LXVII. THE PRODUCTION OF AMMONIA AND UREA IN AUTOLYSIS.

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Since Jacoby [1900] demonstrated an increase of ammonia during autolysis, many workers have studied this question, using varying methods of estimating the NH_3, and obtained very conflicting results, especially on the effect of acid and alkali on the total NH_3 produced, and upon this NH_3 expressed as a percentage of the total uncoagulable nitrogen [Simon, 1910; Bostock, 1912].

The production of urea in liver autolysis, after much contradictory early work, was conclusively demonstrated only by Fosse and Rouchelman [1921], using the xanthydrol technique. They however made no study of the conditions, especially with reference to the NH_3 formed at the same time, and the relation between the two.

In the present work the NH_3 and urea formation in a number of autolysing organs has been studied, and, in some cases, simultaneous determinations of the increase in total uncoagulable and amino N made. The p_H has been controlled throughout.

Methods.

The autolysing digests were made up as follows. 100 g. of the organ, weighed after mincing, were ground fine in a large mortar with or without the addition of sand or ground glass. 25 cc. of toluene were then added, and the mush further ground till a pasty consistency was obtained. 100 cc. of buffer solution were added, the whole thoroughly mixed and transferred to a flask, the residue on the pestle and mortar being washed into the flask with 100 cc. more buffer solution, and well shaken, together with the further addition of 25 cc. toluene. The flask was then corked, and placed in an incubator at 38°. In some cases 50 or even only 25 g. of the organ were used, and the amounts of buffer solution and toluene correspondingly reduced. Using this technique the digests remain perfectly sterile. It is unfortunately necessary to employ some material to prevent bacterial growth, and toluene appears to be the disinfectant which has the least disturbing effect on those processes undergoing investigation, being neutral, insoluble and inert. Samples were withdrawn at once, and again at suitable intervals, coagulated by placing in boiling water, and filtered. A clear yellow filtrate was obtained upon which

the estimations were carried out. This method of sampling is quicker than precipitation with trichloroacetic acid, involves no dilution, and gives satisfactory results.

The buffer solutions used were those of Clark and Lubs, of p_H 2, 3, 4, 5·6 and 7·5. Owing to the buffering action of the tissue proteins and amino acids, the p_H actually obtained with each buffer must be estimated. This was done by the indicator method (using a comparator), on the filtrate on which the estimations were carried out. With the large differences in the series of p_H studied, this method is sufficiently accurate. The p_H of the digests made up in this way did not change appreciably during autolysis, and were as follows:

Table I.

p_H of buffer	p_H of filtrate
2	4·5–4·7
3	5 –5·2
4	5·4–5·6
5·6	5·9–6·1
7·5	6·6–6·8

The method employed for estimating the NH_3 was one devised by Foreman [1920], shortly to be published by him in greater detail and with improved technique. 5 cc. of the filtrate were placed in a steam distillation flask, with 45 cc. 97 % alcohol together with a few drops of phenolphthalein, and soda, approx. *N*, added until the alcoholic solution was faintly pink. Steam distillation was at once commenced into 20 cc. *N*/50 H_2SO_4. Methyl red was used as the indicator in the back-titration of the H_2SO_4 with *N*/50 CO_2-free NaOH. The method is extremely rapid and very accurate.

The urea was estimated by taking 5 cc. of the filtrate, adding 2 cc. urease solution (made by grinding 5 g. soya bean meal with 50 cc. 0·6 % KH_2PO_4, and filtering after 15 minutes), warming for 10 minutes to 45°, and then estimating the total NH_3 present. Estimations of urea by xanthydrol were made in many instances as a check and the two methods agreed very well.

Some of the material used was obtained from animals killed in the laboratory for the purpose (cat, rabbit, hen), but in the main it has been obtained from the slaughter-house immediately after the death of the animal. The effect of cold storage was tried, and found not to influence the results.

Effect of p_H on Ammonia and Urea production.

