THE EFFECT OF DAMAGE ON IN VITRO PROTEIN METABOLISM

by

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Thesis

submitted to the Faculty of

Graduate Studies and Research

in partial fulfillment of the

requirements for the degree of

Master of Science.

McGill University Clinic

Royal Victoria Hospital

Montreal, Canada.

September, 1946.

ACKNOWLEDGEMENTS

This investigation was carried out in the McGill University Clinic. The author wishes to thank Professor J. C. Meakins for the provision of the facilities which made this work possible.

The author is sincerely grateful to Dr. J.S.L. Browne and Dr. K.A.C. Elliot for their guidance and valuable criticism throughout the course of this research.

The supervision of the bacteriological aspects by Dr. Gertrude Kalz is gratefully acknowledged.

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GENERAL INTRODUCTION

This investigation is concerned with the effect of trauma inflicted on the living animal on in vitro protein metabolism. A discussion on the chemistry of protein breakdown and factors affecting it is therefore indicated. This will be followed by a review of investigations concerned with the effect of trauma on protein metabolism and of the role of the adrenal cortex in protein metabolism.

I. CHEMISTRY OF PROTEIN BREAKDOWN.

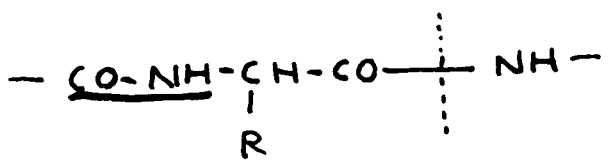
1. Proteolytic enzymes. The first step in the breakdown of the protein molecule is considered to be hydrolysis catalyzed by the proteolytic enzymes.

It was formerly held that the proteolytic enzymes could be classified with respect to the size of the substrate molecule, the proteinases attacking the protein molecule, the peptidases acting on its breakdown products. The proteinases were further subdivided according to the pH ranges at which they acted and theories were developed basing enzyme specificity on the electrical charge of the substrate molecule (Northrop, 1925 - 1926). The peptidases were subdivided further on the basis of the peptide chain length, polypeptidases attacking peptides containing more than two amino acids linked by peptide bonds, the dipeptidases acting on dipeptides.

Extensive research on proteolytic enzyme specificity has led Bergmann to propose a different type of classification based on the position of the vulnerable peptide bond in the protein molecule, and on the nature and position of the amino

acid residues (Bergmann & Fruton, 1941; Bergmann, 1942). The enzymes secreted into the digestive tract as well as the intracellular enzymes (cathepsins) can be fitted into this scheme (Fruton, Irving & Bergmann, 1941). The outstanding differences between the intracellular and extracellular enzymes are 1.) that intracellular enzymes are bound to the cell whereas extracellular enzymes are secreted into the digestive tract, 2.) some of the intracellular enzymes are activated by certain reducing agents. Fruton et al. in their studies on beef spleen and kidney cathepsins observed that Cathepsin I (pepsinase) requires no activators of this type, Cathepsin II (trypsinase) requires cysteine or glutathione, Cathepsin III (aminopeptidase) is activated by cysteine or ascorbic acid, Cathepsin IV (carboxypeptidase) by cysteine.

The so called endopeptidases require an amide linkage adjacent to the carbonyl group of the peptide bond to be hydrolized.



They are, therefore, able to attack centrally located peptide bonds but they can also hydrolyse tripeptides or amides of dipeptides.

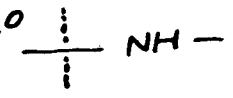
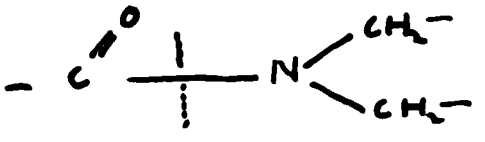
The exopeptidases attack only those peptide linkages that possess a free carboxyl group adjacent to the imido group of the peptide bond (carboxypeptidases; Hofmann and Bergmann 1940) or a free amino group adjacent to the carbonyl group of the peptide linkage to be hydrolyzed (aminopeptidases)

The endopeptidases include the extracellular enzymes

pepsin, trypsin, chymotrypsin, and the intracellular enzymes Cathepsin I (pepsinase), and Cathepsin II (trypsinase). The exopeptidases comprise the extracellular amino and carboxypeptidases and their intracellular counterparts Cathepsin III and IV. The endopeptidases therefore correspond to the "Proteinases" of the old classification, the exopeptidases include the old "Peptidases" but the new terms do not embody the faulty assumption that enzymes splitting protein molecules cannot also attack peptides; i.e., that substrate chain length determines enzyme specificity.

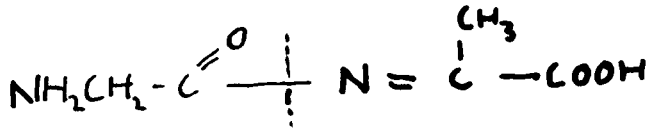
The enzymes in each group are further subdivided according to the nature and position of the amino acid side chain in the following manner: arginine or lysine residues belonging to the carbonyl group of the vulnerable peptide bond are required for optimal, hydrolytic activity of the endopeptidases trypsin (Bermann, Fruton & Pollok, 1939; Hofmann & Bergmann, 1939) and the corresponding intracellular enzyme Cathepsin II. The imido group of the peptide bond to be attacked must be contributed by tyrosine or phenylalanine for optimal pepsin (Fruton & Bergmann, 1939) or Cathepsin I activity. Peptide linkages in which these amino acids contribute the carbonyl group are attacked by chymotrypsin.

Similar sidechain specificity is found among the exopeptidases. Thus, leucine amino peptidase and Cathepsin III attack linkages in which the carbonyl group is contributed by leucine, carboxypeptidases and Cathepsin IV primarily act on peptide bonds in which tyrosine or phenylalanine contribute the imido group.

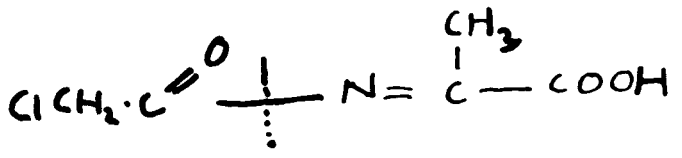
Among the proteolytic enzymes, prolidase and the dehydropeptidases are noteworthy in that they do not act upon the orthodox peptide linkage.  In the substrates for prolidase such as glycyl proline the hydrogen of the imido group in the peptide bond is substituted: (Bergmann & Fruton, 1937). 

In collagen more than one quarter of all peptide linkages belong to this type.

The dehydropeptidases discovered by Bergmann and collaborators are specific for peptides of unsaturated amino acids (Bergmann, Schmitt & Miekeley, 1930: Bergmann & Schleich, 1932a, 1932b). Recent investigations by Greenstein and Leuthardt in 1946 indicate that there are at least two dehydropeptidases, #1 specific for the synthetic substrates glycyldehydroalanine.



or glycyl-dehydro-phenylalanine, #2 specific for chloracetyl-dehydroalanine



These investigators found that dehydropeptidase #1 was present in all tissues tested, #2 in liver, kidney and pancreas.

The existence of dehydropeptidases, and their widespread occurrence lend weight to Bergmann's theory that amino acids may be catabolized while still in peptide linkage (Bergmann et al., 1930). Their powerful activity compared to that of intracellular peptidases hydrolyzing saturated peptides

indicates that an important pathway of protein breakdown may be the dehydrogenation of amino acids in peptide linkage and subsequent splitting of the latter by dehydropeptidases.

2. Other enzymes hydrolyzing the C-N linkage. In connection with nitrogen catabolism in the animal body other enzymes hydrolyzing the C-N linkage should be mentioned. These include enzymes hydrolyzing amide linkages, such as glutaminase, asparaginase with the formation of ammonia and the dicarboxylic amino acid, and the enzyme arginase hydrolyzing the guanido group of arginine with urea and ornithine formation.

3. Deamination. A further step after hydrolysis of the protein molecule into its amino acid components is the oxidative deamination of these amino acids.

Krebs in 1933 observed that kidney and at a slower rate per gram tissue liver oxidatively deaminate d and l forms of alpha amino acids. The corresponding keto acids could be isolated if their further metabolism was blocked by inhibitors. He postulated the existence of two deaminating systems, an l-, and a d- amino acid oxidase. l-amino acid oxidase activity was found in the following tissues listed according to their deamination rate. Kidney cortex, liver, intestine, (especially caecum) muscle, brain and retina. Since destruction of cellular structure and dilution inactivated the l-amino acid oxidase (but not the d-amino acid oxidase) a mechanism involving ternary collision was proposed consisting of activated substrate, activated oxygen and a coenzyme.

The d-amino acid oxidase was later isolated and found ~~to be a~~ flavoprotein. But although enzymes attacking one ~~specific~~ l-amino acid have been extracted, it was not until

The d-amino acid oxidase was later isolated and found to be a flavoprotein. But although enzymes attacking one specific l-amino acid have been extracted, it was not until lately that an l-amino acid oxidase active on a number of naturally occurring amino acids was isolated.

Blanchard, Green, Nocito and Ratner in 1944 were able to extract from rat kidney and liver an l-amino acid oxidase acting upon thirteen naturally occurring amino acids (leucine, methionine, proline, norleucine, norvaline, phenylalanine, tryphtophane, isoleucine, tyrosine, valine, histidine, cystine, and alanine). The process was again oxidative:

$$R\,CH_2NH_2\cdot COOH + O_2 \longrightarrow R\,CO\cdot COOH + NH_3 + H_2O_2$$

The overall equation in the presence of catalase is

$$R\,CH_2NH_2\cdot COOH + \tfrac{1}{2}\,O_2 \longrightarrow R\,CO\cdot COOH + H_2O, \text{ since } H_2O_2 \xrightarrow{\text{catalase}} H_2O + \tfrac{1}{2}\,O_2$$

The enzyme was found to be a flavoprotein with riboflavin phosphate as prosthetic group, (Blanchard et al., 1945) and contrary to the l-amino acid oxidase activity observed by Krebs not inactivated by dilution, the activity decreasing only proportionally to the enzyme concentration. The enzyme oxidizes not only l-alpha amino acids but also l-alpha hydroxy acids.

This enzyme cannot however account for all the deamination occurring in the body since it does not deaminate glycine, serine, and threonine, and the dibasic amino acids lysine, ornithine and arginine. It was further only found ~~to occur~~ in the rat kidney and liver. Cat, dog, guinea pig, rabbit, pig, ox and sheep analyses were negative.

4. Transamination: The transaminases are another group of enzymes concerned with the oxidative deamination of alpha amino acids. The amino group, however, is not formed into ammonia but becomes directly attached to another alpha keto acid. This type of enzyme activity was first descovered by Braunshteĭn and coworkers who distinguished between two enzyme systems: the glutamic aminopherases requiring glutamic acid or its alpha keto analogue (alpha keto glutaric acid), the aspartic aminopherase requring aspartic acid or its alpha keto analogue (oxalacetic acid) as one of the components in the amino nitrogen transfer (Braunshteĭn, 1939). It was held that various other alpha amino acids or their corresponding keto acids, not necessarily dicarboxylic, could act as the other partner in the transamination reaction. While saturated dicarboxylic acids (oxalic, malonic, glutaric, adipic acid) were found to inhibit transamination supposedly by combining with electropositive basic groups in the enzyme, alpha amino acids with a second noncarboxylic acidic group (cysteic acid, homocysteic acid, phosphoserine) could be substituted for glutamic or aspartic acid.

Cohen (1940a, 1940b) was unable to confirm Braunshteĭn's work on several points. He found that only aspartic acid, alanine, and cysteic acid could act as amino nitrogen donators in the glutamic acid formation from alpha keto glutaric acid, and only glutamic and cysteic acid in aspartic acid formation from oxalacetic acid. He was unable to detect direct transamination between oxalacetic acid and alanine postulated by Braunshteĭn. Further, homocysteic acid and phosphoserine were found inactive as NH_2 donators and no inhibition was observed with saturated

dibasic acids.

His investigation led him to the conclusion that two main transamination reactions occurred,

(1) Glutamic acid + oxalacetic acid → α-ketoglutaric acid + aspartic acid

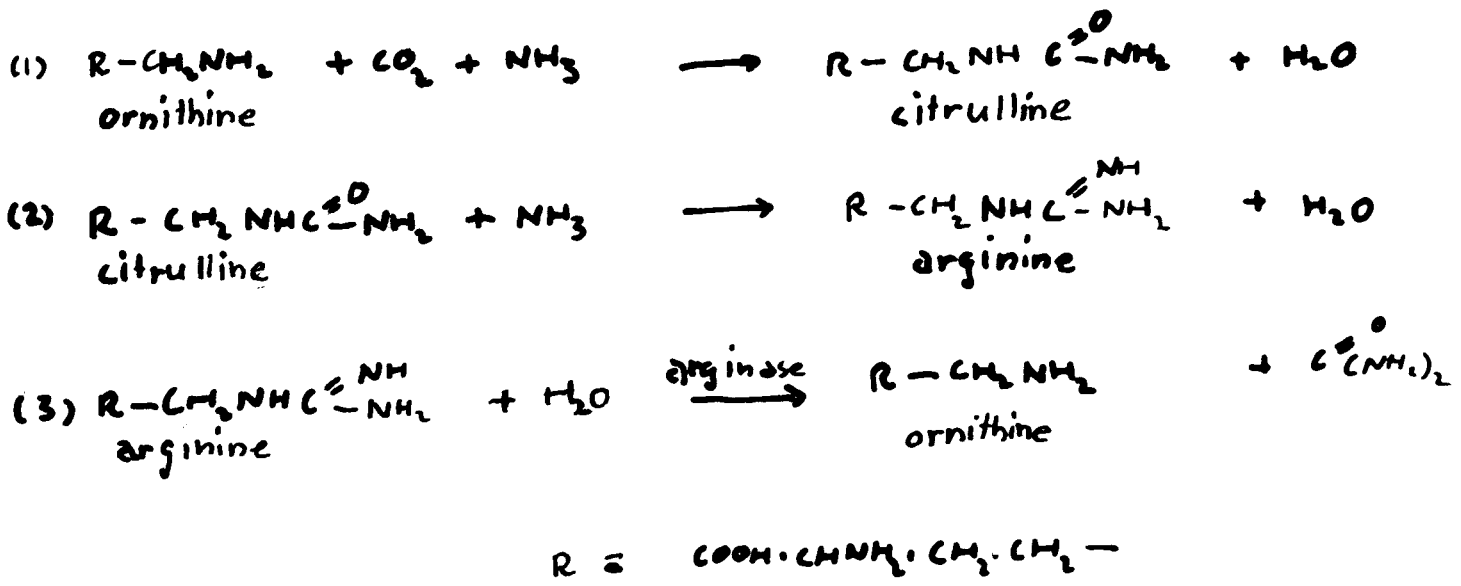
(2) Glutamic acid + pyruvic acid → α-ketoglutaric acid + alanine

and that these reactions might be catalized by the same enzyme, but that the enzyme had a greater affinity for oxalacetic (aspartic) than for pyruvic acid (alanine) since reaction (1) occurred faster than (2).

Green, Leloir and Nocito in 1945 confirmed Cohen's work with respect to the primary substrates involved in transamination. They were however able to isolate two transaminating enzymes from pig heart, the "Aspartic-Glutamic-Transaminase", catalizing reaction (1), and the Alanine-Glutamic-Transaminase" catalizing reaction (2). The transamination between aspartic and pyruvic acid could occur indirectly by the action of both enzymes. They presented experimental evidence pointing to pyridoxal phosphate as the prosthetic group of both enzymes.

5. <u>The Mechanism of Urea Formation</u>: Since urea is the main end product of nitrogen catabolism in the mammalian organism a discussion of some of the experimental work on the mechanism of urea synthesis will be included here.

Krebs and Henseleit in 1932 postulated the ornithine cycle which has since been accepted as the main pathway of urea synthesis. Urea synthesis was pictured in the following manner:

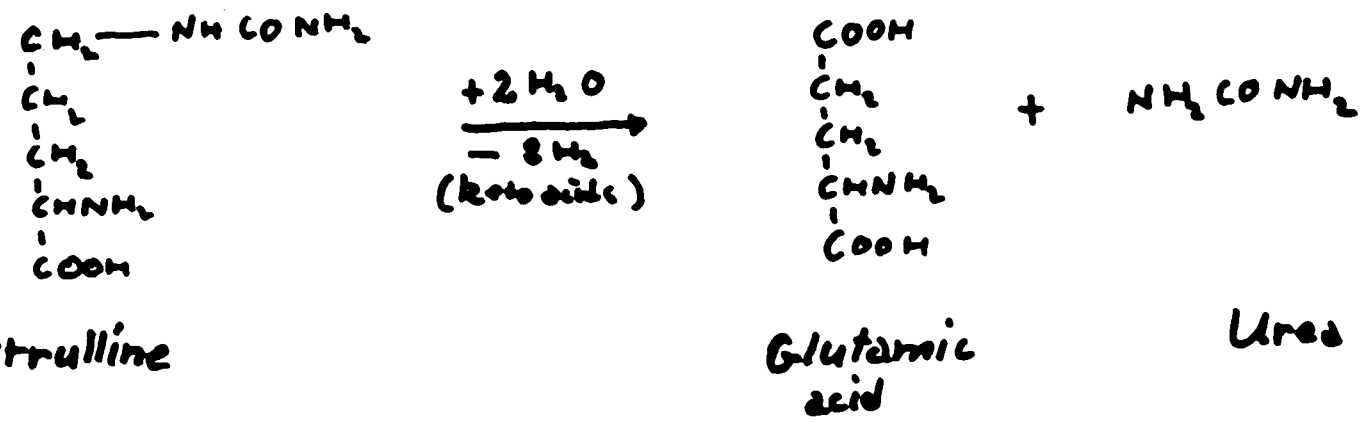


The crucial experimental evidence was the catalytic action of ornithine upon urea synthesis of rat liver slices in a medium containing ammonia and carbonic acid; i.e., ornithine enhanced the rate of urea formation without itself diminishing in concentration. Citrulline was assumed to be an intermediary since urea was formed as rapidly with citrulline as with ornithine provided NH_3 was present, Without NH_3 no urea was formed. Reactions (1) and (2); i.e., the arginine synthesis from ornithine, CO_2 and ammonia was dependent upon the intact living cell. Reaction (3), the formation of urea by arginine hydrolysis could occur with disrupted cell structure. One reason for this is presumably the fact that reaction (1) and (2) are endothermic, and that the energy required for these reactions must be furnished by respiration, glycolysis as source of energy not being able to replace respiration (Krebs 1934). Previously Loffler in 1918 had noted the dependence of urea synthesis upon respiration, and Salaskin & Kriwsky (1931) in liver perfusion experiments found that urea synthesis with Ringer-Locke solution only

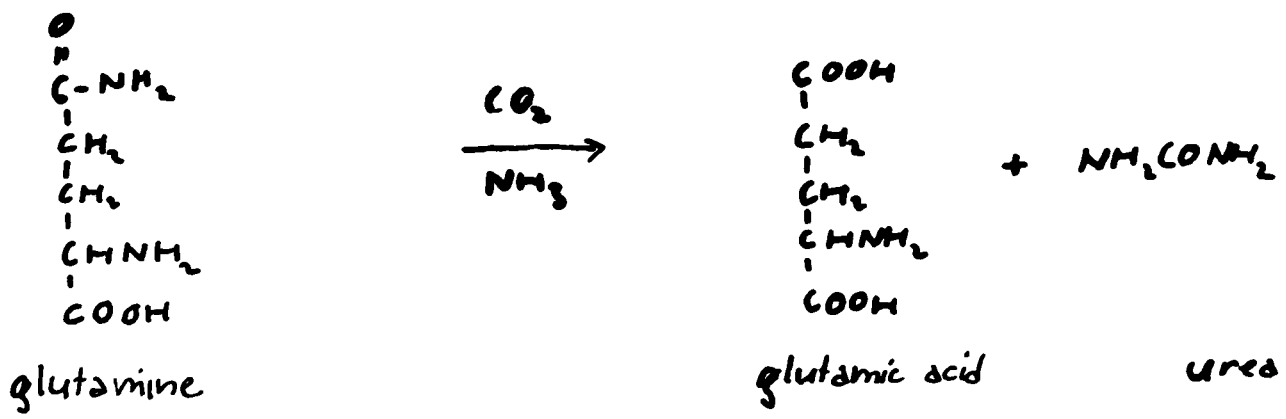
occurred upon the addition of erythrocytes, intact or haemolyzed, or a solution of oxyhaemoglobin. Liver was found to be the only site of urea synthesis from ammonia.

