Evidence has accumulated both for and against the idea that the control of the autolytic mechanism is in some way directly associated with oxidation-reduction levels in the tissue, and is mediated through sulphydryl compounds such as glutathione and cysteine. Grassmann and Dyckerhoff first showed the activating effect of —SH and HCN on yeast proteases (1). Shortly after, Waldschmidt-Leitz et al. (2, 3) reported a similar activation of mammalian tissue proteases. They found an activator which increased as autolysis proceeded, and this was later identified as glutathione (4). Both groups of investigators indicated that the effect of —SH compounds was to increase the range of catheptic activity. Thus certain proteins not digested before the addition of —SH, were digested in its presence. Others were digested as well before activation as after. Kleinmann and his associates (5–7) showed activation of tumor proteinases toward gelatin, but failed to detect it when such native proteins as casein, egg albumin, organ proteins, and the proteins of the tissue from which the cathepsin was obtained were used. Abderhalden and his associates likewise were unable to detect activation toward the native and tissue proteins used, but found gelatin more rapidly digested in the presence of reduced glutathione (8). Mayr and Borger (9) showed that HCN increases the catheptic digestion of various proteins, including gelatin, serum albumin, edestin, and Witte's peptone. Reduced glutathione was more effective, while a combination of glutathione and HCN was the most effective activator found. On the other hand Bierich and Rosenbohm were unable to find activation by reduced glutathione in tumor tissues (10).

Voegtlin and Maver (11) showed that tissue autolysis was inhibited by oxygen, while the removal of oxygen by nitrogen gas permitted more extensive autolysis. They were able to correlate these effects with the loss or maintenance of sulphydryl compounds reacting with nitroprusside. Voegtlin, Maver, and Johnson (12) showed that either protein hydrolysis or synthesis could be produced in an autolyzing tissue hash or by means of papain by properly adjusting the oxygen tension, and that the direction of the reaction was determined by the presence of —SH or —S—S—in the form of reduced or oxidized glutathione. Linderstrøm-Lang et al. (13–15) were unable to repeat this demonstration with papain and attributed the results previously reported to concentration of the digests by
the gas stream bubbled through them, or to cross-links formed on oxidation between the cysteine molecules of peptide chains which may produce compounds precipitated by trichloroacetic acid but which do not represent true protein synthesis. Maver and Voegtlin (16) have published further confirmation of the experiments on protein synthesis which indicates that the balance between substrate and products must be very accurately adjusted for this reaction to occur. Anson (17), working with a purified cathepsin, free from carboxylypeptidase, has shown that the enzyme as prepared is already active toward hemoglobin and that no further activation results by adding cysteine. The enzyme does not digest gelatin. He also showed that polylypeptidase is activated by cysteine. It becomes evident that gelatin, which has been so widely used as substrate material, may not indicate the presence of cathepsin, and that much of the reported catheptic activity is referable to the peptidases of the tissues. Maver has reported the preparation of a cathepsin which is activated by cysteine toward liver globulin (18).

More recently Fruton, Irving, and Bergmann (19) have presented evidence that cathepsin is a mixture of several different proteolytic enzyme factors which behave differently to certain pure synthetic substrate compounds and to activators such as cysteine. Just what the action of these enzyme factors is upon native proteins remains to be determined.

In view of the existing differences in the literature it has seemed to us profitable to reinvestigate the phenomena of activation and inhibition of the autolytic mechanism. In the present study we have concerned ourselves only with the changes which occur in whole liver digests. No foreign substrates have been added. Digestion, as measured by soluble nitrogen and the tyrosine color reaction, represents the action of the liver enzyme upon the liver proteins present. The results reported here are representative and typical examples and have been shown to be easily reproducible. We believe they are of significance in understanding the processes of atrophy, involution, and hypertrophy in the living tissue. Studies of the single purified enzymes of the system upon single pure substrate preparations will eventually be required to clarify the processes of autolysis. But in the meantime it seems desirable to know what happens in the natural substrate milieu of these enzymes as they are in the tissues themselves. Both lines of attack will supplement each other in clarifying the phenomena of changing protein mass in the living organism.

