THE STUDY OF AUTOLYSIS BY PHYSICO-CHEMICAL METHODS. II.

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(Received for publication, May 17, 1910.)

In previous papers\(^1\) were reported the results of studies on the influence of the thyroid and kidney on post mortem autolysis of liver tissue at body temperature. These experiments were carried on by different methods; (1) by ascertaining the proportion of nitrogen which existed in different forms, (a) coagulable, (b) non-coagulable but precipitable by zinc sulphate in acid solution, (c) neither coagulable nor precipitable by zinc sulphate; (2) by determining the amount of depression of the freezing point; (3) by the rise in the electrical conductivity of the autolyzing mixture. The experiments as carried out failed to show that either thyroid or kidney extracts have any influence upon the rate of autolysis of liver tissue in vitro. Finding that the physico-chemical methods offered certain exceptional advantages as a means of study of autolysis, as contrasted with methods in common use, we have applied them in a number of experiments in order to become more familiar with their value and limitations; incidentally, observations have been made upon the rate of autolysis in different tissues, the availability of different antiseptics for the study of autolysis by physico-chemical methods, and the inhibitory action of blood serum upon autolytic processes.

INFLUENCE OF ANTISEPTICS UPON AUTOLYSIS.

It is generally understood that the rate of autolysis is more or less hindered by any antiseptic that may be added and that the ideal method is aseptic autolysis. Difficult or impossible as it is

Autolysis

to keep autolyzing substances free from bacterial growths for any length of time without the use of antiseptics, the difficulties are greatly increased when the autolysis is to be measured by physical methods, especially when it is desired to carry on the autolysis in a conductivity cell. Therefore it is necessary to use antiseptics in practically all experiments, and we first tried various antiseptics to test their availability. Toluene, which is most commonly used, is said by some not to be altogether reliable as a bactericidal agent, but there is usually no difficulty in preventing bacterial growth in fluids if they are shaken thoroughly with the toluene and then a layer of toluene kept on the top. Vandevelde\(^1\) summarizes the results of his investigations on this point as follows: Formalin destroys bacteria but prevents enzyme action, and the effect of phenol and alcohol is quite similar. Thymol is inefficient at body temperature. Toluene permits enzyme action but it is not altogether reliable as a bactericidal agent. Yoshimoto\(^2\) finds that within certain concentrations boric acid and salicylic acid increase the rate of autolysis as compared with chloroform, which we may believe is because of their acidity, since all acids in proper concentrations favor autolysis.\(^3\) Under the most favorable concentration the amount of conversion of the nitrogen of the liver into soluble form with several antiseptics in an autolysis of seventy-two hours duration, was as follows:

Chloroform water, 21.6 per cent; alcohol in 5 per cent solution, 32 per cent; mustard oil in one-eighth saturated solution 39 per cent; boric acid in 1 per cent solution, 40.8 per cent; salicylic acid in one-half saturated solution, 47.4 per cent.

Although salicylic and boric acids seem to be favorable antiseptics for autolysis experiments, yet the amount of crystalloidal substance introduced by the solutions of such concentration is so great as to obscure somewhat the relatively small effect on the freezing point and conductivity that is produced by autolysis; furthermore, the rate of autolysis in acid solution is not a fair measure of autolytic activity, in view of the great influence which weak acids have upon autolysis. For the purpose of physical

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\(^1\) Vandevelde: *Biochem. Zeitschr.*, iii, p. 315, 1907.
\(^3\) See Arinkin: *Zeitschr. f. physiol. Chem.*, liv, p. 192, 1907.
measurements it is preferable to use an antiseptic which does not ionize extensively and which dissolves to but a slight degree in water. Toluene, chloroform and thymol meet these requirements, and if an excess of the antiseptic is always present the solution remains saturated and so there is no change in concentration produced by evaporation. Several experiments were performed, all of which indicate that toluene depresses the autolysis less than the
other two antiseptics mentioned,¹ an extreme case being shown by
the accompanying diagram of the curve obtained in one experiment
(Fig. I). We found that the best results were obtained if water
was saturated with toluene at the temperature at which the experi-
ment was to be conducted, some time before adding it to the
tissue that was to be autolyzed. Toluene possesses one great ad-
vantage over chloroform in that the excess toluene covers the
surface and prevents any changes in concentration of the under-
lying fluid from evaporation, which might somewhat modify the
freezing point and conductivity.