The curves of Fig. 1 are typical of a number obtained from ox spleen, the increase of NH_3 being shown below and of urea above. The increase of urea is often much greater than shown in this figure. The sharp optimum for both NH_3 and urea at p_H 6 is very marked. The curves obtained from the kidney (ox) are identical in general characters with the above, but differ in the following points:—(1) There is a much smaller increase of NH_3 and urea at every p_H. (2) There is a very much higher initial concentration of urea (from 6–12 times) due to small amounts of formed urine in the tubules. (3) The

increase of urea is confined entirely to the first two days, when it may be more rapid than that of the NH_3. (4) The NH_3 curves do not show such a sharp optimum at p_H 6, and resemble more the NH_3 curves of Fig. 2.

Fig. 2 represents one of seven sets of curves obtained from the liver during the winter. The marked differences from the spleen curves are at once apparent. Whereas the optimum p_H for NH_3 is the same but not so marked,

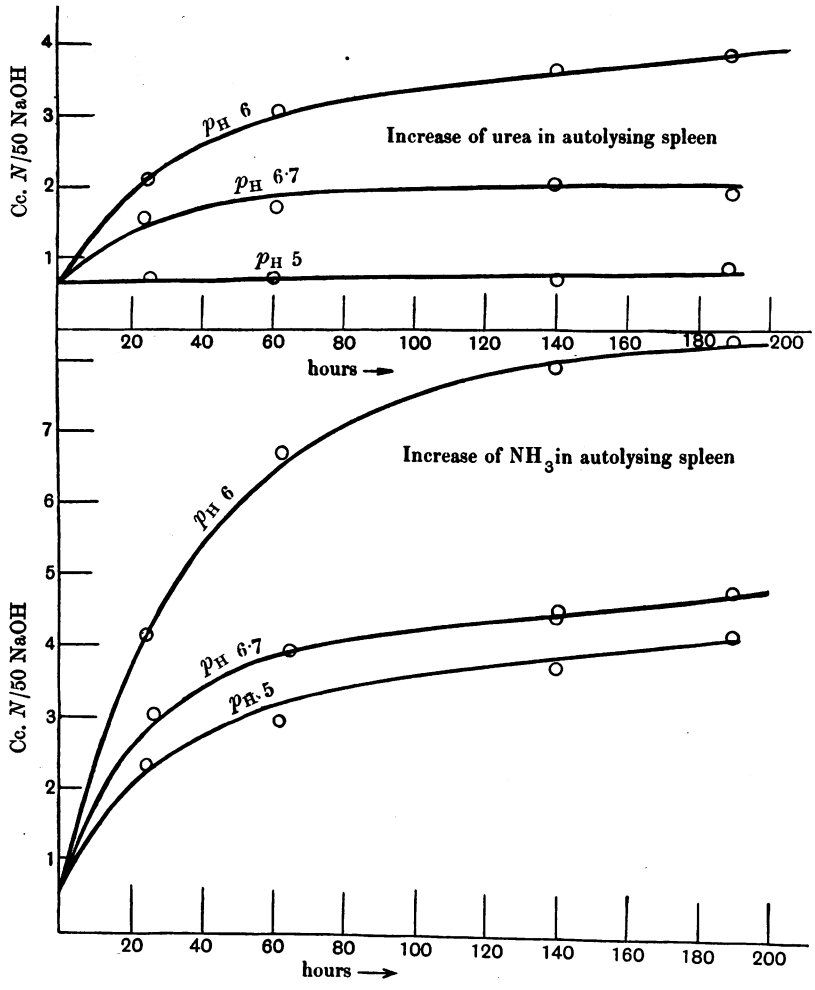

Fig. 1.

there is a greater production of urea on the acid side, at a p_H far from the optimum for NH_3. The urea increase at this p_H is more rapid at first than that of the NH_3, falling off almost to nothing after two days, while the NH_3 increase, slow at first, does not show the same falling off.

