DISCUSSION

Question: Dr. Gross, in your demonstration of this collagenolytic activity, you assume, or the explanation assumes, that the activity is hydrolytic in nature. Is there any reason to suppose that it is so?

Dr. Gross: We believe it is hydrolytic because this protein, with a molecular weight of 300,000, is largely broken down to dialyzable fragments, at physiologic pH and temperature, with the release of large numbers of *N*-terminal groups. Depolymerized or gelatinized collagen is non-dialyzable.

Question: Particularly in studying protein synthesis, there is considerable evidence that the protein, in a different position, goes to amino acid derivatives; it is sufficient, I think, to recall the story of glycogen, where it was assumed that the process was purely hydrolytic, but eventually it proved that there were several intervening stages, which were not of a hydrolytic type.

Dr. Gross: I am afraid I do not see the relationship with glycogen hydrolysis. As I mentioned, most of the collagen is broken down to peptides with release of amino groups. This means hydrolysis of peptide bonds.

Dr. Leon Richelle (New York): When you showed the various types of cross-linkage between alpha chains, as for instance between alpha 1 and alpha 2 chains, you did not show any association between two alpha 2 chains. Is there any reason for this, or any explanation of the fact?

Dr. Gross: None of the several laboratories which have been intensively studying this problem has ever found a dimer of alpha 2.

Dr. Marvin R. Dunn (Philadelphia): Dr. Gross, in your explants of skin from the tadpole tail, do you know which cells are producing collagenase?

Dr. Gross: Living cells are required for production of collagenolytic enzyme in culture. Freezing the tissue, which presumably kills it, abolishes ability to produce the enzyme. Whether the producing cell is completely intact we do not know.

Dr. Zacharias Dische (New York): What is the level of hexose in collagen and do the additional components about which you have speculated give an anthrone reaction?

Dr. Gallop: The anthrone reaction on purified collagens is given by the hexose components which are in general galactose and glucose. The results of Gross and his coworkers and the Grassmann group and others have shown this. Dr. Blumenfeld in our laboratory is investigating the quantity and mode of attachment of these hexoses to the protein (see below).

In addition, there are several components which are not detectable with anthrone

and which are probably not impurities. After deesterification an aldehydic component (about 3 to 6 per α unit) can be detected and released from the proteins upon treatment with mild acid. Spectrophotometrically, about one keto-acid group per α unit (approximately 100,000) can be detected with 2, 4-dinitrophenyl hydrazine, apresoline (2-hydrazinophthalazine · HCl) or *N*-methylbenzothiazolone hydrazone. There is also a component (perhaps a degradation product of the above) which upon direct treatment with thiobarbituric acid gives a small amount of 532 mμ chromogen; this chromogen was first observed by Landucci and his coworkers.

Dr. David J. Smith (Albany): You have speculated that transesterification might take place in the maturation of collagen. In my opinion this is probably an enzymatic process. Do you agree?

Dr. Gallop: We speculate that it is probably not enzymatic; this is based on Dr. Gross's results in which he can produce *in vitro* more β component in certain collagen fractions which at the time of isolation from the animal showed little if any β material.

We postulate that collagen when freshly synthesized has built in along each α chain 6 ester bonds occurring in three pairs. In each pair one ester is more reactive. This ester may transesterify intramolecularly to an adjacent α chain to form a β component or to a neighboring tropocollagen molecule (intermolecularly) to help form "insoluble" collagen. This is, of course, a speculation, but it offers an explanation of how progressive cross-linking in an extracellular fibrous component may result without the intervention of additional tissue components. This implies that the mechanism for a subsequent cross-linking process is already inherent in newly synthesized tropocollagen.

In lathyrism, Dr. Gross and Dr. Piez and their coworkers have shown the presence of a defect in the cross-linking process. The nature of all lathyritic agents suggests that they are carbonyl reagents and one can speculate that these agents may alter the synthesis or incorporation of aldehydic or other reactive carbonyl components into tropocollagen.

Dr. Melvin Levin (Syracuse): On the basis of the interesting data on the overlap of subunits (about 30,000), I would like to know whether these subunits occur in all strands which are later converted to insoluble collagen?

Dr. Gallop: We know little as yet about the nature and number of such collagen subunits as we propose. We find that upon deesterification with various reagents, the molecular weight seems to level at about 25,000 molecular weight, the intrinsic viscosity levels at a value of 0.18 from an initial value of 0.4. We know from the work of Dr. Piez and his colleagues that there are two α 1 chains and one α 2 chain, each about 100,000 molecular weight; it is reasonable that each α strand is composed of four subunits held in a linear array by three pairs of ester bonds. The nature and weights of these subunits are unknown but they average about 25,000 molecular weight based upon sedimentation equilibrium studies by Dr.