Alternative mechanisms of urea formation have been proposed. Leuthardt in 1938 suggested that the amide group of glutamine might enter into urea synthesis without intermediate NH_3 formation. That urea synthesis from glutamine did not occur over the ornithine cycle was concluded from the following findings. If glucose was the respiratory substrate much less urea synthesis occurred from ammonia than if pyruvate or lactate was applied. From glutamine, however, the same amounts of urea were formed with glucose as with pyruvate or lacate as respiratory substrate, Much more urea synthesis occurred from glutamine than from ammonia in the liver of starved animals. Ornithine was found to have hardly any influence on urea synthesis from glutamine. In his experiments with glutamine as substrate he found only 0.2-0.5 mg % free NH_3. He concluded that the reaction velocities of urea synthesis from ammonia at these concentrations of ammonia were too small to account for the urea actually formed.

Bach, 1939, proposed the following mechanism of urea synthesis: (1) "Oxidative Hydrolysis" of citrulline; end products glutamic acid and urea, with keto-acids acting as oxidizing agents.



(2) The synthesis of urea from glutamine, CO_2 and NH_3; end products glutamic acid and urea.



Ornithine was believed to act as precursor in urea formation by oxidation to glutamine. Glutamic acid could act as substrate after its conversion to glutamine by glutaminase.

The following observations were the basis for these postulates:

In the presence of citrulline and ammoniumlactate rat liver slices formed urea without an increase in amino N concentration. If ornithine had been regenerated an increase would be expected due to the delta amino nitrogen. (If citrulline was replaced by arginine the amino nitrogen increased markedly, presumably due to ornithine formation). The ratio of ammonia and nitrogen consumed to urea nitrogen produced was one instead of one-half, the theoretical figure for urea synthesis via the ornithine cycle.

In the presence of ammonium chloride, alpha keto-glutaric acid, and citrulline, a rise in amino and amide N occurred. The increase in amide N was interpreted as indicating glutamine synthesis brought about by the oxidising action of the keto acid (see reaction (1)) (the increase in amino N was presumably due to reductive amination of alpha keto glutaric acid by ammonium ions).

A small but distinct synthesis of urea was observed with citrulline and alpha keto-glutaric or pyruvic acid in the absence of ammonium ions, presumably after reaction (1).

Glutamic acid and glutamine both yielded urea in the presence, but not in the absence, of ammonium ions.

Ornithine, like citrulline, entered into urea formation in the presence of alpha keto-glutarate and ammonium chloride. Again an increase in amide nitrogen was observed. Urea synthesis did not occur, however, if ammonium chloride was absent. The increase in amide nitrogen and the necessity for ammonium chloride fitted in with the hypothesis of ornithine oxidation to glutamine, and urea synthesis after reaction (2).

In the presence of alpha keto glutaric acid and NH_4Cl urea synthesis occurred three times faster from arginine than from ornithine and 1.5 faster than from citrulline. Again little change in amino nitrogen was observed with citrulline, none with ornithine, but a marked increase occurred with arginine as substrate.

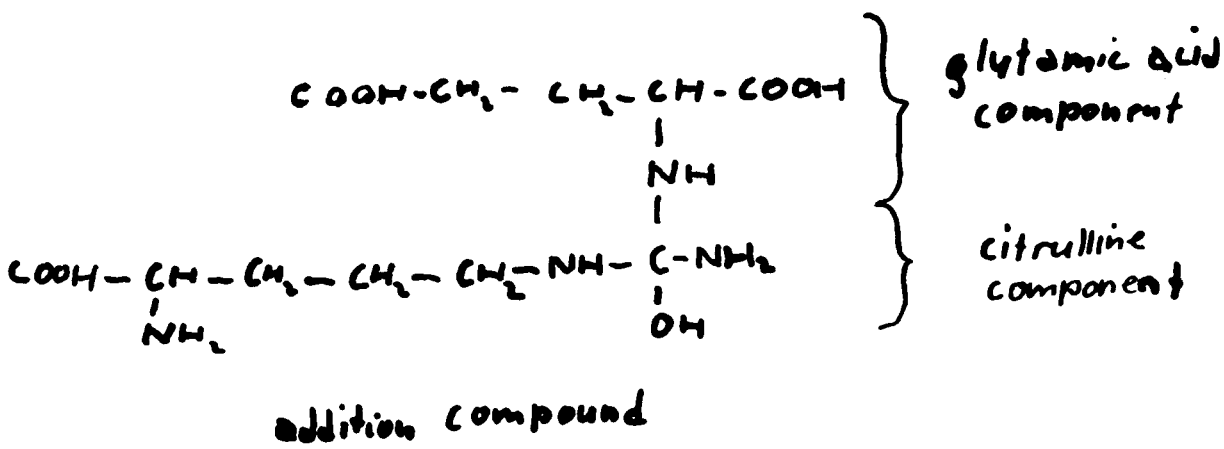
Archibald in 1945 claimed the enzymatic synthesis of urea from glutamine by cell free liver extracts from dog, beef, guinea pigs and humans (rats were not tested). The enzyme was differentiated from arginase by its lack of response to $MnCl_2$ which activates arginase, by its pH optimun at 7.0 or less (arginase pH optimun 9.4) and by the failure of borate to inhibit the urea formation.

Since the urea synthesis through the ornithine cycle depends upon intact cell structure and respiratory energy it could not play a part in the urea synthesis by the cell free liver extract. Accordingly, addition of ornithine to the

glutamine containing medium did not increase urea synthesis, and substitution of ornithine for glutamine completely prevented it.

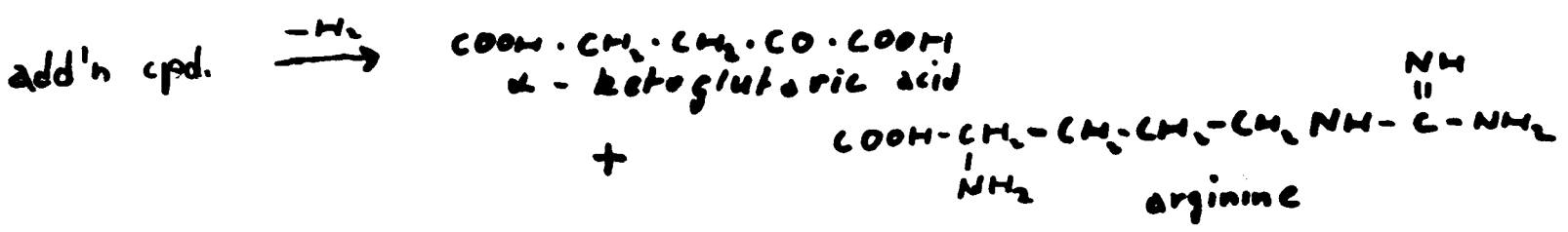
The extract was heated to 50° C in order to prevent the hydrolyzing activity of glutaminase. The production of urea from glutamine under conditions designed specifically to prevent the hydrolysis of the amide group seems to confirm Leuthardt's contention that the conversion of the amide group to ammonia is not involved in urea synthesis from glutamine.

In 1941, Borsook and Dubnoff, studying the guanidoacetic acid formation in the kidney, observed that among other substances citrulline could act as precursor. Citrulline was found to be converted to arginine which then donated its amidine group to glycine with ensuing guanidoacetic acid formation. Citrulline received the imino group not from ammonia as in the ornithine cycle but from the amino group of glutamic (or aspartic) acid through the formation of an addition compound.

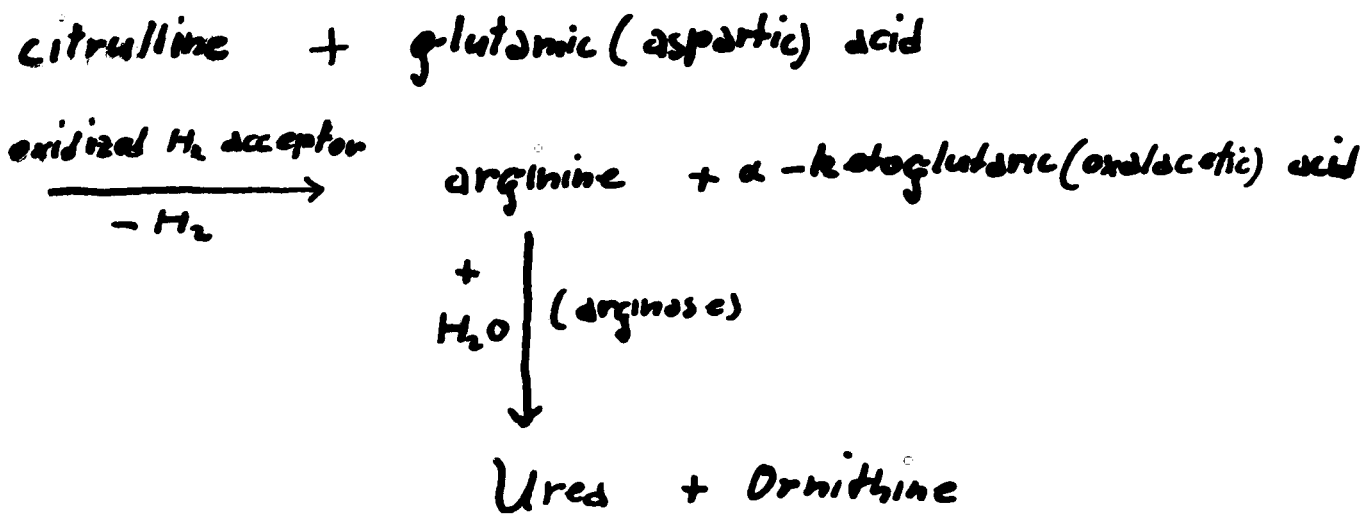


addition compound

This compound was then oxidized, with alpha keto glutaric acid and arginine formation.



This mechanism suggests another way of urea formation.



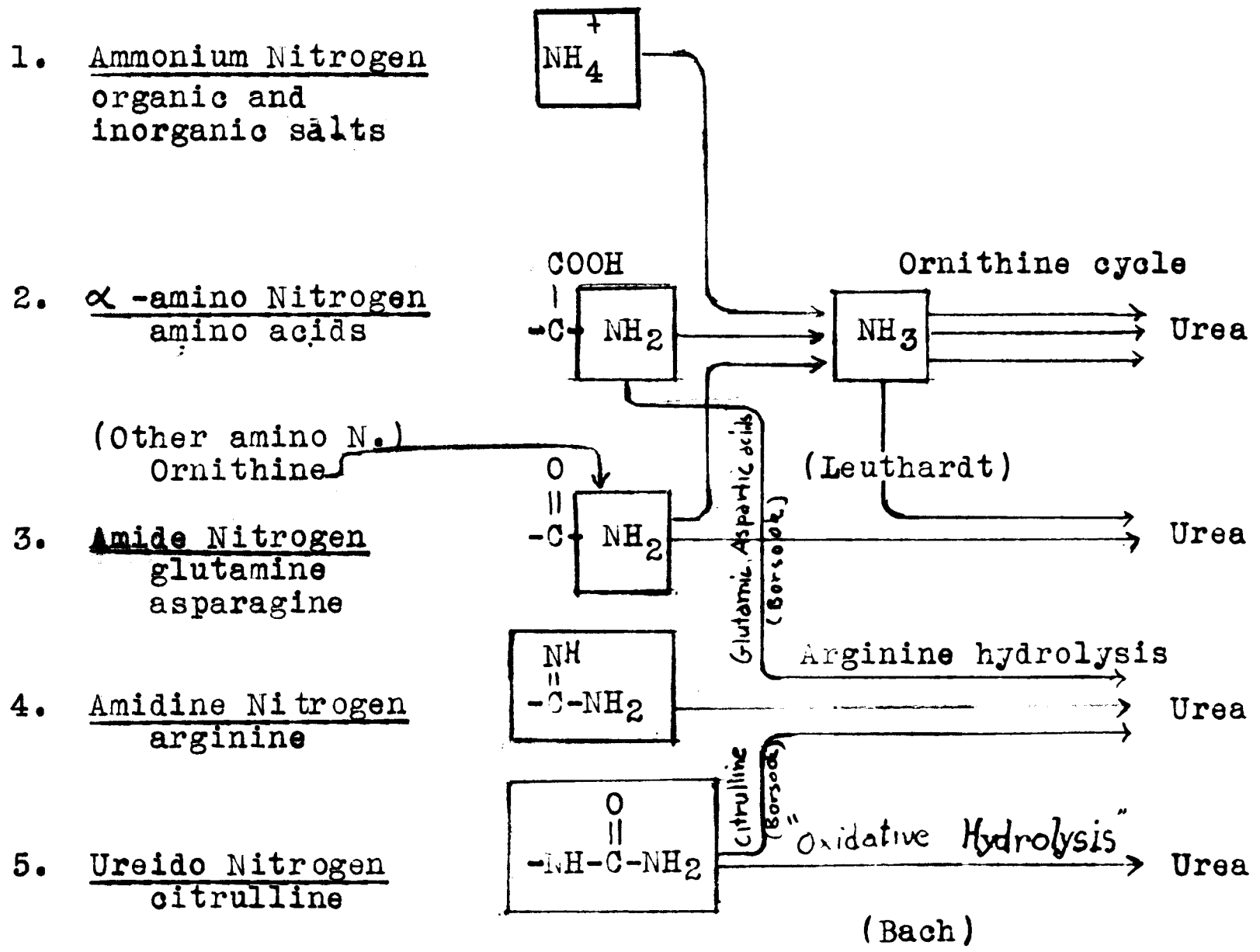
Some of these new pathways of urea synthesis are still debated. Krebs in 1942 took up Leuthardt's and Bach's work in detail and offered the following criticisms based upon his own experimental evidence. Although the rate of urea formation in livers of starved guinea pigs was from two to three times greater with glutamine as substrate instead of NH_4Cl the rate of urea formation from ammonium glutamate was of the same order as that from glutamine. Krebs assumed ammonium glutamate to be an intermediate in urea synthesis from glutamine over the ornithine cycle, (donating its ammonium ion to ornithine).

$$\text{Glutamine} \xrightarrow{\text{Glutaminase}} \text{Ammonium Glutamate} \xrightarrow[\text{Cycle}]{\text{Ornithine}} \text{Urea}$$

Ammonium lactate had a similar effect as ammonium glutamate or glutamine. Either of these substrates were believed to enhance the rate of urea synthesis indirectly by stimulating liver respiration. Krebs was unable to confirm the accelerating effects of the keto acids pyruvate and alpha keto glutarate.

Borsook's work was fully confirmed by Krebs. He suggested the possibility of urea formation from arginine, synthesized from citrulline and glutamic acid after Borsook's scheme, as one way by which glutamic acid could yield urea without intermediate NH_3 formation (see above).

In summarizing one might group the pathways of urea formation and the nitrogen donators involved, in the manner suggested in the following diagram.



Recent findings of the abundance of glutamine in animal tissue may emphasize its possible importance as a urea precursor whatever the pathway may be by which it contributes to urea formation. In muscle tissues it constitutes around 50% of the total amino acids, in blood 15 to 25%, in liver 20% (Hamilton 1945).

It is possible that glutamine may be catabolized and contribute to urea formation while still in peptide linkage. Melville in 1935 observed that the amide groups in d-glutaminyl-glycine, d-glutaminyl-d-glutamic acid and d-glutaminyl-glycyl-glycine were as labile as in d-glutamine. The possibility of catabolism of amino acids in peptide linkage has already been discussed in connection with dehydro peptidase activity. Urea and other nitrogenous products of protein catabolism might conceivably be obtained through enzymatic activity upon larger breakdown fragments of proteolysis.

6. **Factors affecting Protein Breakdown**: The factors known to affect directly or indirectly the chemical reactions involved in protein catabolism are so numerous that it can hardly be attempted to discuss them comprehensively. It was decided to single out the mechanism of proteolysis to illustrate the various ways by which enzymatically catalysed reactions may be affected.

(a) Factors influencing the rate of enzyme activity. The role of the molecular structure of the substrate in determining its susceptibility to proteolytic enzyme action has already been discussed in the section dealing with proteolytic enzyme specificity. In addition, the stereochemical configuration (Bergmann & Zervas, 1934, Bergmann, Zervas & Schleich, 1934), as well as the shape of the molecule, the degree to which the

peptide chains are folded(Haurowitz, Tunca, Schwerin & Göksu, 1945: Rice, Ballou, Boyer, Luck, and Lum, 1945) are decisive factors in determining its vulnerability to enzyme attack. Further, a substrate ordinarly immune to the action of a specific enzyme may become hydrolyzable if it is linked to another molecule, a so called co-substrate (Bergmann,1942). The presence of activators is also said to influence the range of enzyme activity although in this case the possibility that more than one enzyme is involved must be kept in mind.

(b) Factors influencing the rate of proteolysis: as with any chemical reaction the rate of hydrolysis of a protein or a peptide is dependent upon its concentration, and the concentration of end products . It is also dependent upon the concentration of the catalyst of the reaction, the specific proteolytic enzyme. Since the study of proteolytic enzyme kinetics has shown that in all likelihood the enzyme must combine with the substrate before the latter is hydrolyzed (Abderhalden & Michaelis, 1907: Grassmann & Klenk, 1929-30) the concentration of the enzyme substrate complex is of primary importance. Thus, the concentration of the substrate will not affect the reaction rate provided it is sufficiently high to saturate all the available enzyme.