During the summer the NH_3 curves are unchanged, but the production of urea at p_H 5–5·4 falls to a value often lower than that at p_H 6. Six sets of

curves were taken in the summer, and no exceptions found, the summer ones all differing from the winter. Attempts to explain these marked differences have so far failed. It is not due to the youth of the animals killed during the summer (calves or lambs), and attempts to reproduce the differences by imitating in laboratory animals (rabbits) the summer and winter feeding of cows and sheep were failures. Seasonal variations in other enzymes [Burge and Leichsenring, 1922] have been described, and may afford the explanation in this case also.

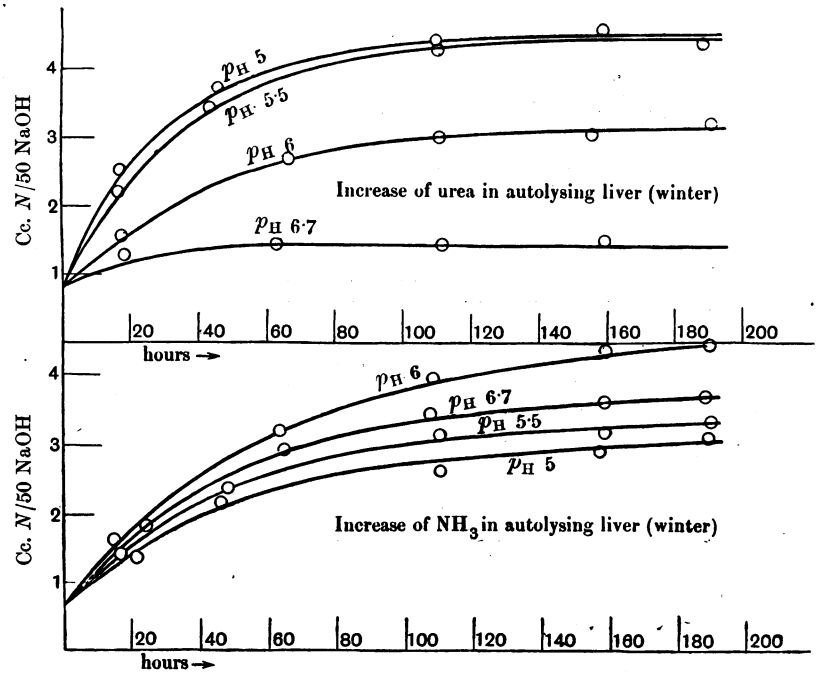

Fig. 2.

Effect of gaseous oxygen, hydrogen or nitrogen on production of Ammonia and Urea.

The effect of these gases on autolysis has been studied by Laqueur [1912], Bellazzi [1908], Morse [1916] and Shima [1922], the first of whom obtained marked inhibition of autolysis by oxygen measured by total soluble and amino N. He used no buffers, and often oxygen under pressure. On the other hand the other three did not obtain this effect, and Morse (estimating total soluble N) certainly took up the work with the idea that oxygen might inhibit autolysis.

Since it seemed that oxygen might increase urea formation by promoting oxidation of amino acids, and possibly other compounds, the effect of oxygen and hydrogen on autolysis, and especially urea formation, was studied in the following way. Two autolysing digests were made up at the same p_H, and,

usually, but not always, evacuated in Buchner flasks or desiccators. Oxygen and hydrogen were then led into each, respectively, from cylinders, after passing through water and toluene. The treatment was continued (with shaking) for about one hour a day for the first three days, and thereafter less frequently. An interesting observation, made repeatedly during these experiments, is the high resistance of reduced haemoglobin to such treatment, its spectrum being visible after several days, whereas oxyhaemoglobin very soon disappears. To ensure the genuineness of the results given below, one experiment was carried out with oxygen made by heating $KClO_3$ with MnO_2, with exactly similar results. A set of these is given below (Table II). Nitrous oxide was also tried, and found not to influence in any way the rate of autolysis, or the effect of oxygen on autolysis. The effect of carbon dioxide is only one of p_H; if this remains constant, this gas has no effect.