Blumenfeld. Since there are three α strands there could be as many as twelve subunits per tropocollagen. Since two α chains are perhaps identical, there can be as many as eight different subunits, but there are may be much less as repeats of subunits could occur.

Obviously, one must start with pure α 1 and α 2 components, deesterify and isolate, and characterize the subunits. This will be a difficult job but one which must be done.

Dr. Karl Meyer (New York): What is known of the attachment of hexose in collagen and its relation if any to cross-linking? Can you summarize your ideas on the nature of the aldehydic component in collagen?

Dr. Gallop: Dr. Blumenfeld has recently shown after isolation of the separated hexose components and the application of glucose oxidase and galactose oxidase, that there are about seven galactose and five glucose residues per ichthyocol tropocollagen (320,000).

From a careful investigation with galactose oxidase (this enzyme is also active on various galactosides) and with periodate oxidation on intact ichthyocol it was shown that galactose is attached to the protein by an *o*-glycosidic bond at carbon position 1, and that the 2,3,4, and 6 hydroxyl groups are free. Accordingly, galactose can *not* participate in cross-linking. With glucose, the attachment to the protein is (from results based on periodate experiments) also *o*-glycosidic at position 1 and the 2,3,4 hydroxyl groups are free and the 6 hydroxyl is probably also free. Accordingly, both hexoses are not directly involved in cross-linking and are most likely attached as monosaccharides to the protein. Most likely, the aldehydic component serves in the capacity of cross-link cementing material.

The properties of the aldehyde have been investigated by Dr. Paz, Dr. Blumenfeld, and me. It is very labile to both acidic and basic treatment. In acid, the aldehyde and various of its derivatives seems to dehydrate to form an α,β unsaturated aldehyde reminiscent of compounds such as aldol and β-hydroxypropionaldehyde. In addition, formaldehyde can be detected.

Under alkaline conditions in the presence of various trapping agents it fragments, possibly by a reverse aldolization in equal parts to acetaldehyde and to another still unidentified aldehyde. It is most probable that the aldehydic component is between an aspartyl group of one subunit (attached by an ester bond) and attached to another subunit as a glycoside or some type of acetal.

Dr. Meyer: My second question is to Dr. Gross, about his intriguing and exciting experiments on the tadpole tails. He calls this a collagenase. Is this a specific proteolytic enzyme? I mean, did you try denatured hemoglobin, which is usually the protein used as a substrate for your protease?

Dr. Gross: We have succeeded in isolating collagenolytic activity from that of proteolytic activity (on casein) by differential precipitation (Nagai, Lapiere, and Gross, to be published).

Dr. Meyer: Could you use your medium incorporating denatured hemoglobin, which is completely insoluble? You could see it more easily than you could see your collagen gel.

Dr. Gross: We have done it with fibrin. We have put the cultures on fibrin gels, and they produce a very small degree, some of them, of lysis in the fibril, but nothing comparable to what we see in the collagen.

Dr. Meyer: The mechanism is that the cells produce a diffusible proteolytic enzyme in addition to substances which cause the denaturation or hydration of the tight structure of the collagen monomers, or of the association of the molecules, which actually convert the collagen locally into gelatin which then would be the substrate of proteolytic enzyme liberated or secreted by the cells.

Dr. Gross: We have isolated a collagenolytic enzyme from lyophilized culture medium which had a pH optimum between 7 and 8, which is reversibly inhibited by EDTA, denatured by heating, and destroyed by trypsin. This material contains no low molecular weight substances.

Dr. Gallop: Didn't you try synthetic substrate?

Dr. Gross: Dr. Nagai is studying the specific requirements of this enzyme with synthetic peptides.

Dr. William L. Farrison: I might be repeating Dr. Meyer's question, but have you tried this collagenase, say, on slices of beef tendon, or something like this, to see whether it would attack native collagen? Franklin in England, I think, has used a different homogenate and got viscometric changes in reconstituted collagen gel at a different pH.

Dr. Gross: Franklin's homogenate from liver lysosomes acts on collagen at pH 4 and only to a limited degree. The animal collagenase we have works at physiologic pH, and degrades all the collagen to peptides.