Factors affecting the concentration of substrate, enzyme, and end products will affect the rate and extent of hydrolysis. Thus conditions favouring, or preventing, chemical reactions which remove the products of hydrolysis, such as deamination, and transamination will inhibit or hasten the establishment of an equilibrium between the products of the reaction and the reactant.

Among the factors influencing proteolysis by their effect on active enzyme concentration are temperature and pH which affect enzyme stability, inhibitors which render enzymes inactive by combining with them, and activators such as metal ions or sulfhydryl compounds which render them active. (Smith, Berger and & Bergmann, 1941;/ Johnson, 1940). Oxidizing agents such as molecular oxygen or redox systems with a more pegitive potential than the SS-SH system also influence proteolysis by their effect on active enzyme concentration, since they render the sulfhydryl activators inactive by oxidizing them.

Oxygen may affect proteolysis not merely through the oxidation of activators. Irving, Fruton and Bergmann (1942) mention the following mechanisms through which it may act. 1. In enzymes that are activated by SH compounds oxygen may inhibit by oxidizing these compounds thereby destroying their capacity as activators. 2. Oxygen may act on the enzyme protein itself. For example, the beta form of papain trypsinase can be converted into the active form by HCN or a coenzyme. Oxygen, however, converts the beta form into the alpha form which cannot be activated by HCN. 3. Under aerobic conditions, H_2S activators (cysteine, glutathione) bring about enzyme-inhibitor complex formations. This was found the case with carboxypeptidase, aminopeptidase, and trypsinase.

The hydrogen ion concentration greatly influences the rate of enzymatic proteolysis (independent of its effect on enzyme stability). The optimum pH is not only governed by the type of proteolytic enzyme but also by the nature of

the substrate. Thus pepsin, with optimal activity at pH 1.5-2.0 toward proteins is most active around pH 4.5 if the substrate is carbobenzoxyglutamylalanine (Fruton & Bergmann, 1939).

If proteolysis is measured in a medium containing a variety of substrates as well as a variety of enzymes, it must be kept in mind that the overall rate of proteolysis is determined by the sum of the individual reaction rates, all of which are affected to a different degree by such factors as have been mentioned.

Similar considerations may be applied to all other enzyme reactions occurring in the course of nitrogen catabolism. A change in concentration of reactants and end products will change the reaction rates. The effect will be dependent upon the order of the reaction one deals with. Thus destruction of cell structure which may affect the chemical reaction due to the ensueing dilution of reactants will be disruptive to a degree depending upon the number of molecules interacting. pH will affect the reaction rate according to the location and range of optimal enzyme activity on the pH scale. Oxygen will be an indispensible requirement when it constitutes a reactant, or it may inhibit where it is not involved as in the hydrolysis of arginine by arginase (Edlbacher, Kraus, & Leuthardt, 1933). Here the inhibition of oxygen is also influenced by the hydrogen ion concentration, being most marked at optimal pH (9-10), still appreciable at pH 7, and of no effect at pH 5. It is further influenced by substances with an affinity for oxygen (H_2S, ferrous compounds) which protect arginase activity by combining with oxygen.

Specific inhibitors and activators exist for all these reactions. Using arginase as an example all l-alpha amino acids were found to inhibit its activity (Hunter & Downs, 1945). The inhibitors and activators may themselves be inhibited or activated (e.g., activation of d-amino acid oxidase inhibitor by anaerobic conditions) and so forth.

The discussion of the chemical reactions concerned in protein breakdown and the factors affecting them has been included in this paper dealing with the effect of damaging stimuli on protein metabolism in order to show the variety of channels through which such stimuli may exert their effect.

II. PROTEIN METABOLISM AFTER DAMAGE.

If a normal, well fed, animal is subjected to a sudden damaging stimulus such as bone fracture, exposure to cold, or formaldehyde infection it will show characteristic changes in its blood, urine and other body constituents which point to an accelerated rate of protein breakdown.

1. Blood Changes: In rats exposed to cold, formaldehyde injection, and forced exercise a rise in blood NPN was observed amounting, in many cases, to 100%-200% of the initial value (Schenker, 1939).

In cats and dogs subjected to head injuries and crushing wounds of the limbs a rise in blood amino nitrogen and a fall in blood pressure was noted by Lurje in 1936. Traumatic shock induced in dogs and rats by mallet pounding or placing in a revolving drum caused an increase in serum potassium in these animals indicating increased cellular permeability or cellular breakdown.

A nearly two fold increase in alpha amino nitrogen and glutamine was found in the plasma of dogs subjected to trauma (Hamilton, 1945). In rats subjected to 300-700 revolutions in a revolving drum Neufeld, Toby and Noble in 1943 observed a rise in NPN of the blood from 38 mgm% to 60 mgm%, in the blood amino nitrogen from 9.4 to 13.4, urea from 14.7 to 21.6, uric acid from 0.82 to 1.48, creatine from 1.86 to 2.94 and rest nitrogen from 5.4 to 10.2 mgm%. A slight rise in potassium concentration was also noted. These changes were not related to blood volume changes. Engel, Winton and Long in 1943 observed a rise in blood amino nitrogen in rats subjected to a shock inducing hemorrhage, but only if it was severe enough to be fatal. The rise was observed regardless of whether the animals were intact, adrenalectomized or adrenaldemedullated. It was associated with the decrease in blood pressure and therefore with a decreased blood supply to the liver.

In experiments with eviscerated rats and cats exposed to hemorrhage these investigators noted a high negative correlation between the blood amino nitrogen level and the portal venous oxygen saturation as well as arterial blood pressure. The arterial oxygen saturation remained normal (Engel, Harrison and Long, 1944). The authors concluded that a decreased ability of the liver to deaminate as well as an increased rate of amino acid production by protein breakdown were responsible for the rise in blood amino nitrogen. To rule out the factor of impaired deamination by the liver, experiments were performed in the same laboratory on rats

whose gastrointestinal tract, pancreas, spleen and liver had been removed (Russell, Long and Engel, 1944). Rats treated in this manner showed a greater rise in blood amino nitrogen after bleeding than similarly treated rats not subjected to hemorrhage. Furthermore, in intact rats subjected to hemorrhage a greater increase in blood amino nitrogen was observed than in liverless rats subjected to hemorrhage in spite of the fact that removal of the liver impairs deamination. Since the liver, because of its size and supply of amino acids from the intestine, is the main site of deamination (Bollman, Mann and Magath 1926; Krebs, 1935) (although other organs are able to deaminate, the kidney cortex at a greater rate than the liver, (Krebs, 1935)), blood amino nitrogen rise elicited by bleeding the liverless rats indicated mainly an increased peripheral protein breakdown even though an impaired deamination ability of the remaining tissue might have been a contributing factor. Decreased renal function was not believed to be responsible for these results because of the short duration of the experiments.

In view of the close relationship of protein and carbohydrate metabolism some findings on the effect of damage on the levels of blood sugar and intermediates of carbohydrate metabolism will be mentioned briefly. Neufeld et al. in 1943 noted a rise in blood sugar, pyruvic and lactic acid content of the blood in rats subjected to the revolving drum. Engel, Winton and Long in 1943 (see also Engel, Harrison and Long, 1944) observed an initial rise and a subsequent fall in blood sugar, a rise in blood lactate and pyruvate, and an increased lactate/pyruvate ratio in rats subjected to shock by hemorrhage.

A fall in blood sugar and rise in blood keto (and amino) acids also occurred in the absence of the entire adrenal. The initial rise in blood sugar only occurred in the presence of the adrenal medulla and in well-fed animals with adequate liver glycogen stores. In eviscerated, demedullated rats shock also elicited a fall in blood sugar and a rise in blood pyruvate and lactic acid.

These findings can be interpreted in various ways. An increased mobilization of liver or other glycogen would induce the initial rise in blood sugar observed. Since mobilization of glycogen is one of the main functions of adrenalin, and the rise did not occur in adrenal demedullated animals this seems a plausible explanation. Increased gluconeogenesis and decreased utilization of glucose may be contributing factors. Since a drop in blood sugar occurred shortly after the initial increase such mechanisms are either of short duration or superimposed by others; e.g., increased breakdown of glucose.

The rise in lactate/pyruvate ratios may indicate a shift from oxidative glucose breakdown to glycolysis. The increase in blood pyruvic and lactic acids was attributed by Engel et al. to a decreased oxidation of these substances as a result of decreased oxygen supply.

Long and his collaborators pictured the effects of shock on protein and amino acid metabolism to occur by the following mechanism. A decrease in circulating blood volume and diminished blood flow result in diminished oxygen supply to the tissues. Most affected are the peripheral tissues, especially the extremities, and the liver. The anoxia

causes an increased rate of protein breakdown; the breakdown products accumulate in the blood since they are not taken up or deaminated sufficiently rapidly by the liver owing to:

a. A greater supply than the liver can normally handle;

b. An impaired ability of the liver to handle them due to damage caused by liver anoxia.

In man similar changes in the blood picture have been observed. After short or prolonged exercise (15 minutes stairclimbing; 2-3 hours bicycling) Rakestraw (1921) noted an increase in blood uric acid concentration, after prolonged exercise a rise also in blood urea and creatine concentration. Surgical trauma, severe head injuries and perforations of the stomach were found to be associated with a high amino nitrogen level in the blood (Lurje, 1936). It is of interest to note that this investigator observed a rise in blood amino nitrogen following surgical trauma only in cases with intact liver innervation. If operations were associated with partial or total exclusion of liver innervation; e.g., paravertebral or splanchnic anaesthesia, no increase in blood amino nitrogen was detected. Animal experiments confirmed this finding. The author interprets the rise in blood amino nitrogen to be the result of a "reflex provoked endogenic breaking down of protein".

Cuthbertson (1929b) was, however, unable to detect any rise in blood NPN and urea during the first two weeks following bone fracture and injury to nonbony parts although subsequently the values approached the upper limits of normal. In many cases he observed a rise in inorganic phosphorus.

The elevated phosphorus and nitrogen levels were not coincident with the rise in nitrogen, phosphorus and sulphur excretion in the urine, the latter occurring within the first two weeks.

Comparatively little has been done on the effect of sudden trauma upon the concentration in the blood of enzymes concerned with protein breakdown. Pfeiffer in 1914 noted an increased proteolytic activity in the serum of burn cases. Zamecnik, Stephenson and Cope in 1945 studied the peptidase activity of lymph and serum and consistently observed an increase in enzymatic activity towards the substrate l-leucylglycylglycine in the lymph draining from the areas subjected to burn or muscle crushing. An increase in activity toward this substrate was also observed in the serum of cats, rats and calves following burns. In lymph the activity paralled the protein concentration. Since the rate of lymph flow from burned areas is greatly increased, the increase in enzyme activity is even more marked per unit time. Since muscle, skin and subcutaneous tissue, and erythrocytes all showed 3 to 20 times greater activity towards the tripeptide than normal lymph it was concluded that the increased activity in lymph and serum following trauma was due to a release of the enzyme from the traumatized tissues. As the optimum pH was approximately 7.4 it does not seem unlikely that the enzyme release may be partly responsible for the protein catabolic process. In this connection the observation that dissolution of lymphocytes can be induced by fasting is of interest.

Iyengar, Sehra and Mukerji in 1942, published findings on the tryptic activity of plasma under various pathological conditions. They observed a significant increase in free trypsin content; i.e., trypsin not bound to an inhibitor, of plasma in nephritis and suggested that the trypsin activity might be an index of enhanced protein catabolism. An increase in blood polypeptides depending on the clinical severity of the nephritis had been observed by Puech and Cristol (1929). Again using leucylglycylglycine as a substrate Grassmann and Heyde in 1930 noted an increased proteolytic activity (pH optimum 7.4-7.5) in the serum of patients with fever.

From the examples mentioned it seems possible that increased proteolytic enzyme activity in the blood may be associated with increased protein catabolism. It is interesting to note that under conditions in which new tissue and therefore protein synthesis occurs a decreased proteolytic enzyme activity has been observed in blood. In cancer patients a decreased tryptic activity has been noted, (Iyengar et al, 1942; Vercellana, cited by Iyengar et al). During tumor growth and pregnancy in rats a fall in plasma proteinase activity was observed by Weil and Russel in 1938.

2. Urine Changes: The changes in urinary constituents following various forms of trauma are in agreement with the hypothesis of increased protein catabolism. As early as 1904 Hawk and Gies observed an increase in the nitrogen and sulphur containing products in the urine of dogs subjected to external haemorrhage amounting to 3 to 3.5% of body weight. Transient glycosuria was also noted.

Cuthbertson, McGirr, and Robertson in 1939 noted a rise in urinary nitrogen sulphur, phosphorus, potassium and creatine excretion following fracture of the left femur in rats which attained a maximum at 3-4 days postoperatively. The loss of muscle substance due to atrophy of the injured limb was not sufficient to account for the increased nitrogen excretion, and the investigators concluded that there was a general increase in catabolism to meet the exigencies of the enhanced metabolism of the recuperative process. This was corroborated by the fact that supplying additional carbohydrates to the diet exercised a definite sparing effect on the general loss of tissue substance, although the local atrophy could not be prevented. Phosphorus, sulphur, nitrogen, potassium and creatine excretion were all reduced although the increased excretion of these substances following femur fracture could not be entirely suppressed by supplying extra carbohydrates. However this response to femur fracture did not occur if the rats had been on a low protein diet for several weeks prior to the operation (Munro and Cuthbertson, 1943). Both the rise in nitrogen excretion following femur fracture in the rat with a maximum 3-4 days postoperatively and the lack of such a response in protein depleted rats have been confirmed in this laboratory, (Cohen 1945). A heightened urinary creatine excretion in rats during exposure to either cold, muscular exercise or formaldehyde injections was observed by Browne, Karady and Selye in 1939, the maximum creatine excretion occurring 24-48 hours after beginning of exposure to the stimulus.

Cuthbertson (1929b) observed an increased rate of nitrogen and sulphur excretion in man following bone fracture as well as injury to nonbony parts. A rise in urinary nitrogen excretion rate after bone fracture and burns as well as other types of trauma has been noted in many cases in this laboratory, (Schenker, 1944). The period following trauma during which the nitrogen output exceeded the intake and nitrogen was therefore lost from the body was termed 'protein catabolic phase', since such large quantities of nitrogen loss could only be explained by assuming a loss of body protein. This period was always followed by one during which the amount of nitrogen ingested exceeded the amount excreted; this phase was termed 'protein anabolic' on the assumption that nitrogen was being laid down as protein. It was concluded that in the first phase, immediately after trauma, protein catabolism outweighed the anabolic process, in the second phase the rate of protein anabolism exceeded the catabolic rate.

Withdrawal of food may also elicit changes in urinary excretion products that may indicate an accelerated rate of protein breakdown. Cohen in 1945 observed a rise in urinary nitrogen excretion upon withdrawal of food in rats kept on a protein free diet for a prolonged period. Various investigators have observed a rise in nitrogen excretion during the second to fourth day in starvation, (Lucian, Munk, Johannson, Benedict, Cathcard; cited from Cuthbertson, 1929b).

It may be well to mention that Cuthbertson also noted a rise in urinary sulphur, nitrogen and phosphorus excretion in patients as a result of immobilization (1929a). The nitrogen increase was due to a rise in urea excretion Mainly. The

sulphur/nitrogen ratio suggested a sulphur-rich source, presumably muscle protein. He ascribed this to irritation of the muscle by nervous stimuli.

In connection with the assumption that damage stimulates protein catabolism the observation that patients postoperatively excrete increased amounts of cortin like material with gluconeogenic properties is of interest (Wenning, Hoffman and Browne, 1943).

c. Tissue Changes: The effect of fasting on liver protein levels has recently been studied by Harrison and Long in 1945. A 32% protein loss was observed in rat livers during a 48 hour fast. Unless one assumes that the proteins were shifted to some other part of the body, these results indicate that fasting is a marked stimulus to liver protein catabolism. Kosterlitz in 1944 noted that rats on a protein free diet lose 15% of their initial liver cytoplasm on the first day, 7% on the second, 5% from the second to the seventh days and a further 5% in the second week on the protein free diet. From protein, phospholipid and nucleic acid ratios he concluded that chromophilic particulates; e.g., mitochondria, were lost as well as interparticulate protein of the cytoplasm. This is of interest since enzymatic activity of the cell is associated with cytoplasmic particulates and may thus be diminished by eliminating protein from the diet (or by complete withdrawal of food). It has been shown that liver arginase activity decreases with decreasing protein content in the diet (Lightbody and Kleinman, 1939).

The same effect of fasting on liver protein levels has been observed in this laboratory (Hoffman, 1946). It was also found that fracturing the femur of a rat at the onset of the 48 hour fast caused a greater reduction in liver protein than the 48 hour fast alone, suggesting an increased catabolic response due to the operation.

In 1932 Krebs and Henseleit in their famous paper on the mechanism of urea synthesis via the ornithine cycle showed that urea formation from added ammonium ions proceeded less rapidly in liver slices from fasted animals (24-48 hour fast) than from well fed ones. This was ascribed to lack of energy-furnishing substances in the starved livers.

Leeffler's results in liver perfusion experiments however seem to indicate that urea formation is greater in livers taken from dogs starved eight days than from dogs which had been fasted only two days (1918). Leuthardt in 1938 observed that in guinea pig liver slices previous fasting caused a fourfold increase in urea production from glutamine. He found that urea formation from glutamine was not dependent upon the 'respiration substrate' in contrast to urea formation from added ammonium ions. These and other considerations led Leuthardt to postulate an alternative mechanism of urea formation besides Krebs ornithine cycle which has been discussed in a previous section. This would make the opposite results of Leuthardt and Krebs on the effects of starvation compatible, since different mechanisms of urea formation might be influenced in different ways by starvation. It will be remembered that Leuthardt's hypothesis of urea formation is still contended.

Since urea formation from amino acids is an energy consuming process one might expect a decreased supply of oxidative energy and therefore a decreased oxygen consumption in tissues under conditions that inhibit the deaminating power of that tissue. In the experiments in Long's laboratory mentioned earlier the rise in blood amino acids following shock induced by hemorrhage was attributed partly to a decreased deaminating power of the liver. In vitro studies on the oxygen consumption of liver and kidney slices from rats in hemorrhagic shock showed that the Q_{O_2} (mm^3 oxygen consumption per milligram dry weight per hour) was depressed in proportion to the severity of shock using the blood amino nitrogen level as an index of the degree of shock (Russell, Long and Wilhelmi, 1944). Beecher and Craig in 1943 however were unable to observe any changes from normal in the oxygen consumption of slices of brain cortex, heart muscle, kidney cortex and liver from cats in profound hemorrhagic shock.

Comparison of Q_{O_2} from fasted with those from fed rats in Long's laboratory (unpublished work cited by Craig, 1943) indicated a sharp depression in oxygen uptake in the livers taken from rats fasted for 24 hours. A similar effect of partial food withdrawal on liver Q_{O_2} was observed by Tipton in 1941. Craig himself noted an increased Q_{O_2} due to fasting. These results are of dubious value, however, since the investigation did not take into account the loss of liver glycogen due to fasting which resulted in an increased concentration of respiring liver tissue. Fuhrman and Field in comparative studies on fasted and well fed rats directed

attention to this source of error (1945). They were able to relate the $\dot{Q}_{O_2}$ to the glycogen content of the liver. This work will be discussed in a later section.