Table II. *Spleen Autolysis. Incubation* 120 *hours at* 37°. *All determinations were made on the filtrate after boiling and refer to* 5 *cc. of this filtrate.*

	Oxygen	Hydrogen
Total soluble N in cc. *N*/10 NaOH	27·25	27·25
p_H	5·9	5·9
NH_3 in cc. *N*/50 NaOH	8·4	8·7
Amino acid N, Sörensen titration in cc. *N*/10 NaOH	12·4	12·9
Urea in cc. *N*/50 NaOH	3	5·8
Urea in g. xanthydrol urea compound	0·015 g.	0·028 g.

The results are quite typical. The total soluble N is always the same, and also the p_H. The NH_3 is sometimes the same and sometimes as here a small percentage higher in the hydrogen filtrate. The urea is always very much greater in the hydrogen filtrate, and the amount of urea produced in an autolysis in hydrogen is the same as that in an autolysis with no gas present, so the difference is due only to an inhibition by oxygen, and not to a simultaneous acceleration by hydrogen. Nitrogen and hydrogen give identical results.

Effect of Adrenaline on Urea production.

The results of the experiments with oxygen led to a repetition of some work of Marie [1922], who found that the addition of suitable amounts of adrenaline to an autolysing liver doubled the amount of urea produced, and attempted to relate this to carbohydrate mobilisation and oxidation. Unfortunately his results could not be repeated either with buffered or unbuffered autolysing ox livers (trying several different p_H) or with varying concentrations of adrenaline; and his suggestion that the urea has its origin in carbohydrate oxidation in presence of NH_3 is rendered unlikely also by the experimental finding, that the effect of oxygen is to reduce, not to increase, the production of urea.

Independence of the sources of Urea and NH_3.

It might appear from Fig. 1 that the precursor of the urea formed was the NH_3. That this is not the case, however, is shown by a number of facts

and considerations. (1) If a balance existed between the concentrations of NH_3 and urea, subject to the laws of mass action, one would expect the ratio between them to remain the same throughout autolysis. This is not the case; and it is possible to alter the concentration of NH_3 or urea in the autolysing digest, either by adding the substances themselves, or suitable precursors, without altering in any way the rate of production of the other. (2) In the liver and kidney autolyses, the urea in the early stages increases more rapidly than the NH_3 at some values of p_H, but later more slowly, while the NH_3 curve is smooth and regular throughout. (3) The effect of oxygen, which may greatly reduce the urea production and leave the NH_3 practically untouched, also points to their separate origin. Their production may therefore be considered separately.

Production of Urea.

Unlike the increase of ammonia which is common to all autolyses to a greater or lesser extent, that of urea occurs only in certain organs. It has been found in all the mammalian livers tested (bullock, sheep, pig, rabbit), in all mammalian kidneys (bullock, sheep, pig), and mammalian spleens (bullock, cat). It has not been found in any mammalian pancreas (bullock, pig), lung, skeletal muscle (bullock), or heart muscle (sheep). Nor has it been found in avian liver (hen), which is interesting.

True optimum p_H for Urea Production.

Advantage has been taken of the fact that there is no mechanism for urea formation in the pancreas to study the optimum p_H for urea production, uncomplicated by the disturbing factor of the simultaneous hydrolytic cleavage which is inevitable during autolysis.

450 g. of spleen (ox) were sliced thin, and added slowly to 750 cc. of boiling distilled water. When cool, the tissue was minced and pulped with toluene, as described above, in the same water in which it was boiled, made up to approx. p_H 8 with Na_2CO_3, and 100 cc. pancreatic extract added. Other 100 cc. pancreatic extract were added later, and incubation continued 14 days at 38°, when the whole was made slightly acid, boiled, and filtered. The filtrate was now titrated to p_H 6, using a comparator, and some of the filtrate further titrated to p_H 7. Autolysing spleen digests were made up as described at p_H 6 and 6·8.

