroitin sulfate is decreased. When thyroxine is administered the concentration of both mucopolysaccharides reverts toward normal levels.

In the hypophysectomized animal, we find the same picture as in the hypothyroid animal: The hyaluronic acid concentration is increased, and the chondroitin sulfate concentration is decreased. When growth hormone is administered to these animals, the concentration of hyaluronic acid increases markedly. Our normal values run about 800 micrograms of hyaluronic acid per gram of dry skin. In the hypothyroid and hypophysectomized animals, they run about 1200 to 1400 micrograms of hyaluronic acid, and in the hypophysectomized animal treated with growth hormone, they run about 2000. Under the influence of growth hormone, the level of chondroitin sulfate is restored to normal.

When the concentration of both mucopolysaccharides is decreased, as in the diabetic animal and in the animal treated with adrenal steroids, the defect may reside in the early stages of biosynthesis, probably at the level of glucose transport or phosphorylation. In the hypothyroid and the hypophysectomized animal, a defect higher in the biosynthetic pathyway is anticipated.

It appears that UDPGA and UDP-*N*-acetyl glucosamine are the precursors for hyaluronic acid. Jacobson and Davidson have isolated, from the skin of rabbits, an isomerase that converts UDPGA to UDP iduronic acid, and another isomerase

that converts UDP-*N*-acetyl glucosamine to UDP-*N*-acetyl galactosamine. Both are dependent on DPN and inhibited by DPNH. It is possible that the increase in concentration of hyaluronic acid found in the hypothyroid rat is due either to an increase in DPNH, which inhibits the conversion of the UDGPA to iduronic acid, or to a defect in activity of both isomerases.

Dr. M. M. Kini (New Haven): I will address this question to Dr. Holtzer. In your spinal cord or notochord experiments, is the morphologic integrity of the tissue necessary or does the dialyzed homogenate cause a similar effect on chondrogenesis?

Dr. H. Holtzer (Philadelphia): If the notochord is cultured by itself for approximately 10 hours it loses its tubular configuration. If somites are added to such a collection of notochord cells, cartilage is not induced. I have not worked with extracts which simulate the action of the native inducers.

Dr. T. T. Tsaltas (Philadelphia): I wish to refer this question to Dr. Thomas and Dr. Holtzer. We have been investigating a lipemia produced in adult rabbits by the intravenous injection of papain. During the study of this lipemia we observed that some of the animals receiving papain developed some very peculiar lesions in the aorta. In these lesions the aortic tissue becomes very rich in basophilic material.

Eventually the aortic tissue changes into cartilage and the cartilage then proceeds to become bone with well formed trabeculae. The center of such bone is filled with active bone marrow. Even though we are publishing these data (*Nature,* December 8, 1962) we have no explanation at present for this peculiar metaplasia of the aortic tissue.

I wonder whether Dr. Holtzer or Dr. Thomas could offer any explanation for this phenomenon; whether it has anything to do with the possible mechanism of the induction of cartilage formation or whether it could be explained on the basis of the studies that Dr. Holtzer has reported here.

Dr. Lewis Thomas (New York): I am puzzled too.

Dr. Hamerman: This is a very important question, and I would like to extend it a little further. Dr. Thomas, perhaps you have some thoughts on the relationship of vitamin A to metastatic calcification. If you administer vitamin A to your rabbits over a period of time, do they develop metastatic calcification, as might be the case in long continued administration of vitamin A to human beings?

Dr. Thomas: We have not seen it. I must confess that we have not done this for very long periods of time. We would like very much to find out. I am familiar with the lesions Dr. Tsaltas has in mind. He has shown some sections to me, and they are quite impressive.

Dr. Hamerman: The question is whether proteolytic enzymes, in degrading the protein moiety of polysaccharides, are, in effect, accounting for the turnover of the entire protein polysaccharide moiety, thus influencing calcification. Dr. Schubert,

do you want to expand on this for a moment? I know I'm taking you somewhat by surprise but, as someone else said, that is the chairman's prerogative.

Dr. Schubert: Well, we did some experiments a year or two ago that may have some bearing on this, but I'm not sure I understand them yet. The experiment is something like this:

You take calcium ion and phosphate ion in a buffered solution of pH 7, at such concentrations that you get a very light precipitate of calcium phosphate as a control experiment. You get a definite precipitate of calcium phosphate that you can measure.

Then in a similar situation you dissolve the calcium and the phosphate in a solution containing the protein polysaccharide compound. You can let such a solution stand for days and nothing will precipitate. Of course, at first, we thought it was simply due to the fact that the polyanion would decrease the calcium ion activity, so that you would get no precipitation, but this is not the case, because if you add the equivalent amount of chondroitin sulfate alone, not the protein compound but chondroitin sulfate, then you get the precipitation again.

On the other hand, if you take the protein polysaccharide compound and mix it with the calcium phosphate and let it stand for days, you get no precipitation. If you add either trypsin or hyaluronidase, you again get immediate precipitation. This was rather intriguing in connection with the whole process of endochondral calcification; at the time of disappearance of the protein polysaccharide compound from the endochondral plate in the area where the big cells are, that is where calcium phosphate seems to be deposited, not as bone but as calcification.

That is a piece of experimental evidence which, as I say, I don't know how to interpret. We are beginning to think of it in terms of what I talked about a little this morning—excluded volume—that you may have areas of incipient crystallization, but the crystals can't get together.

Question: I would like to come back to the interesting data of Dr. Holtzer. There is another instance in which cartilage cells lose their roundness, and that is in the hypophysectomized animal, a hypophysectomized growing rat. They become flat, I guess, because of the morphologic arrangement. One of the first effects of injection of growth hormone is that they become round again.