Various effects on tissue enzymes due to trauma have been observed. As has been mentioned earlier one of the conditions associated with shock is anoxia which might be expected to favor proteolytic enzyme activity by a variety of mechanisms among them the reduction (and hence activation) of proteolytic enzyme activators. Anoxia is also held responsible for a decreased deaminating power of the liver in shock (Engel, Winton and Long, 1943; Engel, Harrison and Long, 1944). Liver slices placed in an atmosphere of nitrogen subsequently show a reduced oxygen consumption under aerobic conditions (Russell, Long and Wilhelmi, 1944) and a decreased ability to metabolize amino acids (Greig,1944b). The experiments of Russell et al. indicated that one of the effects of anoxia might be a destruction of coenzyme factors since supplying these factors in a liver Kochsaft preparation partly counteracted the effect of anoxia on the $\dot{Q}_{O_2}$ provided the shocked animals had been in 'good' condition.

In extensive investigations on the effects of shock induced by hemorrhage conducted by Govier, Grier, Greig and De Turk the following observations were made. Coenzymes active in the catabolism of various intermediary metabolites were inactivated by the effects of shock. Thus a decrease in thiamine diphosphate (cocarboxylase) by dephosphorylation to thiamine monophosphate or thiamine as a result of shock induced by hemorrhage was noted in muscle, liver and duodenum

(Greig and Govier, 1943). After thiamine therapy cocarboxylase was resynthesized; these findings help to explain the earlier observations of a beneficial effect of thiamine therapy on survival time in shock (Govier and Greer, 1941a), of a rise in blood keto acids in shock and fall after thiamine treatment - since the metabolism of keto acids through the tricarboxylic acid cycle requires cocarboxylase - (Govier and Greer, 1941b) and of the correlation of plasma thiamine content with resistance to shock (Govier, 1943). The degree of cocarboxylase dephosphorylation by liver tissue was found to vary directly with the severity of cellular damage, (Govier and Greig, 1943).

A decrease in diphosphopyridine nucleotide (coenzyme I) in tissues following hemorrhagic shock has also been reported by Greig (1944a).

Of more immediate interest in connection with nitrogen metabolism is the decrease of an isoalloxazine nucleotide reported by Greig (1944b) in tissue of animals subjected to shock. The coenzyme tested was alloxazine adenine dinucleotide (Flavine Adenine Dinucleotide), a prosthetic group required for the oxidative deamination of d-aminoacids as well as for the oxidation of many other nitrogenous and non-nitrogenous substances. It is true that the prosthetic group of the l-aminoacid oxidase in rat liver and kidney isolated by Blanchard and Green is the alloxazine mononucleotide (riboflavin phosphate) but the possibility lies at hand that if shock conditions inactivate the dinucleotide they might also affect the mononucleotide. Furthermore, Green's l-amino oxidase was found only

in the rat and not in the dog, the experimental animal employed by Greig. It may be noted that Greig's experimental evidence for a decrease of DPN and FAD in tissues following shock is not entirely convincing. Greig was able to demonstrate that liver extract contained a substance that inhibited amino acid oxidation by liver slices or by the isolated d-amino acid oxidase. The inhibitor appears to be a globulin-like substance which acts by forming an apoenzyme-coenzyme-substrate-inhibitor complex (Greig and De Turk, 1945a). This complex formation is favored under conditions coinciding with those occurring in shock. Intravenous injection of a partially purified liver extract containing the inhibitor was found to increase the blood amino acid level in dogs by an average of 118% in six hours, (Greig and De Turk, 1945c). The investigators suggest that this mechanism may operate in shock.

A similar but not identical inhibitor of lactic acid dehydrogenase was also detected by these workers (Greig, and De Turk, 1945b).

From the experiments cited so far it seems that anoxia may play an important function in regulating tissue metabolism. Experiments by Bernheim and Bernheim in 1946 on the nitrogen metabolism of tissue slices under aerobic and anaerobic conditions are of interest in this connection. The authors noted that anaerobic conditions caused an increased protein loss from kidney slices, a slight increase in non-protein nitrogen formation, a large increase in amino nitrogen and a decrease in ammonia nitrogen formation over a three hour period. In liver slices an increased protein loss was also observed but NPN and amino nitrogen formation was decreased whereas

ammonia nitrogen formation was correspondingly increased under anaerobic conditions. The findings in liver were thus exactly opposite from those in kidney slices except for the protein loss. The protein loss was presumably due to increased leakage occasioned by cellular damage and increase in cellular permeability. The investigators conclude that in the liver there is an actual inhibition of proteolysis under anaerobic conditions. The increased amounts of ammonia in liver slices incubated anaerobically are ascribed to the decrease in urea production under these conditions. The source of anaerobically formed ammonia was not determined but glutamine was suggested as a precursor. The effect of anoxia on liver slices is unexpected in view of the known inhibitory effect of oxygen on proteolytic enzyme activity (Irving, et al, 1942). All experiments were performed at pH 7.4 and 37 degrees C., and, as has been mentioned, on slices. When broken cell suspensions of liver and kidney were made by grinding the tissues in a mortar with sand, amino acid production was the same under aerobic or anaerobic conditions and in the presence or absence of HCN. These results are in disagreement with those of Voegtlin and Maever (1932) and of Bailey, Belfer, Eder and Bradley (1942). The latter investigators found that autolysis of liver suspensions was inhibited by oxygen even at pH 7.5, although the inhibition was more marked in lower pH regions. Autolysis was also inhibited at all hydrogen ion concentrations tested, by the oxidants KIO_3, KIO_4, I_2, $CuSO_4$. Since these substances also abolished the nitroprusside reaction (a test for SH compounds), the

inhibition was assumed to be caused by oxidation of activators. An inhibition of autolysis in liver suspensions at pH 7.5 by KIO_3 was also observed by Luck, Eudin and Nimmo in 1939.

In connection with autolysis and trauma, experiments on denervated atrophied muscle tissue have yielded interesting results. Chen, Meek and Bradley in 1924 found that atrophied muscle tissue autolysed more rapidly than normal muscle tissue at acid or neutral pH. Autolysis was measured by amino acid production and tyrosine liberation. The changes in pH during autolysis were similar in normal and atrophied muscle and hence not responsible for the different results. Autolysis was presumably not aerobic and at room temperature. This experiment was thus successful in demonstrating increased protein catabolism in vitro in a tissue that was the site of extensive protein breakdown in vivo - the muscle tissue had lost 50% or more of its protein before the animal was sacrificed.

Various degenerative changes in pathological anatomy have been found to be associated with the aftereffects of acute damaging stimuli (Selye, 1940). These include involution of the thymus, lymph nodes, spleen and other lymphatic organs, of the pancreas and thyroid: nuclear pyknosis and degranulation of the anterior pituitary, especially of eosinophils: discharge of the chromaffin granules from the adrenal medulla, and sometimes necrosis of the medulla: decrease in liver size accompanied by focal necrosis and fatty infiltration: and ulcers in the gastro-intestinal tract.

In contrast, the adrenal cortex undergoes a marked

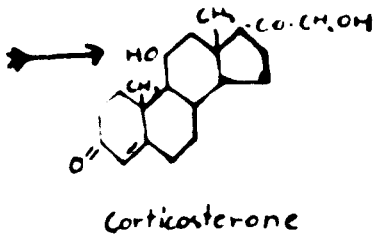
enlargement with hypertrophy of its individual cells and discharge of their lipid granules which are believed to contain the cortical hormones. These changes in the adrenal cortex have been observed in rats, rabbits and guinea pigs as a result of many widely different damaging stimuli, including burns, infectious diseases, intoxication with various drugs, Xrays and ultraviolet rays, exposure to low temperature, and formaldehyde injections (Selye, 1940). They are significant in view of the alleged effect of the cortical hormones on protein metabolism (to be discussed in the following section).

III. THE ADRENAL CORTEX AND PROTEIN METABOLISM: In order to establish a particular function ascribed to an endocrine gland the following proofs are required:

(1) The postulated effect should be absent or at least diminished upon removal of thegland. (2) The administration of its hormones to an animal after removal of the gland should again elicit that effect. (3) The administration of the gland's hormones to the intact animal should increase that effect provided the normal hormones do not already suffice to bring about a maximal reaction.

The hormones secreted by the adrenal cortex may be grouped into three types according to the activity ascribed to them; and their chemical structure.

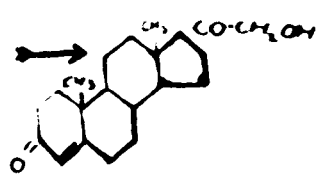
(1) Steroids with an oxygen on Carbon 11. Examples corticosterone, 11-dehydrocorticosterone, 11-dehydro, 17-hydroxycorticosterone. Because of the protein catabolic, and gluconeogenic activity ascribed to them, They are also referred to as



Corticosterone

S-hormones, sugar active hormones, glyconeogenic hormones, protein catabolic hormones.

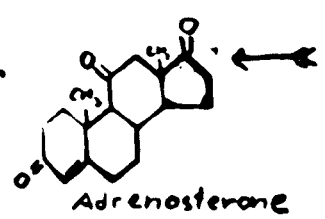
(2) Steroids lacking an oxygen on Carbon 11



Desoxycorticosterone

Example Desoxycorticosterone acetate (DCA)
They are associated with electrolyte metabolism.

(3) Androgenically active 17 ketosteroids or derivatives thereof.



Adrenosterone

Example adrenosterone. Because of their anabolic effect on protein metabolism they are referred to as N-hormones, protein sparing hormones, protein anabolic hormones. They have similar metabolic effects to testosterone.

The following discussion is concerned mainly with the functions associated with group (1).

1. Blood Changes: A great deal of work has been done on the effect of adrenal insufficiency on the blood sugar. From a review of these investigations published by Long, Katzin and Fry in 1940 it appears that while many observers associate a fall in blood sugar with adrenalectomy, there are other investigators who do not confirm this finding. The authors themselves noted that adrenalectomized rats maintained on sodium salts showed reduced blood sugar levels. Fasting caused a much more rapid decline of the blood sugar level in adrenalectomized than in normal rats. The administration of cortical extract or crystalline steroids oxygenated at C_{11} to adrenalectomized and normal animals elicited slight hyperglycemia. The administration of cortical extract to fasted, to depancreatized, and to fasted hypophysectomised depancreatized rats caused a rise in blood sugar.

The findings on the effect of cortin administration in raising blood sugar levels are in agreement with the work of many investigators. Other workers, however, did not find such an effect (Parkins, Hays and Swingle, Harrop and Weinstein; cited by Long et al, 1940).

In patients suffering from Addison's Disease (chronic adrenal insufficiency due to atrophy or destruction of the adrenal cortex) a low blood sugar level is generally but not always concomitant.

A decrease in blood sugar and pyruvic acid content of the blood in adrenalectomized rats has been observed by Schmidt in 1942, although the total bisulfite binding capacity of the blood was increased. The low pyruvic acid levels were believed to be a result of the depletion of glycogen stores (abbaufähiges Glykogen). However, other factors such as increased pyruvate oxidation, and decreased alanine deamination could equally well account for decreased pyruvate levels. Similarly various conditions may affect blood sugar levels, among them changes in mobilization from glycogen, in neogenesis from protein, and in peripheral oxidation.

The effect of adrenal cortical activity on the maintenance of plasma protein levels deserves mention, Levin and Leathem (1942a, 1942b) found that adrenalectomy in cats caused no effect on the total plasma protein content although its concentration increased due to haemoconcentration. The albumin fraction however was decreased both in concentration as well as in total amount. The globulin fraction remained the same in absolute amount. The albumin level also dropped in

hypophysectomized animals. In both cases this drop could be counteracted by cortical extract or DCA administration.

Levin and coll. laborators suggest that part of the protein catabolic action of the S-hormones may be for the resynthesis of vitally important proteins such as serum albumin.

A rise in the serum beta, and especially the gamma globulin fraction has been observed in rabbits following injection of adreno-corticotropic hormone (ACTH) and adrenal cortical extract (White and Dougherty, 1945). These investigators also demonstrated that augmented pituitary-adrenal cortical secretion accelerates the rate of release of antibodies from the lymphoid tissue of immunized rabbits and brings about increased antibody titres in the serum of such animals (Dougherty, Chase and White, 1945). Injection of ACTH into adrenalectomized animals did not evoke these manifestations.

2. Urine changes: Long et al (1940) noted a marked decrease in nitrogen excretion by fasted adrenalectomized animals compared to normal animals under similar conditions. Administration of cortical extract and steroids oxygenated at C_{11} to fasted normal or fasted adrenalectomized rats brought about an increased nitrogen excretion. This was true also in hypophysectomised animals.

The increased nitrogen excretion was found to be sufficiently large to account for the rise in carbohydrate levels of blood and other tissues by assuming the conversion of proteins to carbohydrates. In view of the large amounts of N excreted the source was held to be not only the liver but also other tissues, especially muscle tissue.

A decreased glucose excretion was observed in partially depancreatized rats following adrenalectomy. Cortical extract, and "sugar active" steroids caused an increased urinary sugar excretion in these animals, also in partially depancreatized rats with intact adrenals, and in hypophysectomized depancreatized rats. DCA was much less effective than the steroids oxygenated on C_{11}.

In patients with hyperplasia and tumors of the adrenal cortex glycosuria is a frequent finding.

3. Tissue Changes: In reviewing previous investigations on changes in tissue carbohydrate levels associated with adrenal cortical activity Long et al, again emphasize the disagreement between the results obtained by different workers. Long et al noticed no differences in the liver and muscle glycogen levels between normal and adrenalectomized mice or rats maintained on sodium salts. Fasting, however, caused a more rapid decline in the carbohydrate level of both tissues.

Administration of cortical extract or steroids oxygenated on C_{11} to fasted normal, fasted adrenalectomized, and fasted hypophysectomized animals caused large increases in liverglycogen, but no effect on muscle glycogen. The rise in liverglycogen confirms earlier observations by Britton and Sylvette, but these investigators also noted a rise in muscle glycogen.

From studies on the distribution of known amounts of administered glucose Long et al. concluded that adrenal cortical extract also decreases glucose oxidation and increases liver glycogen deposition. In summarizing their investigations as

well as those of other workers, however, these authors interpret the findings on blood, urine, and tissue changes following adrenalectomy and cortical hormone administration as being due primarily to the absence or presence of the protein catabolic function of the cortical hormones.

In connection with the alleged action on protein catabolism ascribed to the adrenal cortex the striking effect of this organ on lymphoid tissue is worthy of mention (Selye, 1940). After exposure of an animal to damaging stimuli the thymus involution is most marked at a time when the adrenal cortex reaches its maximum development. Removal of the adrenal completely prevents the involution of the thymus. Cortin or DCA administration to normal and adrenalectomized animals elicits thymus involution. It is of interest to note that androgenic hormones (as well as estrogenic) also bring about thymus involution. (It will be remembered that androgenic hormones are excreted by the adrenal). The same protein catabolic effect on an organ is therefore elicited by two groups of hormones that supposedly have directly opposite effects on protein metabolism.

Dougherty and White (1945) observed a depletion of lymphocytes in the thymus and thoracic duct upon injection of adrenocorticotropic hormone or cortical hormones oxygenated at C_{11}. Marked pyknosis of lymphocytes was noted as well as the appearance of phagocytizing macrophages.

Several studies have been made on the effect of the adrenal upon more specific chemical reactions.

Jiminez-Diaz in 1936 observed a lowered deamination

rate of kidney tissue from adrenalectomized animals. These findings have been confirmed by Russell and Wilhelmi in 1941,(a) who also noted a decreased oxygen uptake in kidney slices from adrenalectomized rats maintained on high sodium, low potassium salts. The rate of dl-alanine, pyruvic-, l-glutamic-, alpha ketoglutaric-, and succinic acid but not of citric acid oxidation was impaired in kidney slices from adrenalectomized rats. Cortical extract and DCA raised the $\dot{Q}O_2$ to normal in kidney slices from adrenalectomized animals, above normal in slices from normal animals. The effect was most marked in the presence of alanine and glutamic acid. The $\dot{Q}O_2$ and QNH_3 increased by the proportions to be expected from the overall equation for oxidative deamination; (i.e., $QNH_3/\dot{Q}O_2$ equal 2, since two molecules of NH_3 are liberated by one molecule O_2 in the presence of catalase,(see page 6).

A lowered Q_{O_2} in kidney and liver slices of adrenalectomized animals was also observed by Tipton in 1941. Pyruvate, dextrose and, in particular, succinate were found to increase the $\dot{Q}O_2$ in adrenalectomized rats less than in normal ones. The changes in tissue oxidation of adrenalectomized rats were however largely alleviated by supplying extra salt to the diet. Tipton suggests that the enzymes concerned with the oxidation of pyruvate and succinate are inhibited by adrenalectomy, especially the enzymes concerned with pyruvate oxidation since the latter was suppressed more than succinate oxidation.

The findings of a decreased $\dot{Q}O_2$, a decreased deaminating power of the tissue, as well as the apparent effect on

enzymes concerned with pyruvate metabolism are similar to the observations on liver tissue of animals in hemorrhagic shock discussed in an earlier section.

Russell and Wilhelmi (1941) compared the respiration of kidney slices from normal with those from adrenalectomized animals in the presence of glucose and found no significant difference either in the $\dot{Q}O_2$ or in the rate of disappearance of the added glucose. They further compared the amount of carbohydrate synthesized by kidney slices from normal and adrenalectomized animals in the presence and absence of added substrates. No significant difference between the two types of tissues in the amounts of carbohydrate formed was noted with succinate, or in the absence of substrate. A marked decrease in carbohydrate formation was, however, observed in kidney slices of adrenalectomized rats from the substrates dl-alanine, l-glutamic, and alpha ketoglutaric acid. They conclude that adrenalectomy causes a decrease in gluconeogenesis due to an impairment of the tissue's deaminating power. (This explains the results with alanine and glutamic acid but not with alpha ketoglutaric acid.)

Evans (1941) on the other hand did not observe any difference in the rate of disappearance of intravenously injected dl-alanine and consequent urea formation between adrenalectomized functionally nephrectomized rats and normal rats. This was taken as evidence that deamination in the liver is not impaired in adrenalectomized rats since the liver is the main site of deamination.

