EXPERIMENTAL LIVER NECROSIS; II. ENZYMES.[1]

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The experiments about to be described represent an attempt to determine the relation of the intracellular hepatic enzymes to chemical changes occurring in liver necrosis. Our results are based on a comparison of the variations in the enzymotic equilibrium of the normal hepatic cells with those occurring in necrosis of varying grades of severity. At present the chief and most promising method of detecting such variations consists in determining by means of post-mortem autolysis the condition under which the cell is existing at the time of the death of the animal, and the rapidity, nature and extent of the changes which occur after the commencement of the autolysis. We are well aware that the interpretation of the results of post-mortem autolysis in relation to cellular activity during life is open to objection and may not have the importance usually ascribed to it.

Our investigation of the enzymotic activity of the liver tissue under normal circumstances and in varying degrees of necrosis may naturally be subdivided as follows:

1. A determination in a quantitative way of the degree of autolysis which the tissue undergoes after death.

2. A study of the individual enzymes with reference to the part which they play in the general course of autolysis.

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3. A determination of the products formed as the result of such autolysis. These include the diamino-acids, which have been considered in the preceding paper,[2] where they more properly belong, and the monamino-acids to which, as represented by leucin and tyrosin, we have given considerable attention. It was our intention to determine, by perfusion of livers in various stages of necrosis, the changes in the composition of the blood which might occur, but owing to the great amount of labor entailed in the present studies this has been unavoidably postponed.

Comparative Estimation of Products of Autolysis.—In the study of the changes which the nitrogenous material undergoes during autolysis *in vitro* an attempt was made to carry out a partition analysis of the non-coagulable nitrogen. This method has already been employed with good results by v. Drjewezki[3] to determine the effect of alkalies of varying strengths upon autolysis. His results, as well as those of Wiener,[4] point to the sensitiveness of the autolytic enzymes to changes in reaction, especially those due to alkalies, and these investigators conclude that the alkalies of the serum are responsible for the well-known inhibitory effect of the serum upon autolysis. Baer[5] and his associate, Loeb,[6] admit the inhibitory effect of the serum but are inclined to attribute it to the action of the serum globulin.

These facts, as well as those brought out by Lang[7] concerning the inhibitory effect of large quantities of toluol upon autolytic processes, although other factors may have influenced the results of the latter, all tend to emphasize the fact that in performing experiments of this character too much attention cannot be given

[2] See first paper of this series, "Hexon Bases" in this number of the *Journal.*

[3] v. Drjewezki, A., Ueber den Einfluss der alkalischen Reaktion auf die autolytischen Vorgänge in der Leber, *Biochem. Zeit.,* 1906, i, 229.

[4] Wiener, H., Ueber den Einfluss der Reaktion auf autolytische Vorgänge, *Zent. f. Physiol.,* 1905, xix, 349.

[5] Baer, J., Ueber die Wirkung des Serums auf die intracellularen Ferments, *Arch. f. exper. Path. u. Pharm.,* 1906, lvi, 68.

[6] Baer, J. and Loeb, A., Ueber die Bedingungen der autolytischen Eiweissspaltung in der Leber, *Arch. f. exper. Path. u. Pharm.,* 1905, liii, 1.

[7] Lang, S., Ueber desamidierung im Tierkörper, *Beit. z. chem. Physiol. u. Path.,* 1904, v, 321.

to the attainment of absolutely comparable conditions in all the various experiments. With these points in mind we have endeavored to control our work in every way, as is shown in the following detail of the experiments.

Quantities of the fresh tissue of known weight were ground to such a state of subdivision that when mixed with water or neutral Ringer's solution the mixture could be readily drawn up in a pipette. This mixture, usually consisting of two hundred grams of liver, was made up to 1,200 cubic centimeters and placed in a sterile flask and the mixture covered with a layer of toluol. This latter substance was well shaken in, after which were pipetted off, as controls, two samples of two hundred cubic centimeters each. Both were thoroughly sterilized in the autoclave in order to stop autolysis; one was examined immediately, as an initial control, the other was placed with the original mixture in the thermostat (37.5° C.) and examined at the conclusion of the experiment as a final control. The material in the thermostat was shaken from time to time and at intervals of one, three, five and eight days samples of the main mixture were removed for analysis by the same method as the controls. The analysis of the samples, after they were shown to be bacteria free,[8] took place in the following manner:

The mixture was pipetted into a beaker and sufficient water was added to allow of easy coagulation of the proteid material present. Acetic acid was added to slightly acid reaction after the boiling point was reached. The coagulated proteid was removed by filtration and repeatedly and thoroughly washed with boiling water. The volume of the filtrate and washings was made up to eight hundred cubic centimeters. Of this, twenty-five cubic centimeters served for the determination of the total nitrogen by the Kjeldahl-Gunning method, one hundred for the estimation of ammonia according to the method of Shaffer as applied to the urine, one hundred for the uric acid determination, using the Hopkins-Folin method, and fifty to determine the amount of nitrogen not precipitable by phosphotungstic acid in sulphuric acid solution, the so-called

[8] For this purpose it was deemed sufficient merely to examine stained films though when the final sample of each mixture was taken cultures were made. In this series representing nine livers no contamination occurred.

monamino-nitrogen. An attempt was also made to determine the nitrogen precipitable (proteoses) by zinc sulphate but our results are so incomplete that little can be gained from their discussion. In all cases duplicate determinations were made and the figures given represent their average. Dogs were employed in all experiments.

In Table I are presented the results of the nine experiments which differed in their conditions for the purposes of control, as follows:

Two normal livers (52 and 54) with their usual blood content, the diluting fluid of one being distilled water and of the other neutral Ringer's solution. This solution was prepared in the ordinary way with the exception that the sodium bicarbonate was not added in order to avoid an alkaline medium.

Two normal livers (53 and 58) washed *in situ*, the one with water and the other with neutral Ringer's solution.

One necrotic liver four hours after injection (57). An attempt was made to wash this liver with water but on account of the extensive thrombosis it was only partly successful. It is therefore referred to as "half-washed."

Two necrotic livers forty-eight hours after injection (48 and 56); one unwashed diluted with water; the other washed and diluted with neutral Ringer's solution.

Two livers (43 and 49) five days after injection, both showing necrotic lesions with early repair; one washed and diluted with Ringer's, the other unwashed but diluted with water.

In each instance in which the livers were washed the procedure was begun under ether while the animal was alive. With the exception noted the livers were completely blanched save for slightly tinged areas about the more diffuse foci of necrosis.

The results in Table I, in terms of nitrogen, are expressed in percentages of the total nitrogen of the dry tissue and of the total non-coagulable nitrogen. A critical consideration of the figures presented allows of the following statements:

Non-coagulable Nitrogen.—The inhibiting effect of the blood serum upon the extent of the autolysis of both normal and necrotic tissue is decisively shown. The percentage of non-coagulable nitrogen in the case of the unwashed normal organs increased from 10.7 and 9.7 to 19.9 and 29.1 per cent. respectively, an increase of 100 and 200 per cent., on the eighth day; while in the washed normal livers the average increase at the eighth day amounted to 450 per cent. The increase in the five day necrotic unwashed liver was 127 per cent.; that of the washed tissue 349 per cent. The forty-

TABLE I.

Autolysis; nitrogen partition.

Normal.				4 Hours.	48 Hours. Necrosis.		5 Days. Necrosis.		Duration.
Not Washed.		Washed.		Washed.*	Not Washed.	Washed.	Not Washed.	Washed.	
52†	54	53	58	57	48†	56	43†	49	
Percentage of total nitrogen in non-coagulable form.									
10.7	9.7	13.5	9.5	8.5	12.7	8.3	26.6	18.3	Control
15.5	18.6	35.5	23.6	19.3	20.0	18.5	39.4	41.1	1 day
18.2	27.8	57.6	38.5	24.1	24.9	65.1	51.1	48.5	3 days
19.8	27.8	67.5	49.7	29.6	30.8	75.7	54.3	61.0	5 days
19.9	29.1	70.1	54.2	37.9	32.1	79.4	60.4	82.3	8 days
13.4	11.6	14.0	8.9	7.4	14.5	7.2	22.1	18.1	Final Control
Phosphotungstate-filtrate nitrogen (monamino-acid).									
7.2	6.7	7.4	4.2	5.5	10.7	3.6	15.0	11.3	Control
67.3	69.3	54.8	44.5	64.7	81.1	43.0	56.5	51.7	
12.4	13.7	30.9	18.2	14.7	17.2	16.4	29.7	30.0	1 day
80.0	73.6	87.0	77.1	72.8	86.0	88.6	75.4	73.1	
14.1	21.4	49.8	31.8	19.9	22.6	54.7	37.2	39.3	3 days
77.5	77.0	86.6	82.6	82.5	90.8	84.2	72.8	81.0	
16.2	22.9	56.9	40.3	25.3	26.0	64.3	43.8	53.0	5 days
81.8	82.4	84.3	81.0	85.4	84.4	84.9	80.7	86.9	
16.9	23.9	57.8	46.2	32.3	27.2	70.1	46.9	71.9	8 days
84.9	82.7	80.1	85.2	85.2	84.7	88.3	77.6	87.3	
8.4	8.1	7.6	4.8	6.8	8.9	3.7	11.9	11.9	Final
63.0	70.0	54.1	54.3	90.5	75.4	51.5	53.8	66.0	Control
Ammonia nitrogen.									
0.74	0.49	0.59		0.79	0.80	0.85	2.08	1.10	Control
6.9	5.1	4.3		9.4	6.3	10.2	8.6	6.0	
1.38	0.90	1.41		1.05	1.46	1.50	4.4	2.20	1 day
8.8	4.8	4.0		5.4	7.3	8.1	10.6	5.3	
1.45	1.06	2.45		1.9	2.16	2.78	4.7	3.30	3 days
7.9	3.8	4.2		7.9	8.6	4.3	9.2	6.9	
1.63	1.60	2.52		1.6	2.19	3.25	5.8	3.99	5 days
8.2	5.1	3.8		5.4	7.1	4.3	9.6	6.5	
2.08	1.22	2.45		2.9	2.34	3.01	6.4	5.11	8 days
10.4	4.2	3.5		7.6	7.3	3.8	10.6	6.2	
0.66		0.67			1.02	0.85	2.2	1.43	Final
4.9		5.2			8.6	11.6	10.0	7.9	Control