THE APPLICABILITY OF PHYSICAL METHODS.

Probably the first to study enzyme action by the conductivity
method was Sjöqvist² who found that the hydrolysis of albumins
by pepsin was attended by a decrease in conductivity. Since his
work appeared the method has been only occasionally used. The
freezing point offers what is probably an even better method of
obtaining a long and full series of readings on autolysis, and was
first employed by Sabbatini,³ who studied the freezing point of
various animal tissues, but he did not study autolyzing tissues.
Fredericq⁴ Delrez⁵ and Liagre⁶ have used the cryoscopic method
for studying the rate of autolysis. Its advantage over the conduc-
tivity method is that it records the presence of non-electrolytes
as well as of electrolytes, and affords an accurate measure of the
total number of ions and molecules in the solution, while the con-

¹ R. Chiari: (Arch. f. exp. Path. u. Pharm., lx, p. 256, 1909) claims that
chloroform, alcohol and ether hasten the onset of autolysis by dissolving
the cell lipoids, thus destroying cellular membranes and permitting the
enzymes to have access to the protoplasm. This view is not in harmony
with the observations we have made, namely, that chloroform and toluene
depress autolysis, which is entirely in agreement with the experience of
others who have made comparative studies of aseptic and antiseptic autoly-
sis. It is impossible to tell, from Chiari's paper, whether his observations
were controlled by suitable experiments, but no mention is made of such
controls.

³ Sabbatini: Arch. ital. de biol., xxxvi, p. 440, 1901.
⁴ Fredericq: Bull. acad. méd. belg., Nov. 29, 1902.
⁵ Delrez: Arch. internat. physiol., i, p. 159, 1904.
⁶ Liagre: ibid., p. 172.
ductivity of the autolyzing solution is an index of the number of ions only. Consequently, when the enormous protein molecule breaks down into a number of simpler molecules the freezing point depression is increased in exactly that ratio, it making little difference whether the products of the hydrolysis are electrolytes or non-electrolytes. This gives the cryoscopic method a decided advantage over the conductivity method, for many of the products of protein hydrolysis, especially some of the mono-amino acids, have extremely low conductivities. On the other hand, comparison of the results obtained by the two methods may throw some light upon the nature of the substances that have been formed during autolysis. For instance, the conductivity does not record the carbohydrates and fats; hence, if in a given specimen the curves for autolysis by both the conductivity and the cryoscopic method are parallel, the increase is presumably due to an increase in the amount of dissolved electrolytes.

The conductivity of the isolated products of proteolysis in aqueous solution has been investigated by Walter Neumann\(^1\) in the case of the products of trypic digestion. Peptone he found to be strongly acid, and therefore to conduct well. Diamino acids conduct well, because of their basic character, as likewise do the strongly acid diatomic mono-amino acids (aspartic and glutamic acids.) The other amino acids, such as glycocoll and leucine, have an extremely low conductivity. Bayliss\(^2\), whose results with the isolated amino acids agree with those obtained by Neumann, found that the combined effect of the peptones and diamino acids formed in trypic digestion was sufficient to overshadow greatly the increase in conductivity due to the splitting off of inorganic salts from the protein. He also found that changes of internal friction are insufficient to account for the increase in conductivity which develops during trypic digestion. Gelatin produces a decrease in the conductivity of potassium chloride equal to 3.4 per cent of the original conductivity for each 1 per cent of gelatin added, and caseinogen decreases it 2.7 per cent; but sugar, a non-colloidal non-electrolyte, also produces a decrease of 2.8 per cent in conductivity for each 1 per cent of sugar added; therefore,

\(^2\) Bayliss: *Journ. of Physiol.*, xxxvi, p. 221, 1907.
the two effects are in the same order of magnitude. Moreover, gradually warming a gelatin solution so as to destroy the "gel" condition does not produce a sudden change in the conductivity. During tryptic digestion the change in conductivity is 100 per cent or more, and hence much more than can be accounted for by change in viscosity, and must be due to the products of protein cleavage.