A striking effect of the adrenal cortex upon liver

arginase activity has been noted by Fraenkel-Conrat, Simpson and Evans (1943). They noted that in rats adrenalectomy caused a decrease in liver arginase activity to one third that of normal. Administration of Kendall's Compound E (17-hydroxy-11-dehydrocorticosterone) to adrenalectomized rats caused a decided increase in liver arginase activity. 11-dehydrocorticosterone and corticosterone were also effective (i.e., the type of activity was associated with the S-hormones). Desoxycorticosterone acetate only caused a slight rise. A marked increase in arginase activity of livers from normal and adrenalectomized rats was also noted upon the administration of the adrenocorticotrophic hormone of the anterior pituitary. The investigators think that the effect on liver arginase activity brought about by cortical hormones is the mechanism through which these hormones initiate protein catabolism and gluconeogenesis. Their belief that changes in arginase activity are the cause rather than the result of alterations in protein metabolism is based upon the following facts: increases in arginase levels may be obtained with hormone dosages too low to cause sufficient gluconeogenesis for the maintenance of carbohydrate stores during fasting; 11-dehydrocorticosterone caused an increase in arginase activity in two groups of rats not fasted and in nitrogen balance.

It is of interest to note that an increase in a tissue arginase level has also been associated with the androgenic hormone believed to be directly antagonistic to the action of the adrenocortical S-hormones. This is analogous to the findings in regard to thymus involution. An increase in arginase activity of liver, kidney and intestine upon

implantation of testosterone pellets into castrated male rats was reported by Kochakian and Clark in 1942. A similar increase in d-aminoacid oxidase in liver and kidney was observed by these workers. In the mouse only the arginase contents of the kidney (not of the liver) was affected.

The absence of changes in liver arginase, was somewhat surprisingly interpreted by Kochakian as indicating that those aminoacids providing ammonia to be used in the ornithine cycle for urea formation are not affected by these steroids. (Kochakian,1944). Fraenkel-Conrat challenges both Kochakian's observations and his deductions that increased arginase activity is associated with protein anabolism.

The adrenal cortex was also found to affect the activity of extracellular proteolytic enzymes. Tuerkischer and Wertheimer in 1945 observed that rennin and pepsin activities of the gastric juice were much lower than normal in adrenalectomized rats. The activity could be restored by administration of cortical extract to the animals (not by salt or DCA treatment).

Some studies have been conducted on the effect of the adrenal cortex on in vitro autolysis. An acceleration of thymus autolysis was reported upon the addition of cortical substance (Hammett, cited from Sun, 1929). Other workers have obtained negative results with cortical extract on liver and muscle autolysis. Sun in 1929 noted a small increase (9%, probably not significant) due to cortical tissue addition in amino nitrogen production of muscle pulp incubated at pH 6 and 37 degrees C for 24 hours. (A 30% rise in amino nitrogen production was observed upon the addition of medullary tissue).

Kotlyarov reported a depressing effect on liver pulp autolysis (37°C, pH 4.8). upon the addition of cortical tissue or cortin. Medullary tissue again accelerated proteolysis.

4. RELATION OF THE ADRENAL WITH METABOLIC EFFECTS OF DAMAGE:

It has been suggested that the metabolic effects of damage are mediated through the adrenal. From the similarity of pathological, functional and metabolic changes resulting from widely different damaging stimuli Selye (1940) deduced that these changes are characteristic responses to a "sudden exposure to stimuli to which the organism is quantitatively or qualitatively not adapted." He termed this response "the Alarm Reaction". The alarm reaction is subdivided into two phases, the "Shock Phase" and the "Countershock Phase". The adrenal is believed to take part in the alarm reaction by secreting adrenalin in the shock phase and by secreting cortical hormones mainly during the countershock phase, thereby initiating many responses associated with that phase. In agreement with this view is the initial hyperglycemia during the shock phase, found by Engel to be dependent upon the presence of medullary tissue (see page 23), the hypertrophy of the adrenal cortex and the discharge of lipid granules, thought to contain the cortical hormones, in the countershock phase, changes in pathological anatomy such as thymus involution, lymphopoenia, gonad atrophy, and ulcers all of which are symptoms that can also be elicited by cortical hormone administration (with the obvious exception of adrenal cortical hypertrophy).

The alarm reaction in turn is viewed as part of a characteristic pattern which is evoked in animals subjected to a continuous damaging stimulus. This pattern, the "Adaptation

Syndrome" is subdivided into three stages.
(1) The alarm reaction, a shock phase. b. counter shock phase.
(2) The stage of resistance. (3) The stage of exhaustion.
Adrenal cortical activity is taken to be at its height during the second stage of this syndrome which is characterized by a resistance of the animal to the same or a different damaging agent to such an extent that it does not respond by entering a shock phase. An elevated blood sugar level is associated with this stage.

The metabolic picture in patients who had been exposed to burns, fractures and other forms of trauma is characterized by an initial protein catabolic phase which is followed by a period in which protein anabolism is predominant. A regulatory function of the adrenal cortex has been suggested by Albright and Browne acting in the following manner (Albright, 1942-43). Under normal conditions the nitrogen output is equal to the nitrogen intake; i.e., the individual is in nitrogen balance. The protein catabolic (S-) and the protein anabolic (N-) hormones of the adrenal cortex balance each other out in their activities. Following exposure to trauma, the individual enters into negative nitrogen balance, which is due to a relative predominance of adrenocortical S-hormone activity over that of the N-hormone. During the protein anabolic phase the N-hormone activity predominates and the patient is in positive nitrogen balance. Eventually both hormone actions again balance eachother and the amount of nitrogen excreted equals the amount ingested.

Selye associates the symptoms of "shock" as the

expression is generally understood with the first stage of the alarm reaction, the shock phase. The author does not connect adrenal cortical activity with this first phase. On the contrary he suggests that the shock phase might be an expression of "relative adrenal insufficiency" (presumably including a relative deficiency in adrenalin supply as well.)

It is evident from the variety of adrenal hormones, (adrenalin, S-hormones, N-hormones and hormones influencing electrolyte balance) and the activities associated with them that almost any metabolic finding can be ascribed to adrenal hormone over or underactivity. Herein lies the danger of overlooking other equally plausible explanations for such findings. Thus, many phenomena induced by hemorrhagic shock may be explained simply by assuming deficient oxygen supply due to circulatory disturbances. Of course, one may argue that the reason for these disturbances is "relative adrenal insufficiency" and this may well be so. In such a case one would expect a beneficial result of adrenal hormone therapy which has not been conclusively established. Further, a characteristic aftereffect of trauma such as bone fracture, burns, exposure to a revolving drum, etc. is an increased urinary nitrogen excretion, setting in immediately after the insult has been inflicted. This may be explained by assuming increased secretion of cortical hormones which administered in vivo are known to induce protein breakdown in general (S-hormones), and also to enhance such specific reactions as deamination (cortical extract, DCA) and liver arginase activity (cortical extract, S-hormones only). But during the shock phase there is supposedly a lack of such hormone secretion.

The findings from Long's laboratory indicate that haemorrhagic shock influences protein breakdown by enhancing it, deamination by inhibiting it, both findings explainable by the absence of oxygen, since oxygen inhibits proteolytic enzyme activity, and is required for oxidative deamination. Significantly the Q_{O_2} in tissues from animals in haemorrhagic shock is lowered. Administration in vivo of some of the cortical hormones also enhances protein breakdown, but a drop in Q_{O_2} and deamination rate is effected by adrenalectomy. In vivo administration of cortical extract raises the Q_{O_2} and deamination rate. If increased arginase activity is conductive to increased protein catabolism as Evans suggests, anoxia may induce protein breakdown in shock also by its effect on arginase activity, the inhibitory action of oxygen on arginase activity being absent. As will be remembered adrenal S-hormones also increase liver arginase and the N-hormones increase kidney arginase action. The question is which are the primary factors in inducing the protein catabolic response to damage.

Bone fracture, and fasting are stimuli capable of eliciting the alarm reaction. In this connection the following findings are of interest: Cohen (1945) in this laboratory observed the characteristic rise in urinary nitrogen excretion by well fed rats following femur fracture. When such rats were bilaterally adrenalectomised however, subsequent femur facture did not evoke any rise in urinary nitrogen excretion. In protein depleted, otherwise normal animals the withdrawal of food induced increased urinary N excretion, but femur fracture did not. The possibility was suggested that "the economy

of the depleted rat's metabolism accounted for the lack of increased output after a damaging stimulus (i.e., femur frature) whereas in starvation the cortex now responded with consequent catabolism of it's body tissue". In protein depleted bilaterally adrenalectomised animals not even starvation induced a rise in nitrogen excretion.

These observations point to the necessity of adrenal activity for the rise in nitrogen excretion to occur in response to damaging stimuli.

The recent observations of Ingle (1946) are of interest here. This investigator found that adrenalectomized rats excreted more nitrogen by the third day after adrenalectomy than rats that had been shamoperated. During ten days' postoperative feeding the adrenalectomized rats excreted as much urinary nitrogen as did the controls. Ingle also applied multiple femur fracture to adrenalectomized and sham adrenalectomized animals. Here again the adrenalectomized rats previous to femur fracture excreted more urinary nitrogen than the sham adrenalectomized rats. But similar to Cohen's results, femur fracture, while eliciting increased nitrogen excretion in sham adrenalectomized rats did not evoke any such response in animals lacking their adrenals. When the adrenalectomized rats were maintained on constant amounts of cortin a rise in urinary nitrogen did occur following femur fracture. This indicated that the response was dependent not upon increased amounts of hormone secretion but upon the presence of these hormones. Saline treated adrenalectomized rats responded to burns and also to thyroxin treatment with a rise

in urinary nitrogen excretion. However, during the first twenty four hours following stress, adrenalectomized rats with or without cortin treatment excreted less nitrogen than animals with intact adrenals. Ingle suggests that possibly the thymus and other lymphoid tissue does not regress as rapidly in adrenalectomized as in normal animals following damage.

Ingle concludes from his observations that "at least some of the metabolic adjustments to stress, especially changes in nitrogen balance can occur in the absence of the adrenals."

It may be stated that while there is a good deal of evidence pointing to an active role of the adrenal in adjustment to stress, the extent to which the adrenocortical hormones are active and the mechanism by which such activity may be evoked is not yet established.

The following investigation is concerned with the effect on in vitro protein metabolism of a number of damaging stimuli. No specific experiments were conducted to permit deductions as to the role of the adrenal. A discussion on the interrelation of the adrenal with protein metabolism, and with stress was nevertheless included in this introduction, since the damaging stimuli employed: femur fracture, cold, starvation, are all capable of inducing adrenal hypertrophy and the possibility that the adrenal cortex is responsible for any effects obtained must be taken into consideration.

EXPERIMENTAL

The experimental part deals first with the methods and results of the trial experiments which were conducted to find a satisfactory procedure of determining protein breakdown in vitro simulating in vivo conditions. These include experiments 4 to 11, all performed on normal untreated rats except Nos. 10 and 11 in which the rats were subjected to cold and fasted 22 hours before killing. The modifications in methods for final use in the main group of experiments will also be related in the first section.

The second part deals with experiments on animals subjected to various damaging stimuli, along with normal controls. The manner in which damage was inflicted and its effect on protein metabolism will be discussed in this section.

I. TRIAL EXPERIMENTS:

1. Methods and their Standardization:

(a) Choice of Animals: Male hooded rats weighing 250 to 300 grams were used in the preliminary experiments, rats weighing 245 to 265 grams in the later experiments. Male rats were chosen to avoid any variability due to changes in endocrine secretion associated with the oestrus cycle. The weight was selected because at that stage the animal, although mature, still grows and by confining oneself to a particular weight a uniformity of age could be approximated and at the same time an adequate liver size was assured.

(b) Method of Sacrificing, Excision of Livers: Animals were stunned by a blow on the head and decapitated. It was attempted to rid the animal of as much blood as possible in

in order to obtain relatively blood free livers. The livers were not free of blood however, as was evident macroscopically as well as microscopically. The method of washing the blood out of the liver by injecting physiological saline into its circulatory system was not employed because of the technical difficulties which were the greater since sterile conditions had to be observed in order to avoid bacterial contamination.

The livers were excised using sterile technique: the abdominal hair was thoroughly saturated with a 5% iodine solution. Shaving was found unnecessary. A longitudinal incision through the skin was made with the aid of one pair of sterilized scissors and forceps. The peritoneal cavity was entered with a second, and the liver taken out with a third pair. Approximately half the liver was placed in a modified pyrex test tube containing 3 ml of physiological buffered salt mixture, (Krebs and Henseleit, 1932: Krebs 1933). Two such test tubes, therefore, contained the whole liver which had been taken out of the animal by four or five cuts. The test tubes with the salt mixture and sterile cotton plug standing in a drilled cork stopper had been weighed previously and were again weighed to obtain the liver weight.

(c) Homogenizing procedure: These large liver pieces were then homogenized with a closely fitting rotating glass plunger inserted in the tubes, after the method of Potter and Elvehjem (1936). The time of homogenizing was about three minutes in the preliminary experiments. In the later experiments the time was standardized at two minutes and the number of revolutions was measured by means of a counter in contact with the stirrer throughout homogenization and kept as closely

as possible to 1,000 r.p.m. with the aid of a rheostat in order to get comparable suspensions. The homogenizing procedure was controlled in this way in the hope of standardizing the degree of cellular damage. Potter (1946) pointed out the influence of homogenizing time upon cell breakage.

Homogenizing rather than slicing was chosen for the following reasons: Homogenizing is easier to perform than adequate slicing and is faster. It involves less weighing; it assures homologous samples of tissue and makes possible the use of larger quantities of tissue.

The objections to homogenizing have been emphasized more than the advantages. It is argued that the damage to the cell itself as well as the disorganization of tissue structure involved in homogenizing results in too drastic a departure of in vivo conditions to permit one to draw conclusions from the results obtained, upon in vivo metabolism. While tissue structure is undoubtedly destroyed in a homogenate this is to a lesser degree also the case in slices. The damage to the cells seems overemphasized if one keeps in mind that the tissue is homogenized with a round surface glass plunger-avoiding any contact with metal - and in an isotonic medium. This procedure must not be likened to methods where the tissue is chopped up with a metal grinder or ground with sand and exposed to media that do not have the osmotic requirements to prevent cytolysis.

To determine whether liver cells really survive homogenization the homogenate was examined microscopically in several experiments. Many cells which appeared intact

were found. Figs. 1 - 8 show photo-micrographs of the homogenate used in a control experiment, at the start of incubation, at 3, 6, and 9 hours incubation. The smears were obtained and stained in the following manner: a small drop (about .01 ml) of homogenate was placed on a square coverslip. A second coverslip was placed on the drop and then rapidly pulled off with a sliding motion. A thin and fairly even smear was thus obtained. Immediately after drying the smear was covered with a 1:1 alcohol-ether fixative which was left on for at least 15 minutes. The smear was then stained with haematoxylin and eosin and mounted by the standard method. The solutions were added with a dropper since immersion of the slides into the solutions was liable to cause the detatchment of the smear.

A 16 mm objective was used for the low power photomicrographs, a 4 mm objective for high power. The absolute size of the cells are not comparable from slide to slide since the magnification was not kept constant.

Cells with nuclei and surrounding cytoplasm can be seen even in the sample taken 9 hours after incubation,(Figures 4 and 8). However, after 3 hours incubation there is already a slight reduction in the size of the nucleus (Figures 2 and 6) and at 6 and 9 hours pyknosis is marked, (Figures 3, 4, 7 and 8). In the slide prepared from the homogenate at zero incubation time many apparently naked nuclei were observed. A differential cell count to ascertain the proportion of apparently intact cells and cells that seemed to have lost their cytoplasm, showed 53% intact cells and 47% naked nuclei at the start of incubation. In the later stages of incubation

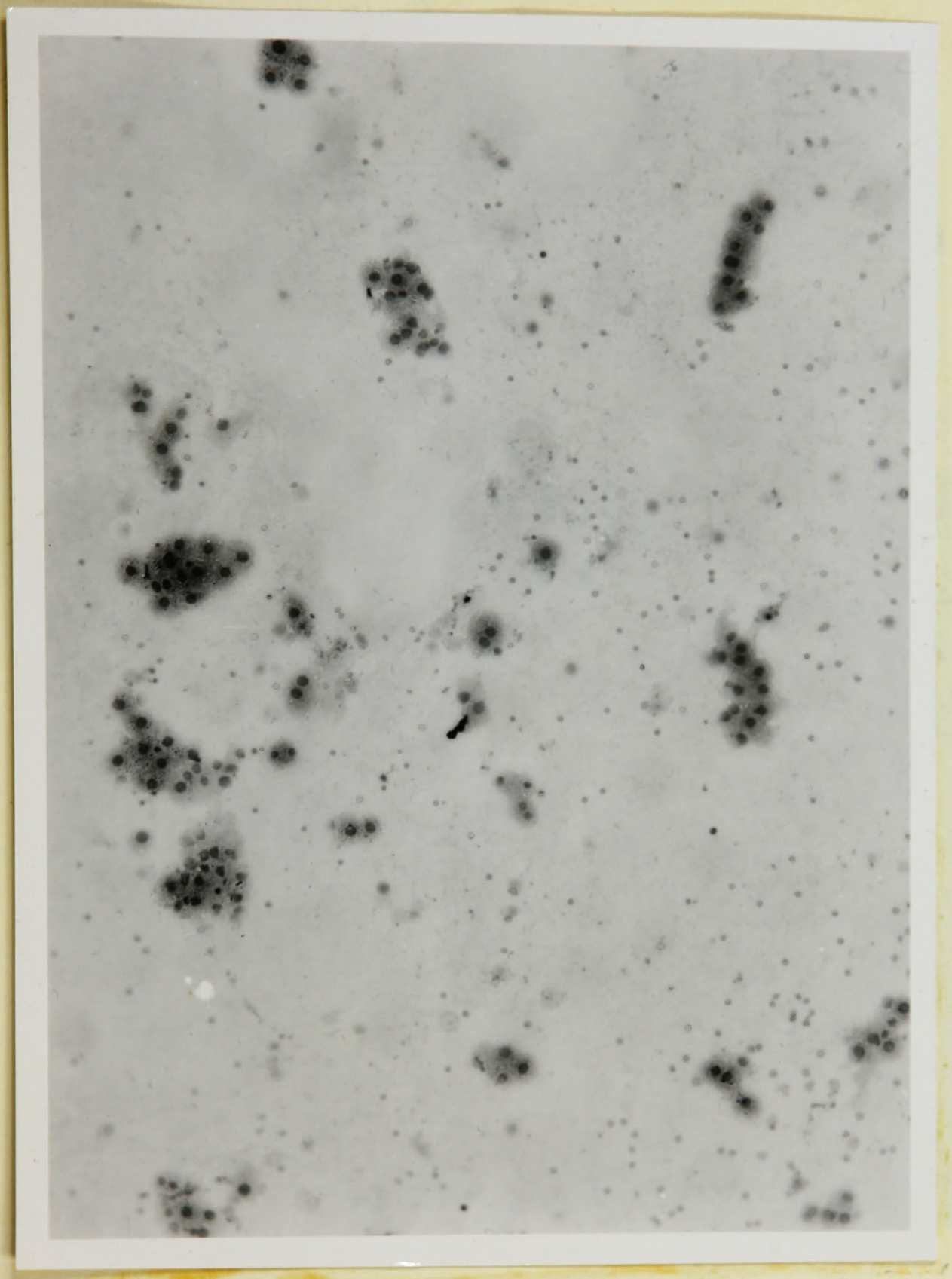


Fig. 1 Low power photomicrograph (16mm. objective) of a liver homogenate from a healthy rat prior to incubation (35 minutes after death). Haematoxylin eosin stain.