* Washing incomplete.

† Distilled water used instead of neutral Ringer's solution.

Figures in upper left-hand corner show percentage of total nitrogen; those in lower right-hand corner, of non-coagulable nitrogen.

eight hour washed tissue (Dog 56) with a very extensive necrosis, showed the greatest increase, equivalent to 856 per cent. of the control. The increase of the four hour experiment (congestion and thrombosis) hardly equaled that of the washed normal.

Wherever water was employed in washing or in diluting, the autolysis was distinctly less than when neutral Ringer was used. (Compare Dogs 52 and 54.)

Concerning the rapidity of the autolysis, it may be noticed that, though the initial increase during the first day in the case of the unwashed tissues is but one half of that of the washed, it represents, as does also the increase of the washed, fifty per cent. of the total autolysis. On the third day, however, the autolysis has reached its maximum in the unwashed tissues, while the washed organs continue to increase until the eighth day, when the autolysis in their case is also apparently complete.

In the forty-eight hour lesions in which, histologically, the autolysis of the necrotic areas would appear to be at its height, we see that the autolytic processes *in vitro* were also very active. The increase at the end of the eighth day in the unwashed liver (Dog 48) was about 150 per cent., but after the removal of the inhibitory action of the blood (Dog 56) the increase rose to almost 900 per cent. The same thing is evident in the fifth day lesions but is not so pronounced.

The rapidity with which the autolysis reaches its maximum is of course dependent upon various factors. The attainment of the maximum signifies that the reaction velocities of the system, made up of substrat, hydrolytic agent and enzyme, have reached an equilibrium, caused, no doubt, by the non-removal of the products of autolysis. Since we must assume that the substrat and enzyme are the same in the normal tissues of both washed and unwashed organs, the varying factor must consist in the hydrolysis which, from the work of Wiener, seems undoubtedly due to the unneutralized acids formed during autolysis as first described by Magnus-Levy.[9] The acids which are formed in the normal metabolism of the cells are neutralized by the ammonia and excess of bases in the blood; hence

[9] Magnus-Levy, A., Ueber die Säurehildung bei der Autolyse der Leber, *Beit. z. chem. Physiol. u. Path.*, 1902, ii, 261.

autolysis does not occur in the living cell. As soon as the serum with its neutralizing power is removed, as in the washed organs or where the acids use up the excess of bases as in the center of a large area of necrosis, the conditions necessary for autolysis are present and hydrolysis of the substrat protoplasm takes place.

Phosphotungstate Filtrate Nitrogen (Monamino-acids).—The phosphotungstate precipitate has been disregarded here, for, as it consists of diamino-nitrogen it has been sufficiently covered in the autolysis experiments in connection with the study of the hexon bases.[10]

By far the major portion of the nitrogen in the filtrate is in the form of monamino-acids.[11] The table indicates the percentage of the fraction in terms of the total nitrogen of the tissue as well as of the total non-coagulable nitrogen. Our figures for the controls indicate that in the normal tissue, washed or unwashed, 4.2 to 7.4 per cent. of the total nitrogen is to be attributed to nitrogen not precipitable with phosphotungstic acid. Of the forty-eight hour lesions, that with the most marked diffuse necrosis (Dog 48) showed 10.7 per cent. of the total nitrogen in that form, while the other, of the focal type (Dog 56), had only 3.6 per cent. or slightly less than the lowest of the normal figures. Also, in the first of this pair, 81.1 per cent. of the non-coagulable nitrogen was in the form of monamino-acid while the other showed only 43.0, again somewhat less than normal.