I wonder whether this would not also explain your earlier experiments in the basement membrane, I think it was, with the somites; that is, we know that nerve cells in the primitive stage produce polysaccharides in a very simple pattern. The polypeptide has, in effect, the same effect which growth hormone has at a later stage.

Dr. Holtzer: At this point I accept anything as possible, for I am quite bewildered with the problem of induction on the molecular level. As long as we were dealing with tissues there was an operational definition of induction. When two tissues were brought together tissue B did something it would not unless it reacted

with tissue A. As you descend to the molecular level the term as used on the tissue level gets lost. But once again, almost as a matter of esthetics, I would predict that the spinal cord and notochord do not act by putting information (*i.e.*, protein or RNA) into reacting somite cells.

Dr. David S. Howell (Miami): I would like to ask Dr. Dziewiatkowski about the radioautograms on normal suckling animal epiphyseal cartilage, for S^{35}-sodium sulfate. The S^{35} was not incorporated into the bulk of the epiphyseal plates, in those sections treated with barium hydroxide, but it appeared that there was a substantial uptake of S^{35} at the metaphyseal junction. Might not sulfate concentrated at this site represent more than a simple exchange with extracellular fluid inorganic sulfate? Could it be released, for example, by a sulfatase from chondroitin sulfate?

Dr. Dominic D. Dziewiatkowski (New York): I believe that the S^{35} which one sees in the metaphyses (lower series of autoradiograms in Fig. 6), very shortly after the administration of the isotope as sulfate, is probably a reflection of the inorganic sulfate which has been incorporated into the mineral phase of the spicules.

The progressive increase in the concentration of S^{35} in the metaphyses, subepiphyseally, which one sees after 24 hours, however, is a reflection of chondroitin sulfate-S^{35} because of endochondral ossification. In other words, the latter S^{35}-labeled material was synthesized in the epiphyseal plate and is retained in part as the cartilage matrix is calcified. Indeed, the strata of S^{35} in the metaphyses of estrogenized rats, repeatedly dosed with S^{35}-sulfate (Fig. 11), are in accord with this suggestion. It was noted that the last dose of S^{35}-sulfate, given 24 hours before removal of the bones, was reflected in the high concentration of S^{35} in the epiphyseal plate; relative to the latter, the concentration of S^{35} in the region of the metaphysis immediately under the epiphyseal plate was low. At some distance from the plate, however, there was a stratum of S^{35} in the metaphyses as a result of the administration of S^{35}-sulfate to the rats a week previously, and at some distance from this stratum, in the direction of the medullary cavity, there was another stratum of S^{35} as a consequence of the administration of S^{35}-sulfate 2 weeks previously.

Question: In the effect of estradiol S^{35} uptake of the bone, are there any data to indicate whether or not this is being mediated through the hypophysis, in particular, by ACTH, or prothrombin inhibition?

Dr. Dziewiatkowski: Although the uptake of S^{35}-sulfate by cartilage is decreased in hypophysectomized rats, it is decreased even further if the rats are given estradiol benzoate.

Question: Then, this would be a direct effect?

Dr. Dziewiatkowski: Yes, it seems that the effect of estradiol *in vivo* is not mediated through the pituitary.

Question: Is there any information or data reflecting the S^{35} uptake of the thoracic aorta, and how that is affected by estrogen?

Dr. Dziewiatkowski: It is decreased.

Question: By estrogen?

Dr. Dziewiatkowski: Yes, the uptake of S^{35}-sulfate by the thoracic aorta is also decreased in estrogenized rats. However, although this effect of estradiol on cartilage is demonstrable within 3 days of treatment, it is necessary to pretreat the rats for 3 weeks before the effect is seen in the aorta.

Question: Are any of these physiologic doses or have all these been high?

Dr. Dziewiatkowski: In the experiments which were described here the doses were massive. We used 2 mg once a week and Priest and his coworkers have been using 0.33 mg daily, 6 days out of the weeks, for a period of 3 or more weeks. That is not to say that at lower doses qualitatively similar effects cannot be discerned. They are discernible but are not as striking as at the higher, very large doses.

Dr. Romano Humberto de Meio (Philadelphia): Dr. Thomas, we understand that vitamin A, from what we heard, seems to inhibit the deposition of the metachromatic-staining substances and that the same effect is seen from cortisone. Now, the combined effect seems to be an inhibition by cortisone of the effect of vitamin A. I wondered how this can be explained.

Dr. Lewis Thomas: I think the fact is that vitamin A is not inhibiting the deposition of the metachromatic material. It brings about its removal, presumably, by causing the release of enzymes in the tissue and of properties similar to those of papain. Cortisone blocks this, presumably, by an effect leading to stabilization of lysosomes. This property of cortisone is quite separate from, and perhaps altogether independent of, the well known and established property of cortisone to inhibit the synthesis of chondroitin sulfate or chondromucoprotein. They are quite separate, and indeed they involve different doses. It takes a large dose of cortisone, in treating a rabbit, to block the effects of the vitamin A, and a similar dose, following the papain experiment, to prevent the reconstitution of the cartilage matrix. But they are, I think, separate events.

Dr. de Meio: Yet I remember, in your slide, the tissue of the animals treated with the combined vitamin A and cortisone still showed metachromatic staining.

Dr. Thomas: Oh, yes. The degree of metachromasia is that of the normal animal. These are short term experiments. You treat with cortisone and administer vitamin A and study the animals for the next 3 or 4 days. This is far too short a time for cortisone treatment alone to have any discernible effect or cartilage effect.