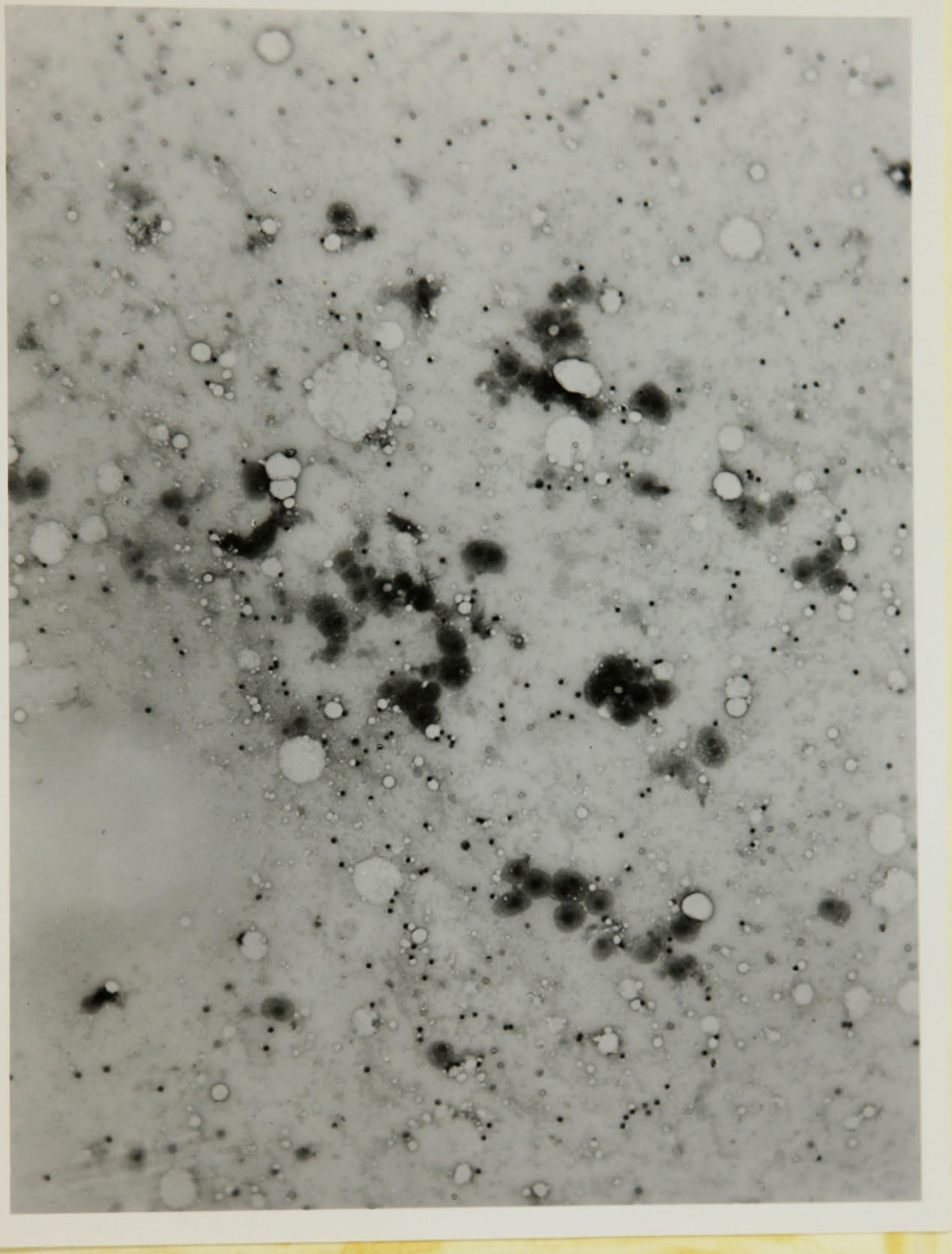


Fig. 2 Low power photomicrograph (16mm. objective) of a liver homogenate from a healthy rat after 3 hours aerobic incubation (Krebs' bicarbonate medium pH 7.3; 37.5°C). Haematoxylin eosin stain.

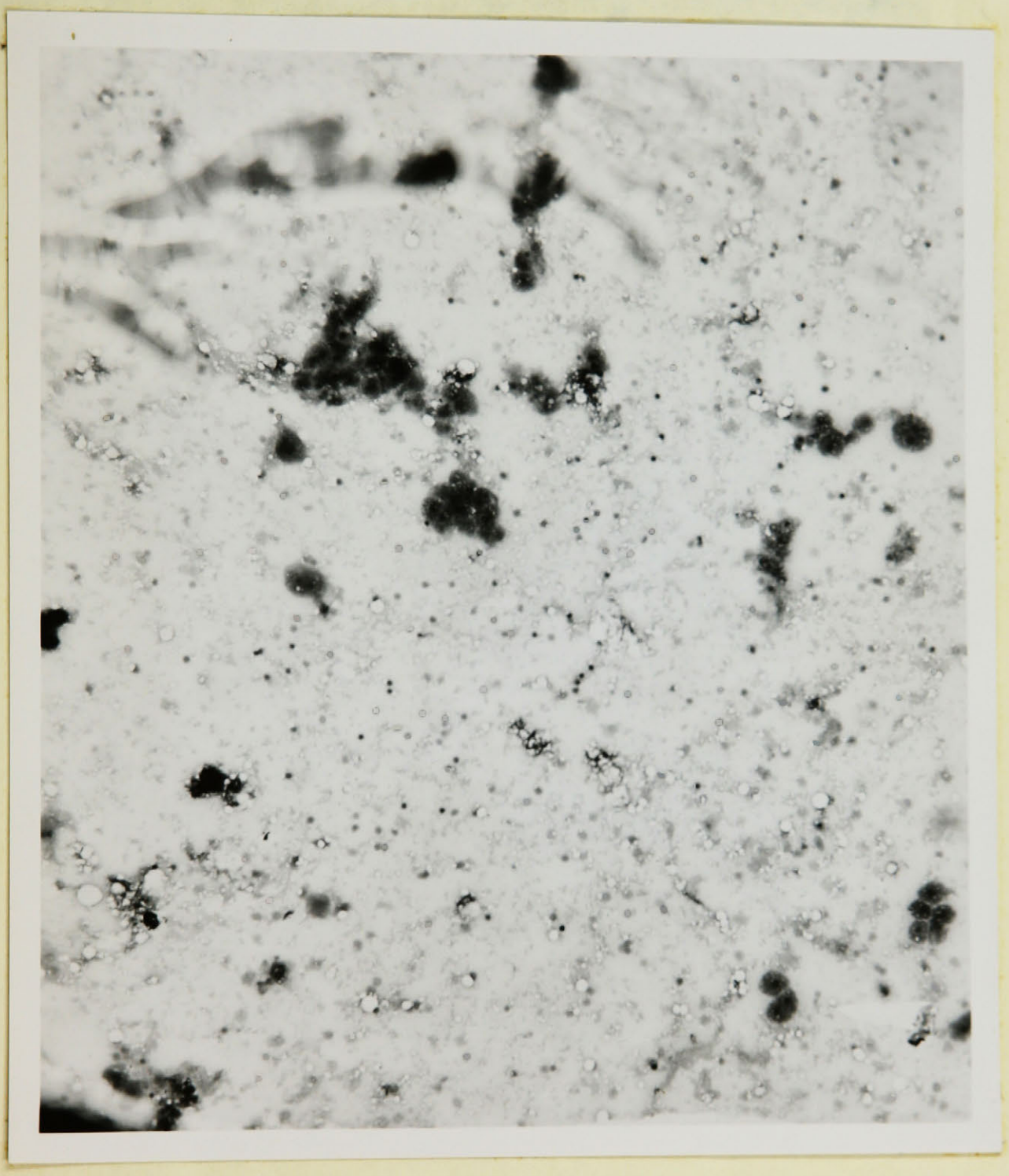


Fig. 3 Low power photomicrograph (16mm. objective) of a liver homogenate from a healthy rat after 6 hours aerobic incubation (Krebs' bicarbonate medium pH 7.3; 37.5°C). Haematoxylin eosin stain.

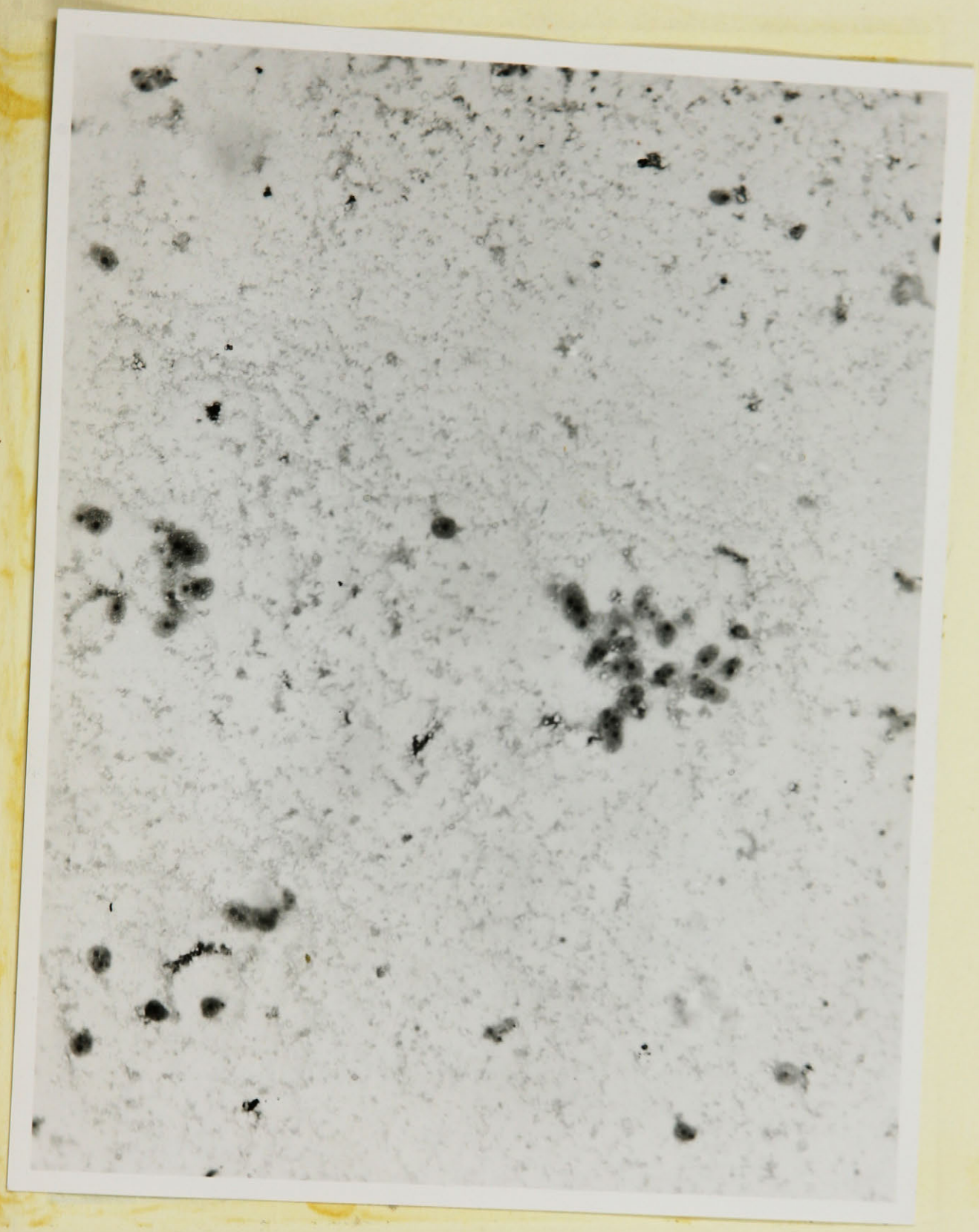


Fig. 4 Low power photomicrograph (16mm. objective) of a liver homogenate from a healthy rat after 9 hours aerobic incubation (Krebs' bicarbonate medium pH 7.3; 37.5°C). Haematoxylin eosin stain.

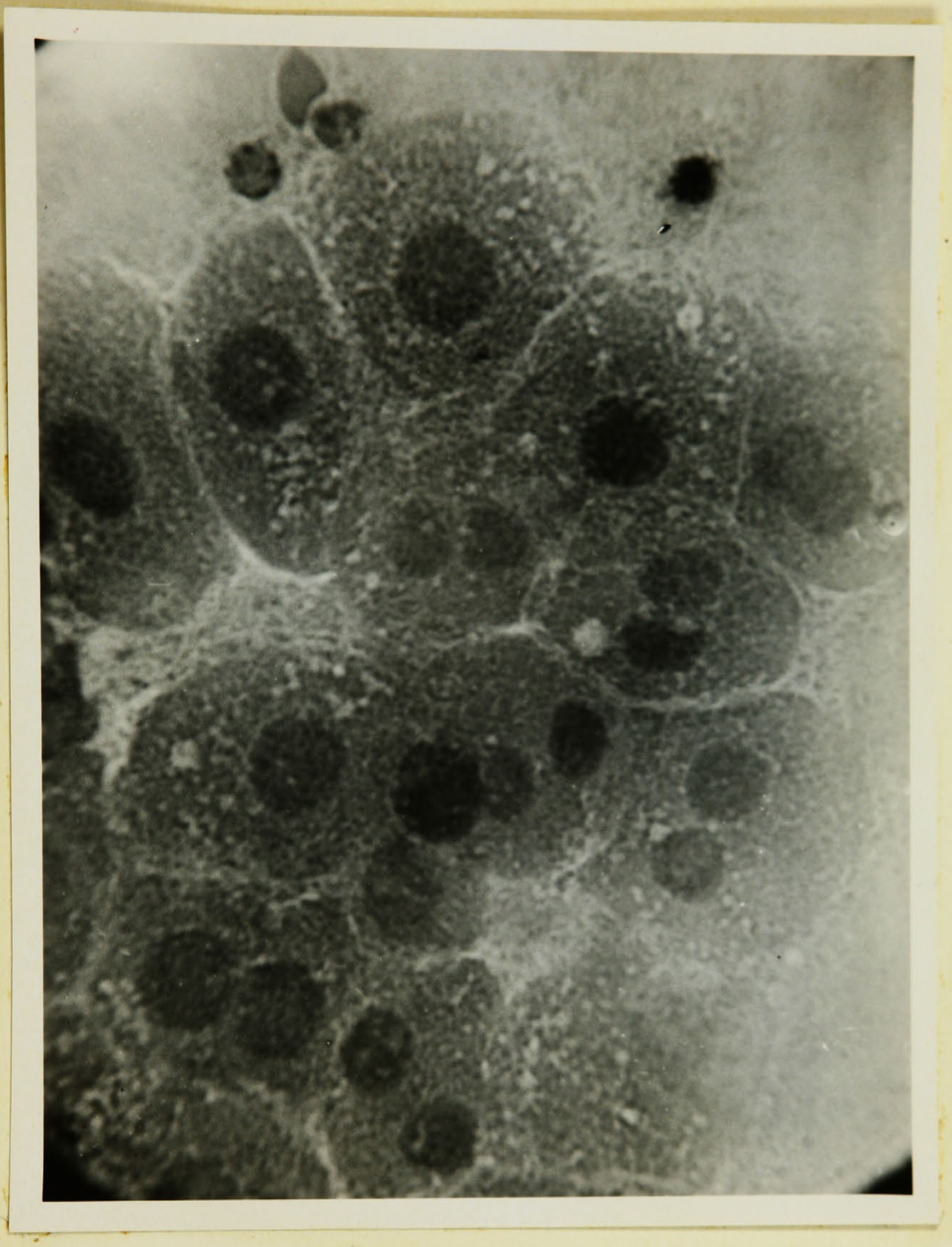


Fig. 5 High power photomicrograph (4mm. objective) of a liver homogenate from a healthy rat prior to incubation (35 minutes after death). Haematoxylin eosin stain.

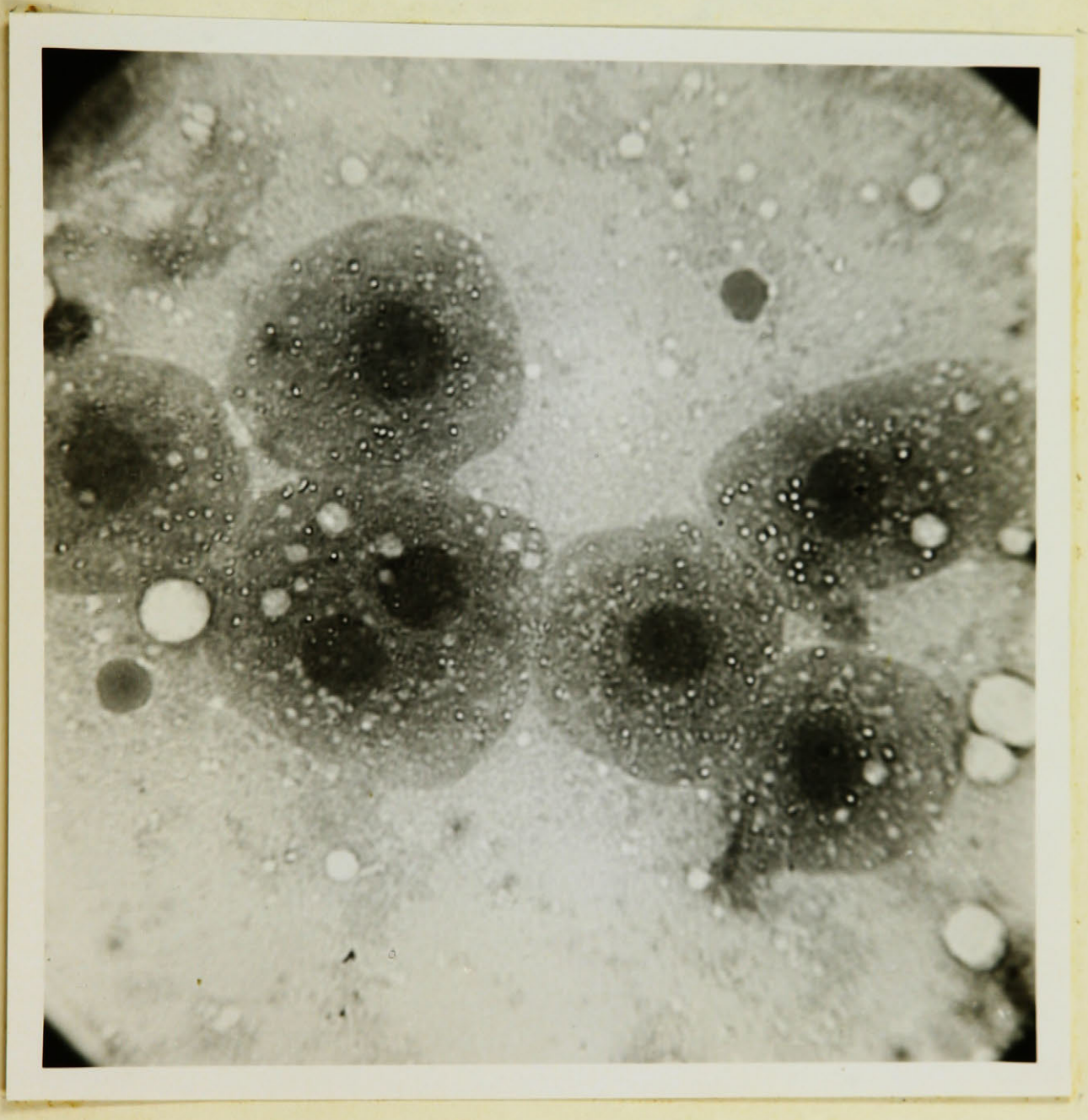


Fig. 6 High power photomicrograph (4mm. objective) of a liver homogenate from a healthy rat after 3 hours aerobic incubation (Krebs' bicarbonate medium pH 7.3; 37.5°C). Haematoxylin eosin stain.