These two experiments illustrate most decisively the point which Taylor's[12] results seem to indicate. That is, there is an absence of monamino-acids in pathological conditions of the liver accompanied by little or no necrosis, while in necrosis of the diffuse type both the monamino- and diamino-acids are present. We have elsewhere[13] suggested that the relation of circulatory disturbances to

[10] See first paper of this series, "Hexon Bases" in this number of the *Journal.*

[11] v. Drjewezki, A., Ueber den Einfluss der alkalischen Reaktion auf die autolytischen Vorgänge in der Leber, *Biochem. Zeit.*, 1906, i, 229.

[12] Taylor, A. E., Ueber das Vorkommen von Spaltungsprodukten der Eiweisskorper in der degenerirten Leber, *Zeit. f. physiol. Chem.*, 1902, xxxiv, 580. On the Occurrence of Amino-acids in Degenerated Tissue, *Univ. of California Publications*, 1904, i (Path.), 43.

[13] See first paper of this series, "Hexon Bases" in this number of the *Journal.*

the removal of the products of autolysis is an all-important factor. This is further supported by the two experiments under discussion which indicate that the organ with the focal lesions contained no more monamino-nitrogen than did the normal tissue. In this case the circulation was very slightly, if at all, impaired, and these acids, if they were formed, were removed immediately by the blood stream. In Experiment 48 the large necrotic areas, the centers of which were remote from circulatory fluids, held the acids as they were produced. That these substances are produced in autolysis of this type *in vivo* in large quantities is also indicated by the fact that 81.1 per cent. of the non-coagulable nitrogen of this liver was present in the fresh tissue as nitrogen non-precipitable with phosphotungstic acid. This value approaches that found in all the other cases after autolysis *in vitro* has proceeded for from one to three days.

The control figures of the five day necrosis, as shown by Experiments 43 and 49, also indicate a high percentage of the total nitrogen of the tissue as monamino-nitrogen. As, however, an unusually large amount of the total nitrogen occurs in non-coagulable form the percentage relation of the monamino acids to the latter is about normal. These lesions were very extensive but of the focal type and the cells at the fifth day were undergoing repair. Hence, although autolytic processes were going on in the tissue at that time, the products were removed as fast as they were formed and no increase in amount occurred in the organ.

The same differences in the velocity and degree of autolysis *in vitro* between the washed and unwashed organs are also very evident and require no discussion since they are due to the same causes. It is interesting to note that the nitrogen occurring as monamino-acids reaches at the end of the first day about 80 per cent. of the non-coagulable nitrogen and then although autolysis may greatly increase, their formation increases only in the same proportion. The exception to this is in case of Dog 48, already discussed, where the percentage was high in the tissue itself and remained at the same level during autolysis *in vitro*. This would seem to point to the fact that as far as monamino-acids are concerned their formation in autolysis *intra vitam* occurs in the same manner and by the same chemical processes as they do in autolysis *in vitro*.

v. Drjewezki found that at the end of seventy-two hours autolysis the monamino-acids took up about sixty per cent. of the total nitrogen of the tissue. This figure is somewhat higher than we obtained at this stage of autolysis but in some instances it was reached on the fifth or eighth day.

Ammonia.—As a result of the work of Loewi,[14] Jacoby,[15] Lang and others, considerable interest has become attached to the power of the surviving liver tissue to produce ammonia, especially in view of the current opinion as to the importance of this product, after it has been split off from the amino-acids, as a step in the formation of urea. This interest has been heightened by the appearance of ammonia in increased absolute and percentage amounts in the urine in certain hepatic disorders, thus apparently bringing these matters into correlation.

We have studied the question in two ways. First, in connection with the nitrogen partition of the autolysis now under discussion, and secondly, after the manner of the discoverer[15] of the ammonia-forming power of the liver. This latter part of the work will be discussed separately.

In the partition tables it is seen that although the ammonia content of the necrotic livers is greater than that of the normal it runs parallel with the increase in the amount of non-coagulable nitrogen. Hence the percentage figures show no regular increase or diminution though variations occur owing to the large limit of error dependent on the small amounts of ammonia formed. A comparison of a forty-eight hour necrotic liver (56), which offers an exception to the above statement, in that it shows a progressive diminution with a normal washed liver (53), is instructive. In 53 the normal percentage of ammonia of the non-coagulable nitrogen runs along at about 4 per cent. throughout the experiment. In 56, however, the control shows a high initial ammonia content (10.2), which on the third day dropped to that of the normal washed liver (53) and remained at that level to the end of the experiment.

[14] Loewi, O., Ueber das Harnstoffbildende Ferment der Leber, *Zeit. f. physiol. Chem.*, 1893, xxv, 511.