Jackson and Pearce1 found that the proportion of nitrogen in different forms in the incoagulable nitrogen of dog’s liver changes during autolysis, as follows:

<table>
<thead>
<tr>
<th></th>
<th>Normal Liver</th>
<th>Autolyzed Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-coagulable nitrogen</td>
<td>9.7</td>
<td>29.1</td>
</tr>
<tr>
<td>Diamino acids</td>
<td>15.9</td>
<td>19.0</td>
</tr>
<tr>
<td>Monoamino acids</td>
<td>6.7</td>
<td>23.9</td>
</tr>
</tbody>
</table>

If previous to autolysis the liver was irrigated with Ringer’s solution, to remove the serum which inhibits autolysis, the increase in mono-amino acid nitrogen they found to be even greater. These figures show another reason why the cryoscopic method gives a better insight into autolytic changes than the conductivity method, for during autolysis the greatest increase is in the mono-amino acids which are for the most part poor conductors. On the other hand, this defect is offset to a greater or less degree by the fact that in autolysis, in contrast to tryptic digestion, there is extensive deamidization of amino acids and purines by amidases, and this results in the formation of ammonia and free organic acids or their salts, which are all good conductors.

The accompanying curves (Fig. II) illustrate the differences in the effects of autolysis upon the freezing point and the conductivity. In this experiment a portion of dog liver was combusted and the freezing point and conductivity of the ash were determined. Both are plotted as one base line, and the freezing point and conductivity of a sample of the same liver undergoing autolysis plotted on the same scale as the ash. The two curves show roughly that autolysis affects the freezing point much more than it affects the conductivity, this being due to the fact that many of the products of autolysis have a relatively low conductivity, and yet being soluble, exert their full influence on the freezing point.

Fig. II.

Freezing point depression of liver

Conductivity of same liver

Fr. pt. & conduct. of combusted liver

10 hours 20 30

Fig. III.

Freezing points of dog's tissues.

Blood Serum XIV

Defibrinated Blood XIII

Lung XII

Myocardium XI

Liver (serum) X

Kidney IX

Spleen VIII

Cerebrum VII

Hours →

2 4 6 8 10 12 14 16 18 20 22 24 26
Consideration of the curves in Figures III and IV, which have been plotted from several different series of autolyzing organs, also shows the differences in the form of the curves of freezing point and conductivity changes. It will be particularly noticed that in the case of the freezing point the most rapid and marked changes occur before the twelfth hour, after which time the changes are in most cases very slight. The conductivity, however, which rises relatively little in the first twelve hours, continues to rise steadily to the twenty-fourth hour, making the curve approach
more nearly a straight line. This fact indicates that at first the newly formed substances are chiefly non-conductors, but that later the new molecules formed are largely electrolytes. Probably the first, comparatively sudden, rise in both conductivity and freezing point depends upon the escape of the cell contents from the tissues and their solution in the fluid. This is followed by a rise due to the formation of sugar from glycogen, fatty acids and glycerin from the fats, proteoses, peptones and amino acids from the proteins, and nucleic acid and free purines from the nucleo-proteins; these substances are, for the most part, poor conductors, and hence the rise in the conductivity is relatively less than the rise in freezing point depression. Later there is introduced the deamidization of the amino acids and purines, which leads to the formation of ammonia from the amino groups, and organic acids from the amino acids, and as at this time the rate of disintegration of the large molecules has greatly slowed down we find the freezing point changing slowly while the conductivity continues to increase.