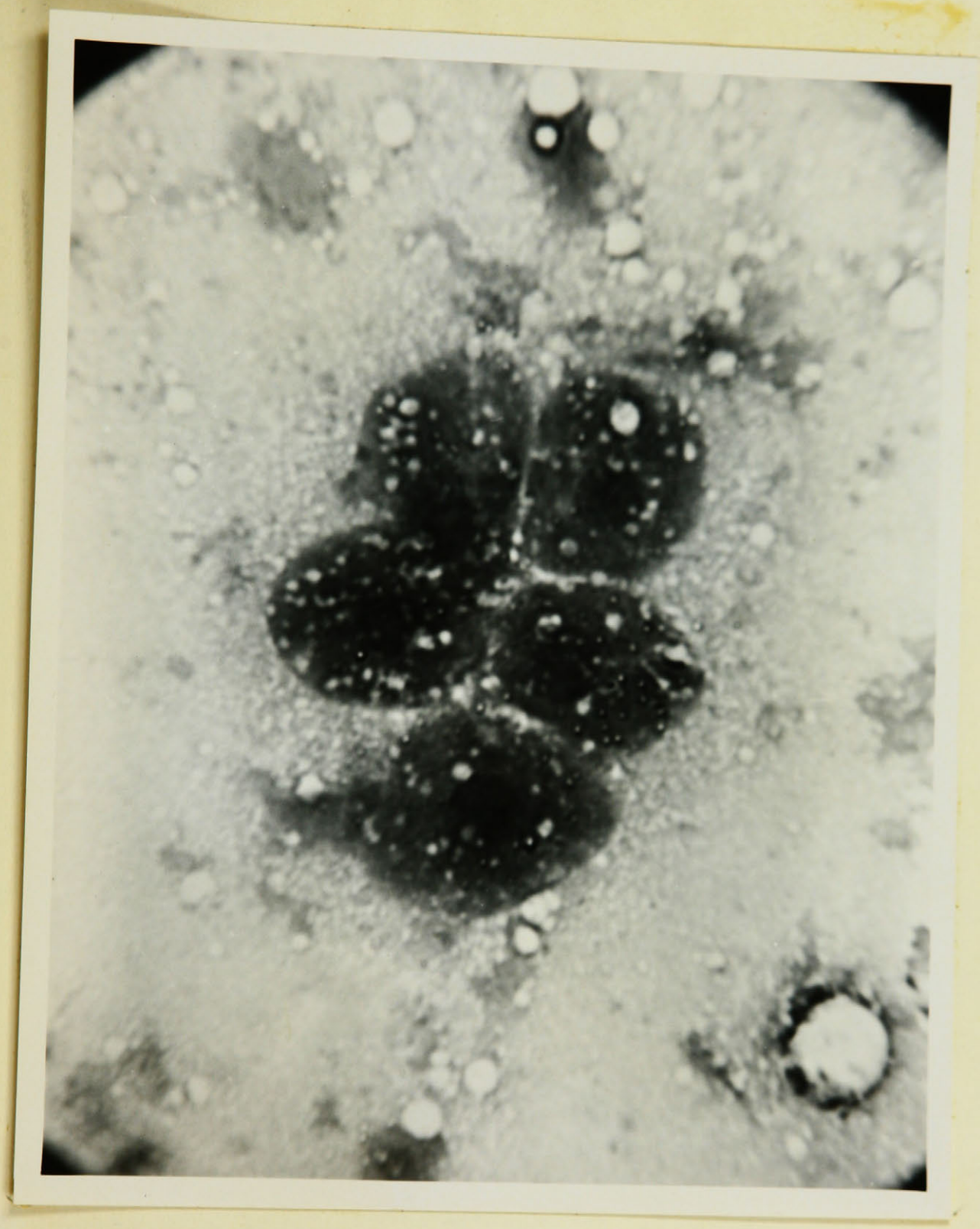


Fig. 7 High power photomicrograph (4mm. objective) of a liver homogenate from a healthy rat after 6 hours aerobic incubation (Krebs' bicarbonate medium pH 7.3; 37.5°C). Haematoxylin eosin stain.

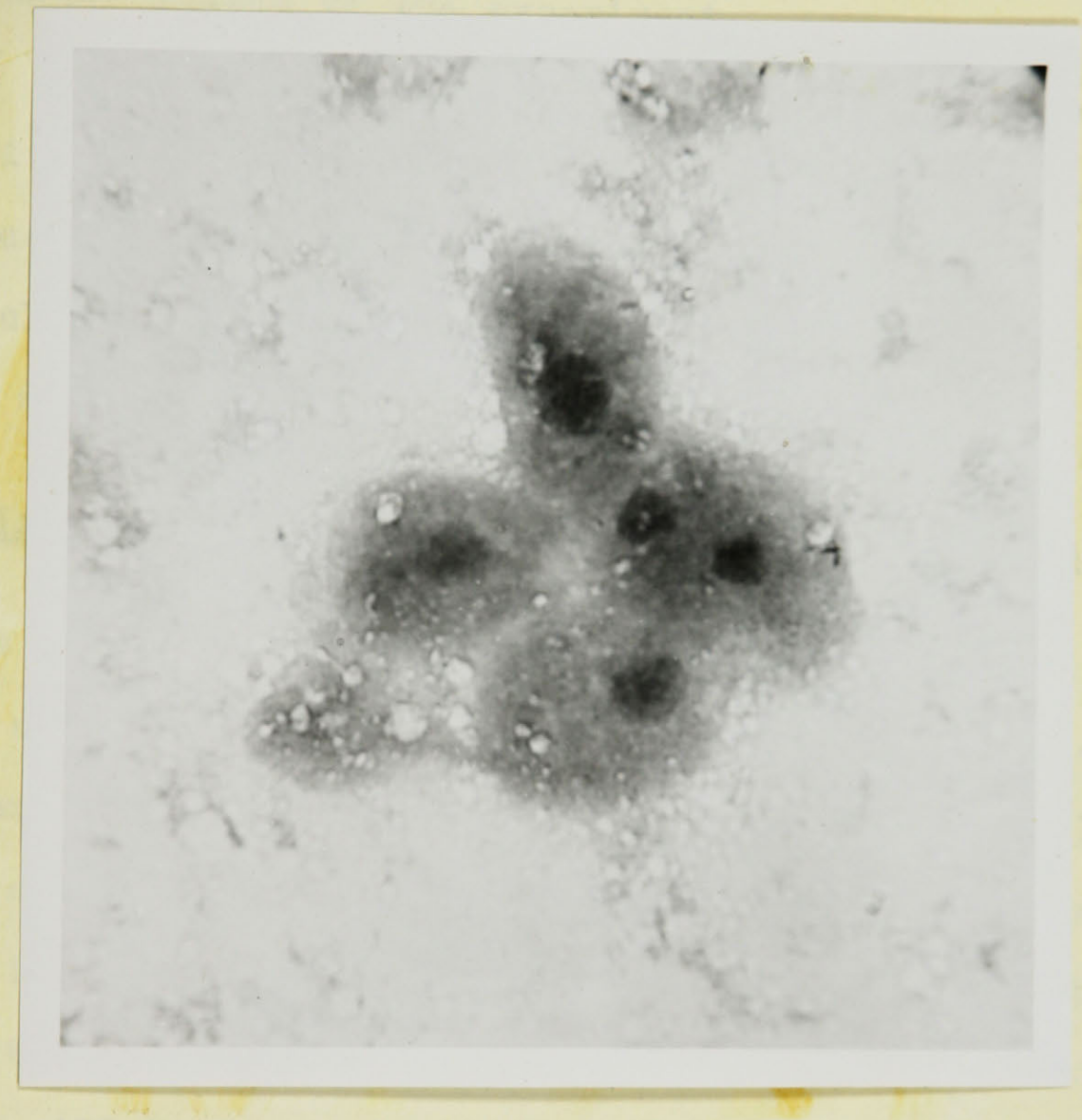


Fig. 8 High power photomicrograph (4mm. objective) of a liver homogenate from a healthy rat after 9 hours aerobic incubation (Krebs' bicarbonate medium pH 7.3; 37.5°C). Haematoxylin eosin stain.

the number of naked nuclei decreased and finally disappeared presumably due to their disintegration. The disintegrated cells probably accounted for part or all of the unorganized protoplasmic debris that was found in the slides especially in the later stages of the experiment.

That microscopic examination can give an exaggerated impression of cell breakage and is therefore not a reliable index has been emphasized by Potter (1946). Potter determined the cytolysis quotient on homogenized rat liver by the succinoxidase method. The cytolysis quotient:

$$100x\frac{\text{(succinoxidase with cytochrome)}-\text{(succinoxidase without cytochrome)}}{\text{succinoxidase with cytochrome}}$$

compares the succinoxidase activity of the suspension with and without added cytochrome c. In intact liver cells there is enough ctyochrome c to saturate the succinoxidase of the cell and further addition of cytochrome c to the medium will have no effect; if the cells are broken up, however, the cytochrome c dissociates from the succinoxidase system and the succinoxidase activity will be increased by addition of cytochrome c. With this method the percentage of intact cells in isotonic rat liver homogenate was estimated by Potter at 60 to 80.

'After homogenizing, the suspension was diluted with Krebs' buffered physiological salt mixture (0.0167M Phosphate buffer in Experiments 1-7; 0.025 M bicarbonate in all later experiments) to contain 0.17 g liver (fresh weight) per ml. The time interval between killing of the animal and placing the homogenate in the water bath was variable in the trial experiments, but standardised at 35 minutes in the main group of experiments.

(d) Incubation: It was decided to incubate the homogenate at body temperature, aerobically, and at pH 7.3 in order to approach in vivo conditions although autolysis of the liver proceeds much more rapidly at a more acid pH (Bailey, Belfer, Eder and Bradley, 1942; Luck, Eidin and Nimmo, 1939), and oxygen is known to inhibit proteolytic enzyme activity, (Voegtlint and Maver, 1932; Bailey, et al, 1942; Irving, Fruton and Bergmann, 1942).

In the earliest experiments it was thought that aerobic conditions could be maintained by incubating the homogenate in a 125 ml. erlenmayer flask (Experiments 4 and 5 "non-aerated".) It was realized later that the layer of homogenate was not quite shallow enough to ensure adequate diffusion of oxygen and it was decided to place these homogenates in a narrow test tube and pass oxygen through the suspension from a glass tube placed with the opening at about .5 cm. from the bottom of the tube. Further, the 0.017M phosphate buffer employed in Experiments 3, 4, 5, and 6 was replaced from Experiment 7 on with the more physiological and efficent 0.025M bicarbonate buffer kept in equilibrium with a 95% O_2, 5% CO_2 gas mixture.

Table I is a summary of the pH determinations on 10 homogenates over the 9 hour period of incubation. The determinations were done on a Beckman pH meter with a gas mixture of 95% O_2, 5% CO_2, bubbling through the homogenate during the pH determination to keep it from turning alkaline due to escape of CO_2. It will be seen that the pH was maintained within 0.3 units.

Aeration by passing gas through the homogenate

TABLE I

pH Changes in Rat Liver Homogenates during Nine Hours of Aerobic Incubation at 37 degrees.

Incubation times-	0	1½	3	5½	6	9 hours.
Homogenate from control animals:						
Experiment						
(18)	pH 7.28		6.90		7.09	6.95
(19)	7.28		7.15		7.10	7.00
(20)	7.20		7.20		7.21	7.16
(24)	7.33		7.30		7.22	7.20
Homogenate from animals subjected to cold:						
(15)	7.30		7.18		7.18	7.04
(17)		7.28		7.10		7.00
Homogenate from animals subjected to femur fracture:						
(21)	7.18		7.38		7.28	7.21
(22)	7.38	7.35			7.20	
(25)	7.35				7.18	7.15
(26)	7.31				6.79	7.04

brought up the problem of foaming and a resulting loss of solid suspension particles on the walls of the test tube. This was reflected in the decrease in total nitrogen with incubation time in Experiments 6 and 7 (Figures 14 and 15) in contrast to the constant level of total N in the "non-aerated" Experiments 4 and 5 (Figures 12 and 13). This difficulty was overcome by coating the wall of the test tube, as well as the glass tubes for aeration, with Russian oil. (Before sterilization the tube was completely filled with Russian oil, the oil was drawn into the tube provided for the gas outlet. The test tube and aeration tubes were then drained thoroughly and sterilized. The tube for the gas outlet was made from a 10 ml volumetric pipette (Figure 9) so that if the homogenate entered the tube the foam broke up when it reached the extended part of the pipette and further rising of the suspension was prevented. Fifteen minutes after incubation foaming usually stopped and the suspension remained in the test tube.)

Russian oil rather than caprylic alcohol was used because it was felt that preventing the homogenate from creeping up on the glass walls by smoothing the surfaces with a relatively inert, insoluble substance was preferable to the more drastic effect of the soluble caprylic alcohol on the surface tension of the homogenate. The disadvantage of using oil was that certain compounds of the homogenate might be soluble in it.

The procedure was effective in preventing the loss of total nitrogen (see Figures 15 and 16: Experiments 9 and 10).

In the preliminary experiments the amount of gas passed through the homogenate was not measured but later it was decided to standardize the aeration thus assuring (a) the same degree of oxygenation, (b) the same amount of agitation of the suspension. For this purpose two 50 ml. pipettes graduated in .1 ml, were placed in a vertical position, connected at the lower ends with a rubber hose, and filled about half the height with water, (Figure 9). A rubber connection passed the gas through one arm of a Y joint to the upper end of one pipette, through the second arm of the Y joint to the glass tube leading to the bottom of the suspension.

The difference in water levels was an indication of the oxygen delivery provided the gas stream encountered the same amount of resistance. The homogenate was therefore always incubated and aerated in the same apparatus. Figure 10 shows the calibration curve obtained by measuring the amount of gas delivered per minute at different manometer levels.

As samples were taken from the homogenate the resistance to the gas stream decreased resulting in a slight increase in oxygen flow. This was reflected in the change in water level (1.4 "ml" for 4 ml. homogenate removed.) Since the change of rate was slight and all experiments were subjected to it, it was thought of no consequence.

The gas tank was made to deliver approximately 100 ml O_2 per minute by adjusting the difference between water levels in the manometer arms, (19.0 "ml" for 45 ml homogenate 17.8 for 40 ml, 16.6 for 35 ml, etc; since the same incubation tube was always used a given volume of homogenate always reached the same height in the incubation tube). This high rate was

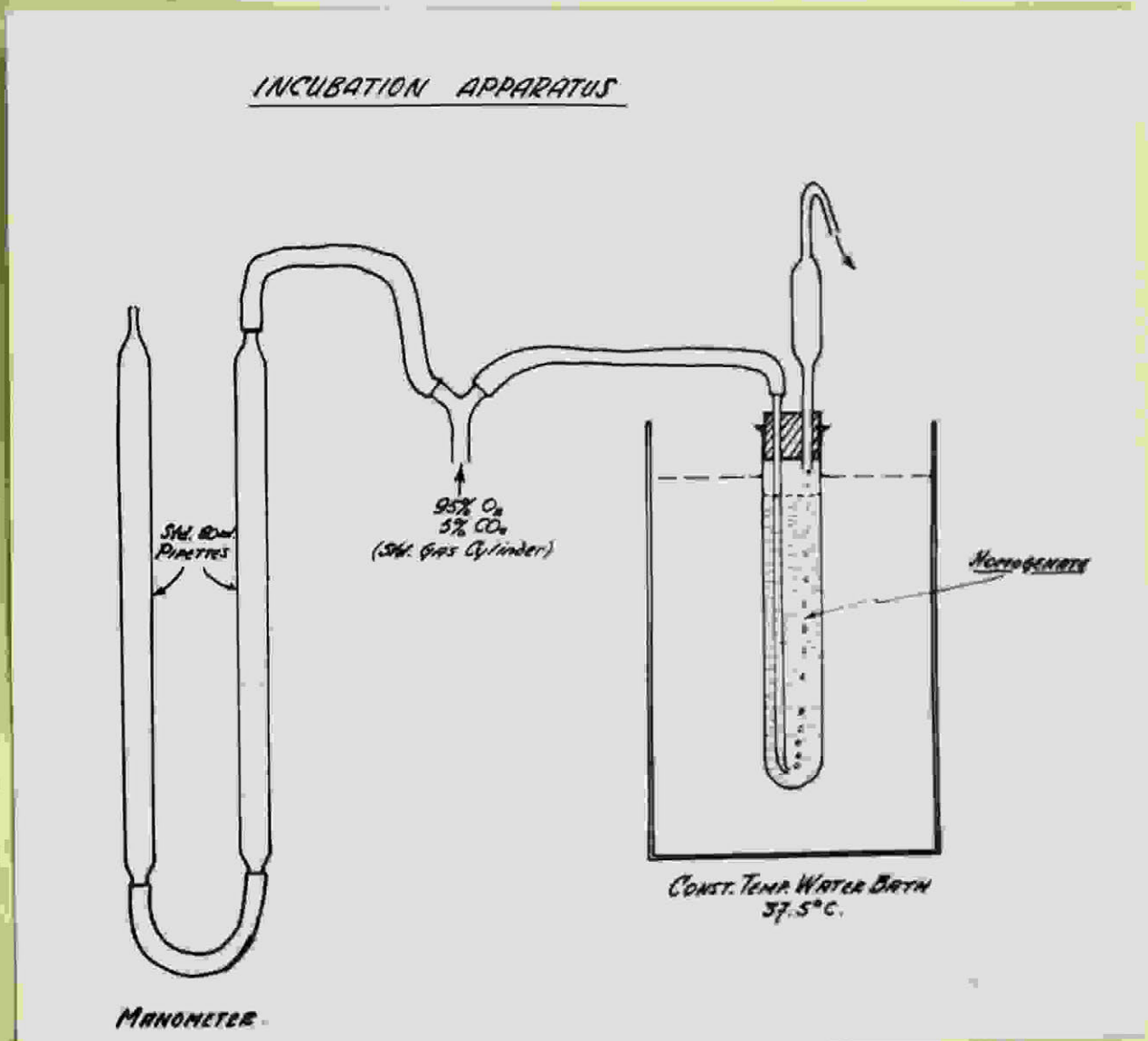


Fig. 9. Incubation apparatus.