[15] Jacoby, M., Ueber die fermentative Eiweissspaltung und Ammoniakbildung in der Leber, *Zeit. f. physiol. Chem.*, 1900, xxx, 149.

We explain this variation in the ammonia formation by the assumption that this tissue contained *intra vitam,* as the result of the necrosis, proportionately larger amounts of ammonia liberating compounds than the normal. As, however, the autolysis proceeded less of these products were formed in relation to the non-coagulable nitrogen than was the case in the autolysis of the normal. Hence the percentage figure dropped. Or if we allow for the initial difference we find that the percentage increase is the same in both cases.

The great increase in the amount of non-coagulable nitrogen which occurred between the first and third day (18.5 to 65.1 per cent.) in Dog 56 could not have included the formation of ammonia compounds, since the increase of these latter bodies in relation to the total nitrogen was so slight that there occurred an actual percentage decrease in relation to the non-coagulable nitrogen.

All this would seem to indicate that the production of ammonia which occurs in the autolysis of the liver *in vitro* is the result of a decomposition of coagulable nitrogen in the cell. That is deamidization of the amino-acids and the splitting of urea does not take place to any greater extent in the necrotic tissue undergoing autolysis than it does in the normal.

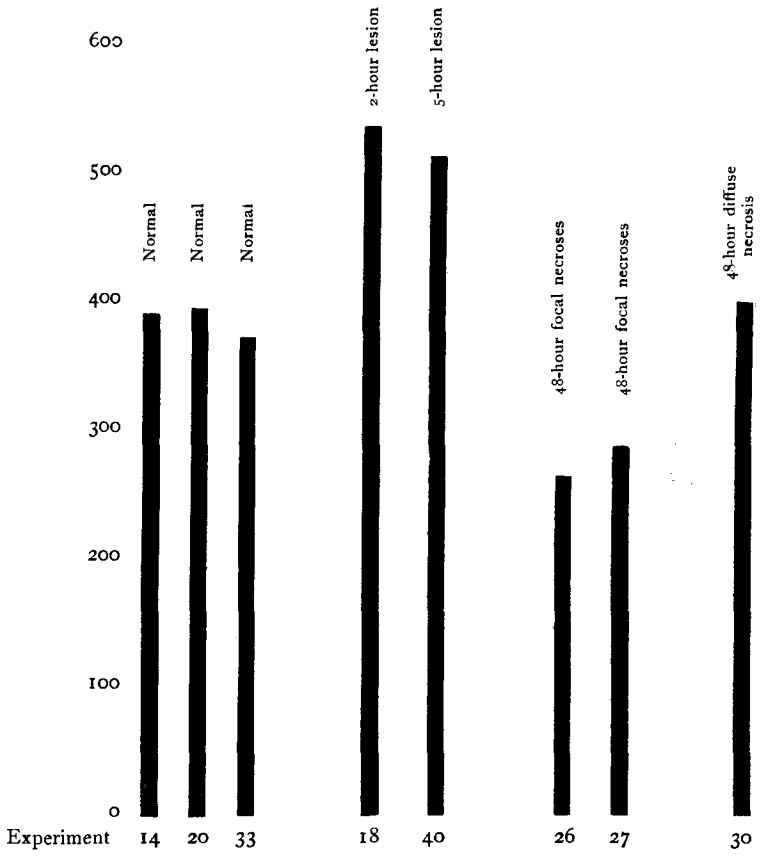
Separate Ammonia Determinations.—These experiments were some of the earlier ones performed and were carried out after the fashion of the investigations reported by Jacoby. A weighed amount, 200 grams, of the finely divided fresh liver tissue was made up to 600 cubic centimeters with distilled water and the well-mixed fluid divided into twelve equal parts. Four of these samples were sterilized immediately for controls. Two were analyzed at once as initial controls, while two, for the purpose of final controls, were placed with the remainder of the portions in the thermostat. At varying periods, duplicate samples were removed for analysis. The ammonia was determined according to a modification of Shaffer's method for the urine. The accompanying diagram indicates in a schematic way the results of the experiments.

It is somewhat difficult to make comparison of these results with those just reported in the partition series since we have no determination of the amount of non-coagulable nitrogen at the various

TABLE II.

Ammonia.

Percentage increase at the end of ten days.



periods; hence the figures represent only the percentage increase of ammonia nitrogen based on its amount in the control sample. Although Jacoby gives in his tables only the amount of ammonia nitrogen formed without taking into consideration the question of dry solids and nitrogen of the dry residue, a recalculation of his figures from average results of our normal livers indicates that more ammonia nitrogen was formed in our experiments than in his. On the other hand, however, our figures are not as high as those re-

ported by Soetbeer.[16] In opposition to the partition series, our figures indicate a larger amount of ammonium compounds in the initial control sample of the normal tissue than in those with varying degrees of necrosis and degeneration. This seeming anomaly we are unable to understand or explain. As, however, the percentage increase figures upon which the diagram is calculated are based upon the initial control as zero, the variable and anomalous control factor is excluded.