Dr. Farrison: Perhaps Mr. Krol will come through with an answer.

Mr. Stefan Krol: I wanted to ask you this question in connection with the work of Franklin in England. Do you consider that these viscometric changes that they have observed—which amount at best to about 20 per cent, I think —are significant enough to conclude that there is an enzymatic action on collagen?

Dr. Gross: From their data, if I remember correctly, they are working with collagen solutions so dilute that the specific viscosity was 1.3, or very low; the fall in viscosity was quite small.

The criteria for collagenase activity require an undegraded substrate and physiologic pH and temperature. The substrate should be degraded to peptides by the enzyme.

Dr. Houck has isolated an enzyme from the pancreas, or from pancreatic juice, which seems to have a depolymerizing effect. Again, it is only a partial effect on extractable collagen, at neutral pH, I believe, but this is a very small effect, and it

is a peculiar enzyme, apparently, since it has a better effect in the cold than it has at body temperature.

Dr. John Fabianek: We found that the requirements for ascorbic acid were increased when the guinea pigs were fed a diet containing a high proportion of protein.[1] We found a similar result for guinea pigs maintained on a diet containing 2 per cent of tyrosine.[2] It appears that ascorbic acid is probably required for the metabolism of certain amino acids or at least of certain aromatic amino acids. Your results show that the biosynthesis of collagen is affected if the diet does not contain any ascorbic acid. Have you any information as to what would happen if you fed guinea pigs a diet containing a high proportion of protein or any amino acid in excess, conditions in which the requirements for vitamin C are markedly increased? Also do you know what happens if the normal diet is very poor in ascorbic acid (a case of chronic scurvy produced, *e.g.*, with 1 mg of ascorbic acid a day)? Would the synthesis of collagen change with time under these situations?

Dr. William van B. Robertson: I don't have the data to answer you, so I can't really say. Just in terms of the relatively large amounts of ascorbic acid that are needed by the animals, compared to other vitamin requirements, one might assume that it was used up in this way. But there are no data that I know of.

Dr. Ward Pigman: I am addressing this question to Dr. Gross. Recently, I came across a Japanese paper which claims that you can take cowhide, treat it with trypsin for a period, say of 24 hours, at room temperature, and then by exposing it to acetic acid you can convert the collagen quantitatively to acid-soluble collagen, which precipitates at neutral pH.

We actually carried out this experiment and got very high yields of a material that behaved in this fashion. The Japanese authors claim that the reconstituted collagen was actually in the form of normal collagen fibrils. We did not test that. But this doesn't seem quite to fit in with the picture proposed, and, if it is true, I wonder whether you have any comment on it?

Dr. Gross: Yes. In 1953, David Jackson described a similar effect of crude hyaluronidase on insoluble beef tendon. He thought it was the effect of hyaluronidase on mucopolysaccharides which were holding the collagen fibers together. He subsequently convinced himself that it was not the hyaluronidase but a proteolytic contaminant.

We tried to duplicate these experiments with crude hyaluronidase and with erepsin, an enzyme complex from the small intestinal tract, and also with crude trypsin. There seems to be some kind of enzymatic activity in these crude preparations

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[1] Fabianek, J., *Compt. rend. Soc. biol.*, 1956, **150**, 88. Fabianek, J., and Lehongre, G., *Compt. rend. Soc. biol.*, 1956, **150**, 520. Fabianek, J., *Arch. sc. physiol.*, 1961, **15**, 141.

[2] Fabianek, J., *Rev. Espan. Fisiol.*, 1960, **16**, suppl. 3, 131; Fabianek, J., *Ann. nutr. et aliment.*, 1961, **15**, 67.

which tends to make insoluble collagen soluble, in acetic acid. Highberger, Schmitt, and I isolated such an enzyme from the pancreas, also.

This may be significant since in lathyrism, and, perhaps, in the normal metabolism of collagen, as Dr. Robertson pointed out, there seems to be a return of the old fibrils to a soluble state.

It could be that an enzyme actually exists in the tissues which is capable of breaking the cross-links between the molecules in the fibrils, reducing those fibrils to a state where they are less loosely packed, and more susceptible to enzyme digestion. The state of "less loosely packedness" is measured by the solubility in cold salt solutions or in acetic acid. These observations may actually have some physiologic significance.

Dr. Schubert: Dr. Gallop, do esterases have any effect on your system to date?