A. At p_H 6·8–7 into three boiling tubes:

(1) 20 cc. filtrate from pancreas digest + 0·5 cc. *N* NaOH } + 20 cc. autolysing spleen suspension at p_H 6·8
(2) „ „ „ alone
(3) 20 cc. autolysing spleen suspension at p_H 6·8 alone

B. At p_H 6 into three boiling tubes:

(1) 20 cc. filtrate from pancreas digest + 0·5 cc. H_2O } + 20 cc. autolysing spleen suspension at p_H 6
(2) „ „ „ alone
(3) 20 cc. autolysing spleen suspension at p_H 6 alone

The six tubes were incubated 48 hours at 37°, and then plunged into boiling water. When boiling, (2) and (3) in each case were mixed, and served as the control. After filtering the urea was estimated.

Figures after correction from the controls:

A. Urea formed at p_H 7 from substances in the pancreas-digested spleen, expressed in cc. *N*/50 NaOH per 5 cc. of filtrate ... ... 1·25 cc.
B. The same at p_H 6 ... ... 0·1

An exactly similar experiment on pancreas-digested liver, with liver enzymes, at p_H 5 and 7, gave the following figures:

A. Urea formed at p_H 6·8–7 from substances in the pancreas-digested liver, expressed in cc. *N*/50 NaOH per 5 cc. filtrate ... ... 4·45 cc.
B. The same at p_H 5 ... ... 1·4

A simultaneous autolysis of both spleen and liver at the same p_H showed, as always, a formation of more urea at the more acid p_H. There must be a series of reactions before the urea is set free, the observed optimum p_H (p_H 6) for urea production in autolysis being the reaction at which these several enzymes can best act; and this is quite in accordance with the work of Hedin [1922] and others, the enzyme termed by him "β-protease," and responsible for primary cleavage of the proteins, acting best at a p_H of 4–5, while the formation of urea according to these results is most rapid at p_H 7.

Possible precursors of Urea.

The mammalian, as distinct from the avian, liver is generally agreed to contain large amounts of arginase, which must account for some at least of the urea formed in such organs. Kossel and Dakin [1904] using isolation methods, Edlbacher [1915] and Fuchs [1921] using the Sörensen amino N titration method for estimating arginine, all failed to detect arginase in the mammalian spleen, and this, coupled with the fact that p_H 5 is the optimum for urea formation in the liver in winter, whereas at this p_H no urea whatever is formed in the spleen, points to the source of the urea in the spleen being other than arginine. The true optimum p_H for urea production is however the same in both spleen and liver. Arginase too was undoubtedly present in all the spleens tested in this work, in a concentration so low, that with the methods employed by previous workers it might not easily be detected. Arginase is present also in the kidney. A number of other conceivable precursors have been tried with consistently negative results, *e.g.* nucleic acid, thymine, cytosine, uracil, creatine and creatinine. The theory that the urea has its origin in the oxidation of glucose or amino acids seems untenable. There is evidence however that arginine is not the sole precursor.

The Rôle of Oxygen.

To elucidate this, the effect of oxygen on a pure arginine-arginase system has been most carefully studied. The experiments were carried out in part with the free base arginine obtained from the British Drug Houses, Ltd. (this was 90 % pure) and partly with a supply of arginine nitrate from

F. Hoffmann, La Roche & Cie, S.A., Bâle. The arginase was sometimes prepared by alcohol precipitation from strained liver suspensions. This method gives a dry powder which can be kept, but the alcohol precipitation must be carried out at a low temperature to prevent destruction of the arginase, room temperature being too high in the height of summer. The powder is ground with water and filtered before use. The method of obtaining an arginase preparation described by Edlbacher was also employed. This yields essentially a dilute suspension of the organ in question. The two methods in this case give identical results. The procedure was as follows: 5 cc. of a 0·4 % solution of arginine, 2·1 cc. of a solution of arginase, and eight drops of toluene were placed in each of two test tubes. Oxygen and hydrogen were led slowly through each respectively for 6–24 hours, and they were then transferred to the incubator at 38°. The extent of the action of the arginase was estimated by the urea formed. The results are summarised in Table III, the figures being the urea produced from 5 cc. of the arginine solution expressed in cc. $N/50$ NaOH.