Considered in this way it will be seen that the three normals showed an increase of ammonia-nitrogen over the control of from 378 to 396 per cent., an agreement which serves well as a basis for comparison of the experiments on necrotic and degenerated tissue. In the case of the focal necroses the increase is less than the normal, amounting only to 265 to 290 per cent. On the other hand, the diffusely necrotic tissue evidenced the same power to produce ammonia-forming compounds as the normal. In the two samples of congestion and thrombosis, the lesion being of two to five hours' duration, more ammonia was produced than in the normal. These results would seem to indicate that during the initial stage of the process when the liver is intensely congested an increase in ammonia output must occur. This, however, is not supported by our metabolism experiments.[17]

Uric Acid.—The investigation concerning uric acid has yielded results of not sufficient interest for presentation in a table. The only point of importance is that a gradual diminution occurs which ceases on the third day. Since in the autolysis of uric acid ammonia is formed, this factor must influence to a slight extent the increase in ammonia observed in the partition experiments.

Arginase.—In the endeavor to explain the results in connection with the hexon bases, reported in the first paper of this series, a few experiments were conducted in the attempt to prepare from normal and necrotic livers an active substance, according to the

[16] Soetbeer, F., Ueber einen Fall von akuten Degeneration des Leberparenchyms, *Arch. f. exper. Path. u. Pharm.*, 1903, l, 294.

[17] See third paper of this series "Nitrogenous Metabolism" in this number of the *Journal.*

method of Kossel and Dakin,[18] which would hydrolyze arginin into ornithin and urea. Preparations were made by both the ammonium sulphate and acetic acid-ether methods outlined by these investigators and solutions of these were added in aliquot parts to an arginin solution of known strength. The determinations were made sometimes upon the phosphotungstic precipitate, sometimes upon the filtrate from this and once upon both. The aliquots were allowed to autolyze for one, three and five days and controls were done at the beginning and at the end of the experiment.

TABLE III.

Arginase.

Experiment.	Lesion.	Method of Preparation.	Estimation on Phosphotungstate.	c.c. N/10 Acid.			
				Control.	1 day.	3 days.	5 days.
15	Normal.	¾ Saturation $(NH_4)_2SO_4$ precipitate.	filtrate.	1.6	2.65	2.75	2.65
15	Normal.	ditto.	filtrate.	4.55	5.95	6.25	6.80
15	Normal.	ditto.	precipitate.	5.75	4.45	4.25	3.65
19	Normal.	ditto.	precipitate.	4.50	3.40	5.00	5.65
42	Normal.	Extraction acetic acid.	filtrate.	5.20	4.45	4.15	4.30
18	2 hours.	Complete saturation $(NH_4)_2SO_4$ precipitate.	precipitate.	11.45	12.10	12.40	9.90
40	5 hours.	Extraction acetic acid.	filtrate.	5.40	4.65	5.30	4.40
16	Focal necroses.	Complete saturation $(NH_4)_2SO_4$ precipitate.	precipitate.	6.75	6.70	6.55	5.70
28	Diffuse necrosis.	ditto.	precipitate.	6.10	6.90	6.95	6.10
28	Diffuse necrosis.	Extraction dilute HCl.	precipitate.	8.05	9.80	8.45	8.60

Our results in regard to the normal liver agree with those of Kossel and Dakin. The preparations from necrotic livers gave negative or doubtful results, thus affording valuable confirmatory evidence of the position which we assumed as a result of our work upon the hexon bases, namely, that in extreme diffuse necrosis where large areas remote from the circulation are undergoing necrosis

[18] Kossel, A. and Dakin, H. D., Ueber die Arginase, *Zeit. f. physiol. Chem.*, 1904, xli, 321. Weitere Untersuchungen ueber fermentative Harnstoffbildung, *ibid.*, 1904, xlii, 181.

there occurs a marked increase in hexon base content of the tissue. In such areas, evidently, the arginin is not split up to any noticeable extent. The experiments of Wakeman[19] do not show the presence of arginin to the marked extent which the author claims, as we have explained in our discussion of the hexon bases.[20]

The table shows that of the ten preparations made from seven different livers, three normal, two necrotic and two with thrombosis, only one, the normal (15), showed any activity. In the others either no results were obtained or were so irregular that the experiments may be considered as negative.