Again comparing the two sets of curves we find that there are certain differences between different organs. The kidney has a high initial conductivity compared with the other tissues, presumably because of the large amount of urinary salts present in its tubules; but its initial freezing point is the same as that of the liver, indicating that the liver is relatively rich in soluble non-electrolytes, which agrees well with our knowledge of the chemistry of these organs. The lung seems to be very poor in soluble materials, and such as there are must be electrolytes, for the $\Delta$ is relatively much lower than the conductivity; however, the rate of autolysis is about the same as that of the liver or kidney. Brain tissue shows relatively more increase in conductivity than in freezing point, indicating that the rate of autolysis is low, but that the molecules formed are mostly good conductors; presumably this is because the protein decomposition in autolyzing brain is relatively small, and the new molecules are chiefly such substances as cholin and fatty acids, which conduct relatively well. On the whole the rate of autolytic change, as measured by the amount of rise between the initial and final readings is about the same in the different organs whether measured by the cryoscopic or the conductivity methods, the liver and kidney leading, closely followed by the lung and spleen, while autolysis of brain and myocar-
The fact that the relative effects of autolytic changes on conductivity and freezing point are comparable with different organs, indicates that the results obtained by conductivity measurements have nearly as much value in comparative studies of the rate of autolysis as have the cryoscopic measurements, in spite of the fact that the latter give a more exact picture of the total change that is taking place in the tissue. Since studies of autolysis are in most cases comparative, the conductivity method can, therefore, often be used to advantage. This is particularly the case when the amount of material available is small, as, for example, in studying autolysis in a single small organ, such as the thyroid or adrenal. The autolysis may be conducted with one 10 c.c. sample in a small conductivity cell, and the readings made as often as desirable with the same sample. Freezing point determinations, however, should be made when possible on separate samples of 10 to 20 c.c. and hence a considerable amount of material must be available in order to secure a series of any considerable extent.

That the freezing point and conductivity measurements actually represent changes that are taking place in the tissues, and are not seriously altered by errors in technic due to the nature of the materials and methods, is well demonstrated by the curves obtained with blood serum and blood (see Figs. III and IV). Repeated readings of the freezing point show that these fluids undergo no appreciable change during twenty-four hours, and even much longer. The conductivity of the blood, indeed seems to fall slightly, the amount becoming quite appreciable if readings are taken for several days. Stewart observed this same phenomenon inconstantly in his experiments, in which precautions to avoid bacterial contaminations were not taken. In our experiments, which were controlled culturally, the decrease in conductivity was observed constantly. Stewart explains this change as due to laking of the blood; the hemoglobin which enters the solution then increases the internal friction, thus

2 Stewart: Journ. of Physiol., xxiv, p. 211, 1899.
reducing the conductivity. Possibly the hemoglobin also depresses conductivity by combining with the free ions. That hemoglobin is probably the cause of the decreased conductivity we have demonstrated by adding to dog serum pure hemoglobin, freshly prepared from dog's blood, and found that it decreased the conductivity considerably, as Stewart had previously shown. The decrease in conductivity which Sjöqvist observed during digestion of protein by pepsin-HCl, is probably also produced in a similar way by the proteins, proteoses and peptones combining with the free ions in the solution, and by the increased internal friction of the augmented number of colloidal molecules.

THE INFLUENCE OF SERUM UPON AUTOLYSIS.

In addition to blood and serum, cerebro-spinal fluid and thoracic duct lymph of the dog have been examined, and found to remain without any appreciable change in the freezing point or conductivity when left standing at 36.8 per cent under toluene for two days. It cannot be said whether this absence of autolysis in these fluids depends upon absence of autolytic enzymes or upon their inhibition by the anti-bodies the fluids contain.