Table III.

Organ	Type of enzyme	Time of treatment with O_2 and H_2 in hours	Time of incubation after passage of gas in hours	p_H	Urea formed	
					Oxygen	Hydrogen
Liver	Alc. ppt.	8	14	6·9–7	5·65	5·65
,,	Dilute suspension	12	24	,,	6	6·1
,,	,,	12	48	,,	6·5	6·5
,,	,,	24	0	,,	5·3	5·4
Spleen	,,	24	14	,,	1·25	1·3
,,	,,	21	14	,,	0·75	0·65
,,	,,	24	14	,,	0·45	0·35
,,	,,	6	24	6·0	1·9	1·8
,,	,,	6	24	6·0 and neutralised before incubation 6·0 and ditto	0·8	0·7

The table shows clearly that the pure arginine-arginase system is unaffected by a treatment with oxygen, which would undoubtedly suppress to a large extent the formation of urea in an autolysis.

The effect of oxygen is not an irreversible oxidation of the precursor of urea. This is shown by an experiment such as the following. Spleen autolysis in oxygen and hydrogen is carried out as already described, and the urea estimated in the filtrates after heating to 100° (A in Table IV). Dilute liver suspension is now added to each, and incubation continued 14 hours. After heating to 100° the urea is again estimated (B in Table IV). The controls have been deducted.

Table IV.

	A	*B*
Urea in 5 cc. filtrate from autolysis in oxygen	1·9	4·1
,, ,, ,, hydrogen	4·2	4·4

Although not converted into urea, the substrate in an autolysis in oxygen remains untouched, and may be converted into urea by the addition of fresh enzyme.

Autolysis of spleen at p_H 5, digestion of liver or spleen with pepsin and trypsin, autolysis in oxygen, and finally acid hydrolysis of spleen or liver, all give solutions, which when filtered and neutralised, are capable of acting as substrates for the urea-producing enzyme systems of the liver. The effect of oxygen can be shown in this way to be on an enzyme responsible for a part of the urea produced in autolysis.

Liver is ground up as for an autolysis at p_H 6, and strained through muslin into two small filter flasks which are evacuated, treated with the gas for some hours, and incubated at 38° for 12 hours; treated with the gas again for two hours, and incubated 12 hours more. The two suspensions so obtained form the enzyme solutions O and H (Table V) with which the following experiment was performed. The controls have been deducted, and the figures indicate the urea present in 5 cc. samples after 12 hours' incubation.

Table V.

					Urea
20 cc. neutral filtrate of autolysis at p_H 5	+ 2 cc. enzyme	O + 10	drops of	toluene	3·95
" " " "	" "	H + 10	"	"	6·65
20 cc. filtrate of autolysis at p_H 5 treated with "pancreatin" 60 hours	" "	O + 10	"	"	5·95
" " " "	" "	H + 10	"	"	7·5
20 cc. filtrate of spleen digested 4 days with pepsin and 10 with trypsin	" "	O + 10	"	"	3·2
" " " "	" "	H + 10	"	"	5·5
20 cc. neutral acid hydrolysis filtrate	" "	O + 10	"	"	(1) 2·2 (2) 4·3 (3) 4·05 (4) 3·6
" " "	" "	H + 10	"	"	(1) 2·1 (2) 4·4 (3) 4·55 (4) 4·