Effects of Varying Oxygen Tension on Liver Autolysis—Hog liver, obtained fresh from the abattoir, was ground to a fine hash and made up as a 20 per cent suspension in water with sufficient toluene to make 5 per cent of the final volume. Such a mixture was then homogenized in a Waring mixer, any inclusion of air bubbles during homogenization being avoided. The
creamy suspension was passed through a fine mesh sieve to remove shreds of connective tissue. Such a suspension may then be accurately sampled with an ordinary pipette. Digests were set up at a number of pH levels by the addition of HCl or NaOH. Rapid streams of oxygen or nitrogen, saturated with water and toluene vapor to prevent evaporation, were passed through these digestes in the cold for 15 minutes. The digestes were then placed in the thermostat at 38° and a slow stream of the gas bubbled through them as long as the experiment lasted. Reflux condensers prevented change in volume of the digestes. Samples were removed from time to time and precipitated with trichloroacetic acid whose final concentration was 5 per cent or approximately 0.3 N. pH values were read daily on the digestes with a glass electrode, and adjustments made so that each digest was maintained at the initial H ion level throughout the experiment. Digestion was determined by the soluble N₂ appearing in the trichloro filtrate, and the increase of tyrosine, according to the Kjeldahl and Folin-Ciocalteu techniques respectively. The usual series of digestes was set and maintained at pH 2, 3, 4, 5, 6, and 7.5. In several digestes in this series oxygen was bubbled through autolyzed mixtures which had been maintained anaerobic with a stream of nitrogen, in the hope of reproducing the synthetic effects observed by Voegtlin. In no instance were we able to discover evidence of protein synthesis under the conditions of our experimentation. Typical results are shown in Fig. 1.

The points of significance appear to us to be the following.

1. The difference between anaerobic and aerobic autolysis, while not large, is nevertheless significant and reproducible. Under anaerobic conditions, the rate of protein digestion was slower than under aerobic conditions. This is evident from the lower set of curves in Fig. 1, which represent anaerobic conditions, in contrast to the upper set of curves, which represent aerobic conditions. The rate of digestion was measured by the increase in soluble N₂ concentration and the increase in tyrosine content. The results indicated that the anaerobic conditions slowed down the rate of protein digestion significantly compared to aerobic conditions.

Fig. 1. The effect of aerobic and anaerobic conditions on the speed of autolysis at pH 4 (upper set of curves) and pH 7 (lower curves).
conditions the digestion rate is highest and the extent of digestion in the 10 day period is greatest. Aerobic digestion is least. In this experiment we confirm the results reported by Voegtlin and Maver (11).

2. The effect of anaerobic or aerobic conditions is perceptible at all pH levels. It is greatest where digestion proceeds at the optimum pH, and least where digestion is always small, namely at pH 2, 2.5, 6, and 7.5.

3. The nitroprusside reaction disappears in 24 to 48 hours. It is most persistent in acid reaction and under anaerobic conditions when digestion is also optimum. This confirms the observation by Voegtlin and Maver (11).

4. The H ion concentration of the digest is the dominant factor in determining the speed and extent of autolysis. The oxidation level modifies this result up or down to a relatively small but significant extent.

5. We were unable to reverse the action from protein cleavage to synthesis by passing oxygen through digests previously subjected to anaerobic autolysis.

Activation of Autolysis by —SH Compounds—In the following series of experiments we have added cysteine and other thiol compounds in order to determine their effects upon the over-all process of tissue digestion under fixed conditions of pH.

As before, digests were maintained at the initial pH levels throughout the digestion period by making the necessary adjustments at frequent intervals during the first 2 days. Nitroprusside tests were made from day to day. The naturally occurring reaction usually persists for about 2 days or less, while in digests to which cysteine has been added the reaction may persist several days longer.

The graph of a typical series resembles very closely the curves of control and N₂ gas as shown in Fig. 1. The addition of cysteine to the liver increases the speed and extent of digestion beyond that of the control at all pH levels tried and for the period of observation. While the effect appears to be upon the final level of cleavage attained and in the 10 day period is indistinguishable from that of the addition of some fragile protein like hemoglobin, the experiments were not carried on long enough to determine whether equilibria had been approximated in all cases. This point will be discussed later.

Similar increases of autolysis were produced by thiocresol and thioacetic acid. Thioglycolic acid produced no increase of autolysis but inhibited the reaction in proportion to the amount added.

Inhibition of Autolysis by Oxidants—Certain common oxidizing agents very rapidly abolish the naturally occurring nitroprusside reaction for —SH of freshly ground tissue. In this category are KIO₃, KIO₄, I₂, and CuSO₄. The first three are believed to oxidize —SH to —S—S— compounds
directly. Copper sulfate is said to catalyze the oxidation by oxygen. These oxidants inhibit the autolytic process at all pH levels tried. The degree of inhibition is determined by the amount of the oxidant added, within narrow limits, but even a very large excess does not completely arrest digestion. Inhibition may be counteracted by the addition of cysteine.

Since these four compounds give very much the same picture, we shall present data from one of them, KIO₃, as typical.