That the serum of animals has a strong inhibiting influence upon autolysis is well known, and this effect is similar to its inhibiting action on tryptic digestion which was first shown by Hahn.\(^1\) Serum inhibits to a greater or less degree the autolysis of the fixed tissue elements, as shown by Baer and Loeb and others\(^2\) and also autolysis and heterolysis by leucocytes.\(^3\)

This inhibitory effect can be demonstrated well by conductivity and freezing point determinations, as shown in Fig. V. In these curves it is seen that during twenty-four hours there has been practically no change of conductivity in the liver emulsion in serum, although in water the increase in conductivity is marked, and what is particularly striking in this experiment is the persistence of the

\(^1\) Hahn: \textit{Berl. klin. Woch.}, xxxiv, p. 499, 1897.
\(^3\) Opie: \textit{Journ. of Exp. Med.}, vii, p. 316, 1905; Opie and Barker: \textit{ibid.}, ix, p. 307, 1907.
inhibition in spite of heating of the serum to 75° for thirty minutes. Opie found that the inhibiting action of serum upon autolysis of leucocytes is destroyed by heating thirty minutes at 75°, but not at 70°. Our results all indicate almost complete inhibition of the autolysis of dog liver by dog serum. Heating serum diluted with an equal volume of distilled water to 60° or 70° for one half-hour produces no visible change in the serum, but if heated at 75° or 80° it becomes more or less cloudy or even jelly-like; such jellied serum, however, retains, either unimpaired or but slightly reduced, its power to inhibit autolysis. Even serum diluted with four volumes of water and heated thirty minutes at 85° and 95°, when added to

1 Schryver (Biochem. Journ., i, p. 144, 1906) has found that this inhibition is not specific, as serum from one species of animals inhibits the autolysis of tissues of other species, and Opie and Barker observed the same lack of specificity in the inhibition of leucocytic protease by serum.
liver emulsion (400 c.c. diluted serum to 67 c.c. of 33 per cent liver
emulsion) was found either entirely to prevent appreciable changes
in the freezing point of the emulsion, or to limit the change to a
small fraction of the change taking place in liver emulsion without
serum. This thermostable character of the inhibiting action of
serum is in harmony with the results which were obtained by Baer
and Loeb; they found that liver autolysis was inhibited by serum
which had been heated to boiling, almost as effectively as by fresh
serum. This inhibitive property was found to reside chiefly in the
serum albumin, which, like the serum, inhibits autolysis equally
well after being heated. Serum globulin, when isolated and free
from albumin seems rather to accelerate autolysis, but this effect
is destroyed by heat. The entire inhibiting effect of serum, how-
ever, is not accounted for by these proteins, and Baer believes
that other unknown factors must enter into reaction. Although
the anti-leucoprotease of Opie also is found to reside in the
albumin fraction of the serum, its thermolabile property indi-
cates that it is entirely distinct from the thermostable substance
which inhibits autolysis of the fixed tissue cells. The so-called
"antitrypsin" of normal serum is also heat resistant.1 In con-
nection with the subject of inhibition of intracellular proteolytic
enzymes by serum, it is of interest to observe that a specific
intracellular oxidizing enzyme, uricase, seems not to be inhibited
by serum.2 Similarly Baer has found that deamidization can go
on in the presence of serum which inhibits earlier stages of pro-
teolytic disintegration, but he did not ascertain whether the
new-formed ammonia was derived from amino acids or purines.
This fact may be taken as evidence in favor of the view that
autolysis does not take place in normal cells under normal con-
ditions, since known normal intracellular enzymes are here found
not to be inhibited by serum, while the intracellular enzymes of
protein destruction are held in check by normal serum.