This experiment makes clearer the part played by oxygen in autolysis. It would appear also to be chemical not physical for nitrous oxide which is much more soluble does not produce any effect whatever. The enzyme whose action is suppressed cannot be Hedin's β-protease, or arginase. It is present in spleen, liver and kidney, as the urea production in all three is suppressed by oxygen. It is more active in neutral than acid solution, and appears to be inactivated more or less rapidly at p_H 5. This may account for its action not being apparent at all at this p_H in the spleen; and in the liver, the production of urea at p_H 5 is limited by the inactivation of the enzyme, for after urea production at this p_H has ceased, addition of fresh enzyme leads to further amounts of urea. Arginase [Hunter and Morell, 1922] has an optimum reaction at p_H 7, so that some of the effects obtained on the optimum p_H for urea production in filtrates from trypsin-digested organs must be attributed to this, but it seems that there may be two different enzymes at work simultaneously, and their effects are here additive.

The Nature of the Substrate of this Enzyme.

If this is not arginine in the free state, the other possibilities are (1) that the arginine is present in some combined form on which this enzyme acts, setting free the arginine, to be decomposed in turn by the arginase. (2) That there is some compound, probably so far undescribed, acting as the urea precursor.

If the former is the true interpretation, the compound containing the arginine must be an interesting one. It can hardly be a simple peptide, and the enzyme a kind of ereptase, for the action of the enzyme is so extremely selective, *e.g.* in an autolysis in oxygen the urea may be half the amount of its control in hydrogen, and the ammonia N and amino N practically the same. It is possible that the compound, if it exists, does not retain the arginine moiety by a peptide linkage. At all events it is not decomposed at all readily by trypsin (Table IV), for these digests all gave the bromine reaction for free tryptophan intensely.

The following experiments, however, point to the substrate not containing arginine. They are done on filtrates from spleen autolysis in oxygen and hydrogen, with a preparation of enzymes from a bullock's liver precipitated by alcohol (Fig. 3). Considerably more urea is produced from the filtrates of the autolyses done in oxygen, as already described, and if the concentration of enzyme is low, and incubation not too long, the enzyme becomes the limiting factor in this urea production. The technique is as follows. 5 cc. of the filtrate substrate, and five drops of toluene, are placed

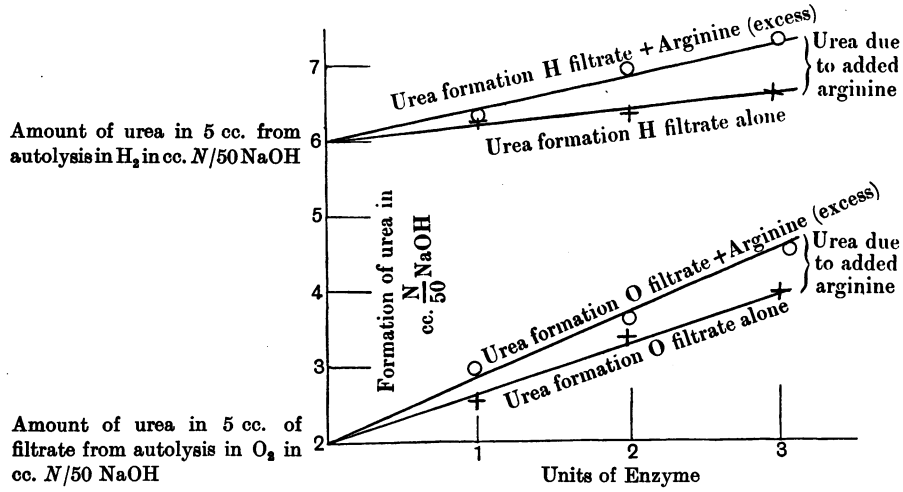

Fig. 3.

in each test tube. 1, 2, 3, etc. cc. of enzyme solution are added, as required, and all the tubes made up to a standard volume with distilled water. After incubation they are heated rapidly to 100°, and the urea estimated. The enzyme preparation contains both arginase and the enzyme which is suppressed by oxygen. If all the urea were due ultimately to arginine, on adding excess of arginine to all the tubes, the rate of urea production should now depend solely on the concentration of arginase and on nothing else, and the curves (Fig. 3) of urea production with excess of arginine present would be parallel. This is not the case at all, and the effect of adding arginine is to get an increased production of urea of the same order of magnitude in the filtrates, both from the oxygen and hydrogen experiments, as the figure shows.