Effect of KIO₃—At pH 4 a preliminary trial showed that 0.75 cc. of 0.2 M KIO₃ in 30 minutes reduced the nitroprusside reaction in 100 cc. of the liver digest to a barely perceptible test. 1 cc. of the oxidant abolished the nitroprusside test completely. Cysteine increased autolysis over the control to a small extent. When increments of cysteine were added to digests in which the —SH reaction had been abolished, digestion was increased in proportion to the amount of cysteine added. In such digests the nitroprusside reaction was brilliant and persisted for 2 days or more. These results are shown in Fig. 2.

Certain other oxidants, when added to liver digests in amounts equivalent to KIO₃, have no effect upon the autolysis and do not abolish the nitroprusside reaction. The compounds in this category tried were KClO₃, KClO₄, and K₂S₂O₈.

There can be no doubt that there is a close correlation between the presence of —SH in a tissue digest and the speed and extent of autolysis at-
tained in limited periods such as 5 or 10 days. It is important, however, to know whether equilibria are being approached in 10 days in all digests, and whether these equilibria are artifacts due to destruction of the enzymes present or not. At pH 4 autolysis is ordinarily so rapid that digestion curves level off before the 10th day and represent substantially the final value of proteolysis. Further, it has been established (20) that the enzymes remain active for several weeks under control conditions, so that cessation of digestion indicates that all of the available substrate proteins have been digested, rather than that the enzymes have become inactive.

In the following prolonged experiment we have studied the behavior of cathepsin in digests treated with sufficient KIO₃ to remove immediately the nitroprusside reaction. The procedure was that previously described (20). A large 20 per cent liver hash was adjusted to pH 4. Sufficient KIO₃ was added to abolish the —SH test. From this primary digest were removed 100 cc. aliquots; they were treated with 2.5 cc. of 0.2 M cysteine solution and allowed to continue digesting. All digests were maintained at pH 4 throughout the experiment.

On the 34th day a secondary digest was set up with hemoglobin added instead of cysteine as an additional check on enzyme activity. The enzyme was found to be still active to hemoglobin (Fig. 3). In this experiment about 80 per cent of the total liver N₂ was solubilized in the presence of cysteine. In the first secondary digest activated immediately, equilibrium was approximated in 10 days. There was very slow digestion following that point and up to 20 days, at which time the reaction

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**Fig. 3.** The heavy curve represents autolysis inhibited by sufficient KIO₃ to abolish the —SH reaction. The lighter curves are for secondary digests activated at the times indicated by cysteine. The dotted curve represents the digestion of hemoglobin without cysteine activation. Activated and oxidized digests approach the same final level of digestion at different rates.
had become stationary. Later secondary digests did not quite attain the level of the first one, but the difference is not great enough to be certainly significant. All the secondary digests approximate 75 to 80 per cent of the total N₅ solubilized.

The primary digest proceeded very slowly but did not level off in the 50 day period. It is fair to conclude that digestion goes on more slowly in the absence of —SH but to the same ultimate level, if the time allotted is sufficiently long. From the slope of the later secondary digests it is apparent that the enzymes are gradually becoming less active and, in a very long digest of 60 days or more, might become wholly inactive. That all the cathepsin in the primary oxidized digest had not been destroyed, however, in 34 days exposure to the conditions set up is clear from the rapid digestion of hemoglobin added at that time, with no added cysteine.

DISCUSSION

So far as the over-all digestion of hog liver tissue is concerned, there can be no question but that the reducing level set up or indicated by cysteine leads to more rapid autolysis. The removal of cysteine as determined by the nitroprusside reaction, by such an oxidant as KIO₃, or merely by providing a high level of oxygen tension, decreases the rate of autolysis. In an extended period, however, the two reactions appear to approach an identical level of protein cleavage, as measured by soluble nitrogen and the tyrosine reaction.

Without more extensive data it will not be profitable to attempt positive interpretations at this time. Suffice it to point out that our results suggest the presence of two enzymes concerned in the primary cleavage of the tissue proteins. One of these is active whether cysteine is present or not, and in the absence of the activator autolysis goes on slowly to reach a cleavage level determined by the active masses of substrate and products. The second enzyme requires sulfhydryl activation. When this is provided, digestion is much more rapid, but the same final degree of cleavage is attained. Thus the effect of cysteine is to produce more active proteinase in the mixture rather than more available protein. This interpretation is in harmony with the results obtained by Fruton, Irving, and Bergmann working with small molecule, specific substrates, from which they conclude that splenic cathepsin is a multiple proteinase, some of whose factors require sulfhydryl activation, and some of which do not. It is also probable that the cathepsin prepared by Anson from spleen, which required no activation to digest hemoglobin, is a single member of this complex.

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