In this one experiment with normal liver in which the precipitate obtained by complete saturation of the three-quarter saturated filtrate with ammonium sulphate was employed, the nitrogen of the filtrate from the phosphotungstic precipitate showed a gradual increase presumably due to the autolysis of the arginin added. These results would indicate that an active arginase can be obtained only from normal tissue. This is in complete accord with the results reported in the paper on the hexon bases in which it is shown that during the autolysis of necrotic liver tissue an increase in the hexon base content of the cell occurs.

Leucin and Tyrosin.—In view of the well-recognized presence at times of monamino-acids in the urine of individuals suffering with hepatic disorders, particularly acute yellow atrophy, and of the varying results reported by the different observers in regard to the question of the presence of these compounds in the liver tissue, it seemed advisable to examine the urine and the liver of the animals under observation as to the presence of leucin and tyrosin.

It would appear to be unnecessary for us to enter into a discussion of the older literature concerning the variation in the results as to the presence or absence of leucin and tyrosin in the urine under many pathological conditions. This subject has been well discussed by Ewing and Wolf,[21] who conclude that the differences observed

[19] Wakeman, A. J., On the Hexon Bases of Liver Tissue under Normal and Certain Pathological Conditions, *Jour. of Exper. Med.*, 1905, vii, 292.

[20] See first paper of this series, "Hexon Bases" in this number of the *Journal.*

[21] Ewing, J. and Wolf, C. G. L., The Clinical Significance of the Urinary Nitrogen, *Amer. Jour. of the Med. Sciences,* 1906, cxxxi, 751.

are most probably due to faulty methods of technique and of confirmation. Of the recent work, to which this criticism cannot properly be applied is that of Taylor,[22] who found these monamino-acids present in the liver of acute yellow atrophy as well as in that of probable chloroform poisoning. In both instances, supposedly, necrosis of varying degree had taken place. Wells[23] in a preliminary communication confirms these results for acute yellow atrophy. On the other hand, however, in other conditions to which he gives the general term of " degeneration," Taylor failed to find these substances. Again leucin and tyrosin usually appear in the urine of persons or animals poisoned with phosphorus and this fact has been associated with the occurrence of the well-known hepatic changes, chiefly fatty infiltration, which are known to occur in this condition.

The recent method devised by Fischer and Bergell, in which β-naphthalin sulphochloride is employed, and Abderhalden and Barker's modification of Fischer's esterification method are so time-consuming that we decided that for the purpose in view, the simpler methods were of sufficient accuracy to warrant their use. Ewing and Wolf in the paper mentioned above criticize severely the lead acetate method, originally employed by Frerichs and Städeler. They claim that the microscopic demonstration of leucin and tyrosin by this procedure is unreliable and the crystals supposed to be leucin may be in reality urates or urea. We have used a modification of the lead acetate method in which after the removal of the excess of lead by means of hydrogen sulphide the filtrate is evaporated to dryness and the residue extracted with several portions of absolute alcohol to remove the urea, after which it is treated with repeated portions of ammoniacal absolute alcohol. The united extracts are allowed to evaporate almost to dryness, when characteristic crystals appear, if leucin or tyrosin is present in the original material. When sufficient quantities were present these microscopic findings were controlled by the usual chemical tests. We feel reas-

[22] Taylor, A. E., Ueber das Vorkommen von Spaltungsprodukten der Eiweisskorper in der degenerirten Leber, *Zeit. f. physiol. Chem.*, 1902, xxxiv, 580. On the Occurrence of Amino-acids in Degenerated Tissue, *Univ. of California Publications*, 1904, i (Path.), 43.

[23] Wells, H. G., The Composition of the Liver in Acute Yellow Atrophy. Communication read at the first meeting of the Amer. Soc. of Biol. Chemists, Washington, May 8, 1907.

onably sure that the substances upon which we have based the following results were leucin and tyrosin.

TABLE IV.

Leucin and Tyrosin in the Urine.

Experiment.	Lesion.	Leucin.	Tyrosin.	Urine of
2	No necroses	—	+	4th day
34	No necroses	+++	+	1st and 2d day
1	Focal necroses	+	+	1st day
5	Focal necroses	—	+	1st day
23	Focal necroses	++	+	1st and 2d day
32	Diffuse necrosis	—	+	1st day
48	Diffuse necrosis	—	++	1st and 2d day
51	Diffuse necrosis	—	++	2d and 3d day

TABLE V.

Leucin, Tyrosin and Proteoses in the Liver.