It is not quite easy to see why the urea production from the filtrate from the autolysis in hydrogen, though much smaller than that from the filtrate from the autolysis in oxygen, should bear a linear relationship to the enzyme concentration, as it appears, from this and other experiments, to do. Apart from this difficulty the experiment points to the precursor of this urea being other than arginine.

Whatever the compound it appears to be destroyed at any rate almost completely by acid hydrolysis. This is shown by Table IV. The enzyme, which is suppressed by oxygen, appears not to have any substrate upon which to act after 30 hours' acid hydrolysis. Arginine is of course not destroyed by acid hydrolysis. The compound however must exist in a state of combination in the cell and be set free by the action of trypsin. This is shown by the consideration of the observed and the true optimum p_H for autolytic urea production.

The production of Ammonia and its possible Precursors.

The amount of ammonia produced varies greatly from one organ to another, being much greater in glandular than in muscular tissues. Earlier workers claimed that its source was to some extent amino acids, though it is now recognised generally to be acid amides present in the protein molecule, and to a small extent the purines of nucleic acid [Jones, 1920]. Glycine, and free cytosine, uracil and thymine have been tried in this work and suffer no deamination.

Optimum p_H for Ammonia production.

This from Figs. 1 and 2 is p_H 6, but this is not the optimum for general autolysis, as measured by total soluble N or amino N in either organ [Dernby, 1918; Bradley, 1923]. p_H 6 is evidently the observed optimum, because at this p_H, as in the case of urea, two or more enzyme systems can act best either independently or consecutively. This is supported by studying the production of ammonia by spleen enzymes from a pancreas-digested spleen, at different p_H, exactly as described above for urea (Table VI).

Table VI.

NH_3 formed from trypsin-digested spleen expressed in cc. $N/50$ cc. soda per 5 cc. of filtrate at p_H 6·8–7	2·45
The same at p_H 6·0	0·1

In this connection treatment of asparagine by tissues is interesting, although asparagine may not be a normal tissue constituent. It is deamidised by both liver [Clementi, 1922] and spleen, and the rate of deamidisation falls off rapidly on the acid side. The following figures have been obtained with liver showing the rates of deamidisation at different p_H. The experiments were done with a 1 % solution of asparagine and a dilute ox-liver suspension.

Table VII.

p_H	NH_3 produced in cc. $N/50$ NaOH per 5 cc.
5	0·2
6	3·3
6·9	8·2

The primary proteolytic enzyme system acting best at more acid reactions than p_H 6, can only deamidise some of the substances present in such an organ as the spleen, and capable of yielding ammonia under the action of its own tissue enzymes. The remainder are only deamidised by an enzyme system acting best at p_H 7. This enzyme system however is not capable of hydrolysing the tissue proteins. The reaction at which these two systems can best combine is p_H 6. At a more acid reaction than this, though hydrolysis is active, the deamidising enzyme system, acting best at p_H 7, is inactive; on the alkaline side of p_H 6 the activities of this system are limited by the small amount of hydrolysis and consequently of substrate.

These experiments show the source of the conflicting results obtained by early workers on the absolute ammonia production, and the same production expressed as a percentage of the total soluble N.

Summary.

(1) The effect of oxygen, hydrogen, nitrous oxide and adrenaline, and of p_H on the formation of NH_3 and urea in autolysis has been investigated.

(2) Ammonia and urea have been shown to be formed by separate processes, and their precursors have been discussed.

(3) An enzyme which forms part of the urea-producing system has been described, which is strongly inhibited by gaseous oxygen. Its substrate has been considered.

(4) This enzyme system is under further investigation.

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