Longcope, studying the histological changes in autolyzing liver
tissue, found that the inhibition of nuclear and cytoplasmic dis-
integration which is exerted by serum, is not impaired by first
heating the serum at 65° for fifty minutes. When heated at 85° for

2 Wells and Corper: this Journal, vi, p. 321, 1900.
twenty or thirty minutes, however, the inhibitory effect was greatly reduced as contrasted with unheated serum; no comparison of the heated serum with salt solution is mentioned. Wells, who studied the histological changes produced by autolysis in the kidney, found that in the presence of serum the rate of nuclear disintegration was reduced to about half the rate observed in kidney tissue kept in salt solution, while the disintegration of the cytoplasm was almost entirely inhibited by the serum. This fact suggests that the intracellular enzymes which act upon nucleo-proteins are inhibited less by serum than are the enzymes which attack the simpler proteins of the cells, a view entirely in harmony with the results of chemical studies. Heating the serum to 80° for thirty minutes (removing the coagulum by filtration) greatly reduced its power to inhibit autolytic changes in the cell structure, although the changes in this menstruum were always somewhat less rapid than the changes that took place in similar tissues kept in salt solution.

From the above observations it would seem that the thermostable element of serum which inhibits autolysis of liver tissue is distinct from the thermolabile anti-leucoprotease described by Opie. However, there seems to be a discrepancy in the results obtained when the autolytic changes are measured by physical or chemical means, and when they are measured by the histological changes which take place in the autolyzing cells.

**GENERAL CONSIDERATIONS.**

We believe that these experiments have, above all, indicated the value of the results obtained by physico-chemical methods in the study of autolytic processes. Not only do these methods permit of a much greater number of determinations being made in less time with less material, and with less labor and expense, but they are also of far more value in giving an idea of the amount of change that is taking place in the autolyzing tissues, than are the chemical methods in common use. Autolysis comprises the disintegration of the cell components, and involves a great number of chemical substances, some of which are coagulable proteins and many of which are not. The customary measure of autolysis is the change in the amount of nitrogen contained in forms coagulable and not coagulable by heat—a measure which is very misleading and of com-
paratively little value for many reasons. In the first place it takes into account only the autolysis of the nitrogenous constituents of the cell which are either insoluble or heat coagulable. Secondly, it measures only a single step of the many changes that take place in these particular cellular elements—namely, their loss of coagulability; this is certainly a very inadequate measure of tissue disintegration, for it gives the same result whether a coagulable or insoluble protein is merely converted into soluble proteoses or whether the most extensive disintegration to the ultimate amino acids has been accomplished. Thirdly, the amount of connective tissue in the organ under investigation may modify greatly the amount of coagulable nitrogen, since during the heating and extraction the collagen will be converted into gelatin and will be estimated with the incoagulable nitrogen equally whether it has been attacked by the enzymes or not. Fourth, it takes no account of the extent of disintegration of the all-important nucleo-proteins, for after the first step of this disintegration is accomplished the products are soluble, and so all the other important later changes in the nucleic acid and the purines are not shown by the nitrogen determinations.

On the other hand the determination of the freezing point gives us an absolute, delicate and reliable measure of disintegration of the tissue, since every step of this disintegration results in the presence of more ions and molecules, no matter whether it is the proteins, the carbohydrates or the fats of the cell that breaks down. A freezing point curve is, therefore, a correct picture of the change that is taking place in the autolyzing tissue, which a non-coagulable nitrogen curve cannot well be. If we supplement the freezing point curve with a conductivity curve we secure information of qualitative changes that is of great value, for the conductivity curve indicates the increase of free ions, which we know are largely supplied by certain products of autolysis, while the difference between the two curves gives us a measure of the new non-electrolytes that have made their appearance as a result of disintegration of other non-electrolytes. The information obtained by these two methods of analysis is, therefore, not only more easily obtained but also much more instructive, and a much better indication of the actual amount of autolysis, than is the mere estimation of nitrogen in coagulable and non-coagulable form, even when in
addition we make determinations of nitrogen in proteose and peptone forms.

Inhibition of autolysis by normal serum concerns only the autolytic disintegration of certain of the cell constituents, particularly the coagulable proteins, and not all the enzymes which have to do with hydrolysis of cell constituents are thus inhibited. As the inhibiting effect of serum upon autolysis of the fixed tissue is not destroyed readily by heat, it is presumably different from the thermolabile constituent of serum which inhibits autolysis and heterolysis by leucocytic enzymes.
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J. Biol. Chem. 1910, 8:61-76.