Experiment.	Lesion.	Leucin.	Tyrosin.	Proteoses.	Age of Lesion.
16	Focal necroses	—	+	+	48 hour
32	Diffuse necrosis	—	+	+	26 hour
48	Diffuse necrosis	—	++	+	48 hour
51	Diffuse necrosis	—	++	+	48 hour

The table giving the results of the examinations of the urine shows that there is no regularity in the occurrence of these compounds. The type of the lesion has apparently no relation to the amount eliminated and the results presented justify the general concensus of current opinion that the appearance of these compounds is not to be regarded as pathognomonic of any one condition such as acute yellow atrophy or phosphorus or chloroform poisoning. In addition to the positive results presented in the table the urine of nine other animals was examined with negative results. In five of these the liver showed necroses, in four none.

The results of the examination of the liver substance point to the occurrence of tyrosin in larger amounts when the lesion was most pronounced; thus in each of three livers with diffuse necrosis it was present, but in only one of the five examples of focal necroses did it occur. A normal liver and also one with degeneration but no necroses were likewise negative. In no condition did we find leucin. In four livers with extensive necrosis proteoses were found in considerable quantities while a normal liver yielded none. All of

this is in agreement with the variable results of Taylor mentioned elsewhere.

It is evident, therefore, that leucin and tyrosin may be formed during the autolysis of the hepatic tissue, but their appearance in the urine or detection in the liver is dependent upon the condition of the hepatic cells not involved in the lesion. If these cells can take care of large quantities of monamino-acids carried to them normally by the portal vein we see no reason why, if they are present in sufficient numbers and properly functionating, that they should not react in the same way with the same acids formed during the autolysis. The appearance of these monamino-acids under any condition then would depend upon the quantitative relation of the necrosis to the actively functionating cells which are unaffected by the lesion.

Of considerable interest in connection with the finding by Salkowski[24] in the urine of various pathological conditions, more particularly a case of yellow atrophy, of an increased amount of nitrogen precipitable by alcohol, is the fact that although in the normal liver the residue remaining after the extraction with absolute alcohol and ammoniacal alcohol is small in amount, in the case of the necrotic tissues this amount is markedly increased. We have examined the residue as to its character and find that it consists mainly of proteoses. The removal of these compounds by way of the blood-stream would cause an increase in the urine of undetermined nitrogen usually ascribed to amino-acids. This occurrence explains those conditions characterized by a high rest-nitrogen without the presence of monamino-acids.

SUMMARY.

1. The presence of blood serum has a decided inhibitory effect on autolysis. Thus in the normal unwashed organs the non-coagulable nitrogen increase was 100 to 300 per cent., while in the washed it amounted to 450 per cent. The washed necrotic livers showed an increase of from 600 to 850 per cent., while that of the unwashed necrotic was only slightly above the normal unwashed.

2. While the initial amount of non-coagulable nitrogen varies it is greater in those livers showing the more extensive forms of

[24] Salkowski, E., Zur Kenntnis der Alkoholunlöslichen bzw. kolloidalen Stickstoffsubstanzen im Harn, *Berl. klin. Woch.*, 1905, xlii, 1581, 1618.

necrosis. The final amount of autolysis is also greatest in livers of this type. As regards the rate of autolysis fifty per cent. of the total occurs in the first day in the normal and in all types of lesions both washed and unwashed. The maximum is usually reached on the third day in the unwashed, while in the washed there is a continued increase to the eighth day. At this time in the necrotic livers about two to three times as much of the total nitrogen is in the form of non-coagulable nitrogen as in the normal.

3. In the necrotic tissue the initial controls show the content of monamino-acids, with one exception, to be practically doubled. In the washed necrotic the final amount is seventy per cent. of the total nitrogen against forty-six to fifty-seven per cent. in the washed normal. In all cases the monamino-acid nitrogen runs parallel to the nitrogen in non-coagulable form, but in relation to the total nitrogen it shows a greater increase in the washed than in the unwashed organs.

4. The ammonia production in the necrotic livers as shown by the partition experiments is greater than that in the normal and this increase corresponds to that of the non-coagulable nitrogen. In the experiments concerning the absolute production of ammonia in the presence of serum a greater amount was produced in the two and five hours' lesions than in the normal livers. On the other hand, the forty-eight hour diffuse necrosis equaled the normal and the focal fell below.

5. Arginase was obtained from normal but could not be isolated from necrotic livers.

6. No constant relation could be demonstrated between the anatomical lesion in the liver and the presence of leucin and tyrosin in the urine. Leucin was found occasionally in the urine, but none in the liver. On the other hand, tyrosin was constantly present in livers with diffuse but rarely in those with focal necrosis. In the instances of diffuse necrosis in which the liver and urine of the same animal were examined tyrosin was found in both.

7. The presence of large amounts of proteoses in the necrotic liver indicates that the elimination of these substances (colloidal nitrogen of Salkowski) under such circumstances may account for a part of the total nitrogen of the urine usually attributed to the monamino-acids.