Dr. Gallop: We haven't tried them. The speaker asked whether trypsin or chymotrypsin would work as esterases, but the point is that these are aspartyl esters, and I'm not sure that we know enough about the specificity of most esterases, or most esterases are not clean enough preparations to be able to interpret whether they would attack in part of the molecule without doing some proteolytic digestion at the same time.

Dr. Myer Fishman (New York): I have a question for Dr. Gross. I believe you said that the extract of your material had a low optical rotation and a low viscosity, but, upon cooling, the rotation increased. Is that correct?

Dr. Gross: No, not quite. If you denature collagen, the optical rotation falls precipitously, and then, if you renature it again, you get what is called "mutarotation." The optical rotation becomes more and more negative for a period of time. Then, eventually, it stops at some level below that of the native structure. But the viscosity does not come back. It returns in the gamma component, one in which the three polypeptide strands are cross-linked together; although they are randomized, they can't get away from each other.

If you quench this by cooling the gamma collagen, not only do you get restoration of optical rotation, which is due to the individual chain becoming a helical structure, but also the individual chains, because they are trapped together by the cross-links, will then wrap around each other and form a rod.

Dr. Fishman: Then, this is essentially a temperature-dependent phenomenon?

Dr. Gross: That's right.

Dr. Fishman: In systems of this kind, is there any possibility that you might obtain the same thing under isothermal conditions, something equivalent to a gelatin, or a sol-gel transformation?

Dr. Gross: It does happen under isothermal conditions. If you set the temperature at a particular level and let it stay, mutarotation progresses to a point, then stops. If you lower the temperature further, mutarotation will continue to another fixed level.

Dr. Fishman: Let me rephrase that. If you maintain the isothermal conditions, having achieved one set of reactions, can you then alter the condition by a pressure effect, for example?

Dr. Gross: I don't know.

Dr. Lewis Lukens (New Haven): I was wondering whether Dr. Gallop had any information on what happens to the alpha chains when you extract collagen from tissue at 90° with trichloroacetic acid. Would you expect these alpha chains to be split into subunits, or not?

Dr. Gallop: I don't know what would happen with trichloroacetic acid under your conditions. The *o*-glycosidic linkages just might break under those conditions at 90°C.

Dr. H. R. Catchpole (Chicago): I think this question should be addressed to Dr. Gross and/or Dr. Robertson. In the early work on collagenogenesis or cytogenesis of collagen, it seems that mucoproteins and/or mucopolysaccharides were rather strongly implicated in the question of the particular spacing that was attained. I would like to ask whether this is still considered to be an important feature of collagenogenesis?

Dr. Gross: If chondroitin sulfate was added to soluble collagen, you could get a long-spacing type of structure. In those days when we all thought that mucopolysaccharides were important in the formation of collagen fibrils, we thought this might be significant. But, then, after we purified the systems, we discovered that a whole host of completely non-specific things, ACTH, boiled TCA-treated collagenase, DNA, or RNA, almost anything picked off the shelf, could all do these things. They could precipitate collagen in various forms.

We concluded that the specificity lies in the collagen molecule. If there is a particular charge configuration in the relatively non-specific additive, this could cause the fibrils to form. We found that we could get perfectly good cross-striated fibrils without any additive, all from pure native collagen. There was no evidence for any hexose amine or uronic acid present at all.

We don't believe that mucopolysaccharides are involved in the formation of the fibrils from collagen molecules.

Dr. Robertson: Isn't there some work by Geoffrey Wood reported in the *Biochemical Journal* showing that the thickness of the collagen fiber could be influenced by the mucopolysaccharide present during precipitation? I believe chondroitin A and C, as well as keratosulfate, caused formation of thin fibers whereas other mucopolysaccharides were without effect.

Dr. Gross: Yes, there is. The size of the fibrils can be greatly modulated by a whole host of conditions, such as concentration of the collagen and the nature of the surrounding protein that might be present.

It is possible, certainly, that the mucopolysaccharides in the ground substance could regulate the rate at which molecules of collagen approach each other, simply

by alteration in the viscosity of the ground substance. This would change the rate of nucleation, which would alter the size of the fibrils. This would be a simple physical blocking factor which would be of physiologic significance.

The point I am trying to make is that in the formation of the striated fibril itself, the interactions between the collagen molecules do not need any kind of a midwife. The molecules are capable of doing this all by themselves.

We have felt that this is an important point. Structure can be formed, because the information is built into the building block itself. Perhaps the rate at which the structure is formed, its location, etc., may be determined by extraneous substances. The mucopolysaccharides play a very important role here.