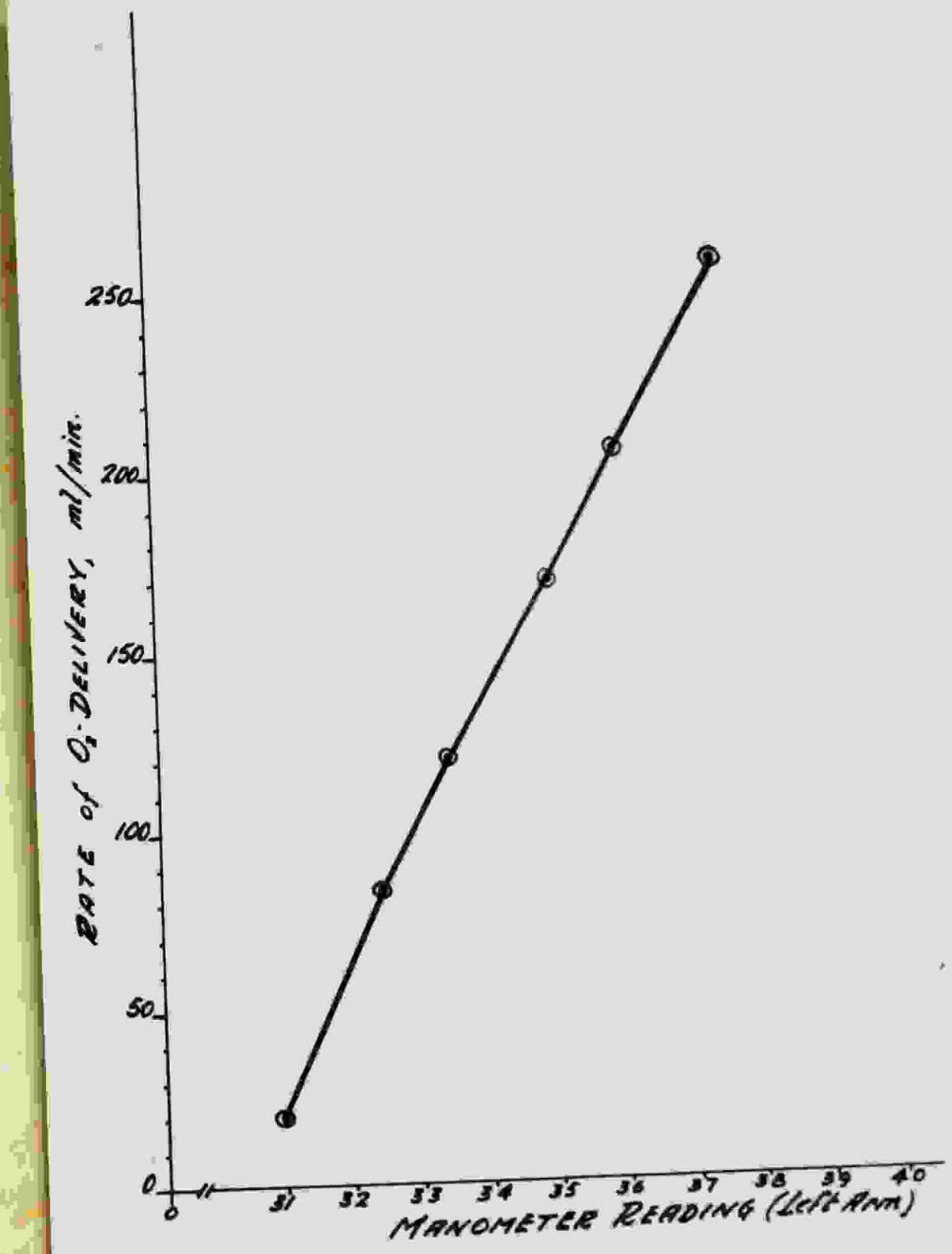


Fig. 10. Relation between oxygen delivery and water level in manometer arm.

chosen mainly to prevent any particles of the suspension from settling out. The oxygen supply was much greater than that required for maximum respiration.

(e) Methods of Analysis: It was decided to follow protein breakdown by determining the increase of non-protein nitrogen and of alpha amino-acid nitrogen with incubation time, from zero incubation time (45 minutes after death of rat) up to 3 days in some of the preliminary experiments, from zero incubation time (35 minutes after killing) until 9 hours incubation time in the main group of experiments. The time was eventually limited to 9 hours in order to assure maintenance of the pH throughout incubation, to make available more material for each analysis, and to obtain results from a larger number of animals by shortening the time of the experiment.

It was realized that the values for alpha amino-nitrogen and NPN did not necessarily reflect the absolute rate of formation of these substances but represented the over-all result of their rate of formation and their further metabolism.

In earlier experiments the "cell" volume or better, the concentration of solid particles in the suspension was roughly measured and the volume of protein precipitate was also noted.

The nitrogen determinations were carried out on the whole suspension as well as on the supernatant phase of the centrifuged homogenate in the preliminary experiments, in the hope of gaining some idea of the distribution of the nitrogenous components extra - and intracellularly and changes in that distribution. However, the NPN and alpha-amino N values

per gram liver showed no consistent difference (except possibly the zero hour samples) whether the whole suspension or only the non-solid part of the suspension was analyzed in spite of efforts to free the intra-cellular N-components by diluting the suspension with distilled water to make it hypotonic, repeated freezing and addition of saponin, measures that should have brought about cytolysis (see Figures 12-16: in Experiments 4, 7, 9, 10, and 11 suspensions were diluted with distilled water, in Experiment 5 suspensions were frozen and thawed twice after dilution with distilled water: in Experiment 6 suspensions were diluted with distilled water, treated with saponin and frozen twice.) All determinations were therefore done on whole homogenate in the main group of experiments. Only with respect to total N was there a difference between the two fractions. These results will be discussed in a later section.

Total N was determined on .5 ml of homogenate diluted five times with isotonic saline. Saline instead of distilled water was used to dilute the homogenate since with distilled water satisfactory duplicate N determinations could not be obtained presumably due to the formation of larger aggregates of solid matter.

To precipitate proteins for NPN and alpha-amino nitrogen determination 4 ml.of the homogenate were diluted with 20 ml. of Na tungstate, H_2SO_4 mixture. In the preliminary experiments 8 ml. of distilled water were added to the homogenate previous to protein precipitation in an effort to induce cytolysis. Then 12 ml. of the protein precipitant: 1 part 10% Na tungstate, 8 parts N/12 H_2SO_4, were added. As has already

been mentioned no consistent difference in NPN or amino nitrogen yield was obtained by any cytolysis inducing procedures. The dilution with distilled water was therefore discontinued in the later experiments but in order to maintain the same concentration of protein precipitant throughout, the latter was diluted with 1/3 its volume of distilled water before addition to the homogenate.

The samples were centrifuged for 15 minutes and then filtered by suction since the oil that had to be employed to prevent foaming in the homogenate tended to collect a film of precipitated proteins which was not carried down by centrifuging. It was found quicker and less wasteful of the deproteinized solution to centrifuge first and then filter, rather than to remove the precipitate by suction filtering alone.

The supernatant obtained from precipitating 4 ml. of homogenate (corresponding to 0.67g liver) was sufficient to do NPN and alpha-amino N determinations in quadruplicate if necessary.

Total N and NPN were determined by the Heidelberger modification of the microkjeldahl technique of Meeker and Wagner (Meeker and Wagner, 1933), which calls for boric acid as receiver of the ammonium hydroxide carried over by distillation and direct titration of the ammonium borate with standardized sulphuric acid using an indicator with a turning point around pH 4.5. This method has the advantage that only one solution, the sulphuric acid has to be standardized and measured accurately. The digestion mixture used was that proposed by Campbell and Hanna (1937) which contains SeO_2 as a catalyst,

cutting down digestion time to 5-10 minutes. Determination on known amounts of glycine (1mg N), yielded 97 to 100% recoveries. Samples of the deproteinized homogenate were adjusted so as to contain 0.3 to 1.0 mg N by using 6 ml. of the filtrate. 0.5 ml of a 1.5 dilution of homogenate was used for total nitrogen determination. (1.0 mg N corresponded to a titration figure of 5.00 ml N/70 H_2 SO_4: a 5 ml microburette graduated in 0.01 ml was used). All determinations were carried out in duplicate. Duplicate NPN determinations usually agreed within 1%, occasionally with a 4% error. Duplicates on the total nitrogen determinations were not so satisfactory since one dealt with the diluted suspension and two pipetted samples of the same volume did not necessarily contain the same amount of solid matter. The average agreement of duplicates was 4%.

Alpha amino-nitrogen was determined after Frame, Russell, and Wilhelmi's (1943; Russell, 1944) modification of Folin, Wu (1922) and Danielson's (1933) technique. The method is a colorimetric one, based on the inter-action of sodium beta-naphtho-quinone-4-sulfonate, with the amino group of amino acids and, to some extent also with the free amino group of peptides. Both amino groups of lysine, and ornithine react quantitatively. Arginine yields quantitative values corresponding to its alpha-amino N contents. Ammonia and uric acid yield a large percentage of color and therefore interfer with the determination.

The following procedure was used: to remove ammonia 1 ml of the deproteinized solutions and also a blank of 1 ml distilled H_2O were aerated for 30 minutes with 4 ml of N/4

NaOH and one drop .25% phenolphthalein indicator. 2.5% boric acid was used to receive the ammonia. Except in the 24 to 72 hour samples of the preliminary experiments the quantities of ammonia were too small to be detected by titration with N/70 H_2SO_4, but they were large enough to interfer with the amino-nitrogen determination. After aeration 1 or 2 ml of the aeration mixture, depending on the amino-nitrogen concentration, were diluted to 5 ml with distilled H_2O in 25 x 200 ml colorimeter tubes. One drop of .25% phenolphthalein was added and the samples brought to pH 9.2 to 9.4 by first acidifying with H_2SO_4 or HCl and then adding NaOH until a permanent light pink colour was developed. One or two ml of the aerated distilled water blank was similarly diluted and adjusted to pH 9.2 to 9.4. One ml of 2% sodium borate solution was added to buffer the solutions. One ml of the freshly prepared aqueous .5% solution of sodium naphtho quinone sulfonate was then added, the tubes covered with drawn glass beads and immediately placed in a boiling water bath. After 10 minutes the samples were cooled in ice water for 3 minutes and the excess colour bleached by adding first 1 ml acid formaldehyde (0.30 N HCL containing 0.04 M formaldehyde) then 1 ml of .05 M sodium thiosulfate. The samples were diluted to 15 ml with distilled water and after 15 minutes the colour intensity was measured on the Evelyn colorimeter (Evelyn, 1936) using a 490 mu filter. The amino-nitrogen values were taken from a curve (Figure 11) constructed from the galvanometer readings obtained with known amounts of glycine. Samples were adjusted to fall between 6 and 17 micrograms.

The advantage of this method over gasometric analysis is its rapidity since 8 to 12 samples can be analysed

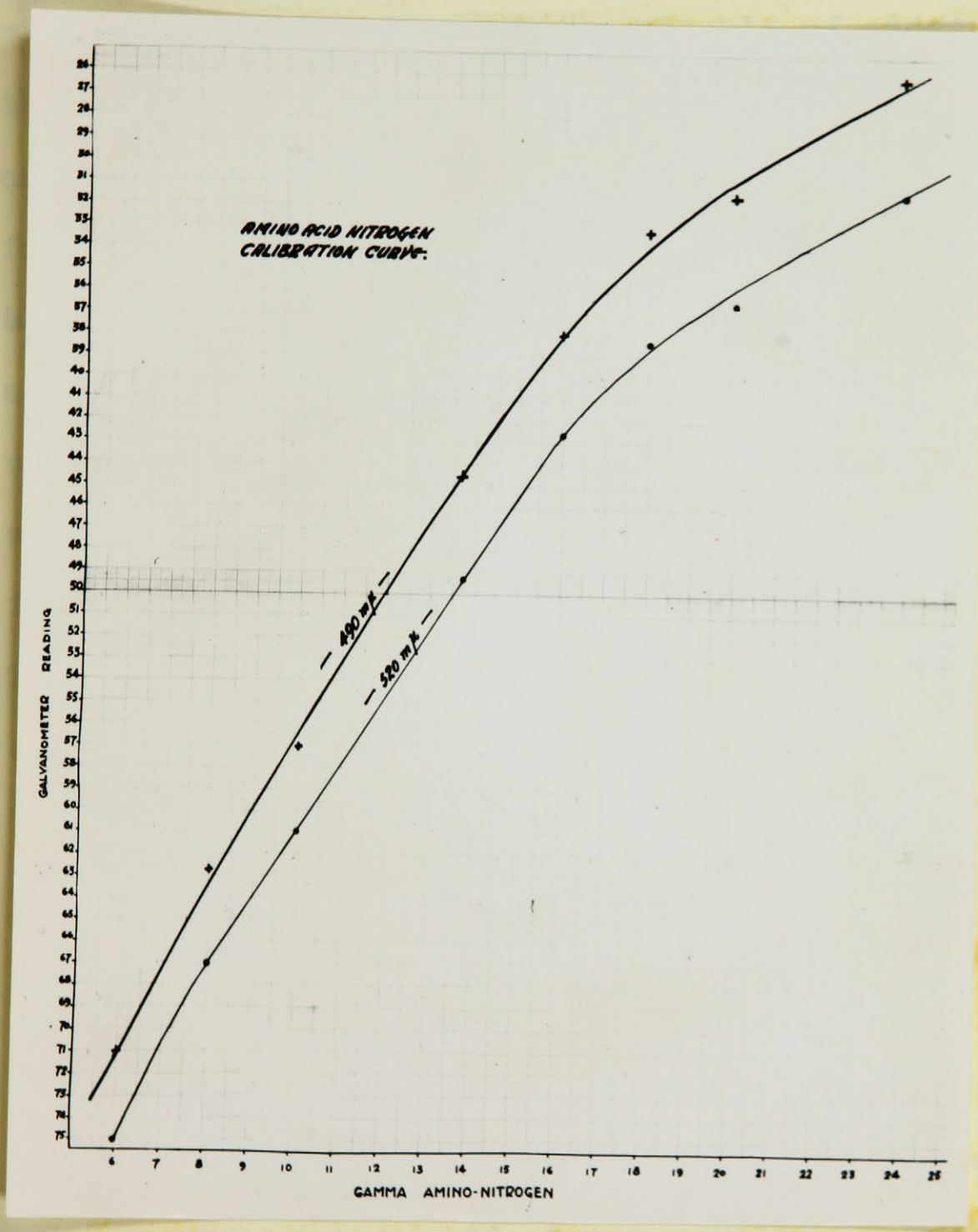


Fig. 11. Amino Acid Nitrogen Calibration Curve.

together if the time relationships are kept constant, and its economy of material since accurate determinations can be done on samples containing as little as 6 amino-nitrogen micrograms. Duplicates usually agreed to within less than 2%, small samples within 4%. Recovery of known amounts of added amino acids to the deproteinized solution was satisfactory. However, known and unknown factors are liable to give faulty results. Known factors are the interference of traces of chromic acid with the bleaching reaction, so that all glassware has to be rinsed in alkaline wash-solution if chromic acid cleaning solution is used. The sodium naphtho quinone sulfonate must be very pure and its solution freshly prepared. Cigarette smoke was also found to interfer strongly by causing a colour development. The amount of indicator added had to be kept constant, an extra drop causing a false higher value. It was further found that more colour was developed in a blank prepared from 2 ml of the distilled water -NaOH aeration mixture than in a blank containing 1 ml of the aeration mixture. The same was true for standard solutions containing the same amount of amino-nitrogen in 1 ml and in 2 ml aeration mixtures. By using blanks prepared from the same amount of aeration mixture as the solutions to be analysed this effect was completely cancelled out as verified from tests on standard solutions. Whether this colour development was related to the extra amount of sodium hydroxide contained in the samples or the quantity of acid necessary for neutralization or some other factor was not determined. Addition of NaCl corresponding to the amount of acid and alkali used caused no darkening. Possibly the acid may have contained ammonia.

To be on the safe side a standard glycine solution was subjected to aeration and colorimetric analysis along with the unknowns in all later experiments, and blanks were set up in duplicate.

(f) Aseptic Technique: Except for the earliest experiments (Experiments 4 ~~and 5~~) sterile technique was employed in order to make sure that the results would not be obscured by bacterial metabolism. The operation procedure has already been described. Operating instruments, the glass plunger and the rubber connections from the gas tank to the homogenate were sterilized by boiling twenty minutes in distilled water. All other utensils were placed for twenty minutes in a steam sterilizer at 120 lbs. pressure. The tubes in which the liver was to be weighed were plugged with cotton covered with cellophane. Pipettes were also plugged to prevent contamination by breathing, and sterilized in a large cotton stoppered graduate cylinder. The test tube in which the homogenate was to be incubated was sterilized with its rubber stopper containing the two glass tubes for aeration (plugged with small pieces of cotton) and with the Russian oil coating. The rubber stopper was lined with cellophane to avoid sticking to the tube.

The physiological salt mixture containing the phosphate buffer was also sterilized in the steam sterilizer, but when bicarbonate buffer was used the bicarbonate solution was passed through a Seitz filter since the CO_2 would have been driven off by heating. Usually four 50 ml lots of unbuffered salt mixture in 125 ml erlenmeyer flasks were sterilized at one time, enough for 4 experiments. Sufficient bicarbonate

solution was seitz filtered to last for about 10 experiments. The appropriate amount of bicarbonate was added with a sterile pipette to the salt mixture immediately before the start of the experiment.

Wherever possible the openings of flasks, tubes and the cylinder containing the pipettes were flamed when they had to be exposed, just after removal of the stopper, and just before re-insertion.

The measures were satisfactory since tests performed in the Department of Bacteriology on the homogenate occasionally at the start and always at the end of the incubation period showed mostly no contamination whatsoever, occasionally slight growth of non-proteolytic bacteria and only in two experiments was there contamination by proteolytic bacteria. These latter experiments were discarded. Even after 50 hours incubation the homogenates were still sterile. Samples were tested on brewer's meat and blood agar plates and where growth did occur the cultures were transferred to media designed to test proteolytic activity.

2. Results: It must be borne in mind that in the early experiments conditions were not yet satisfactorily standardized with respect to homogenizing time, aeration rate, asceptic technique, prevention of foaming, etc. Also since homogenates were analysed during incubation periods some times as long as 3 days in the early experiments, involving over 100 analysis on one rat liver, less material was available for each determination. Nevertheless, certain deductions may be drawn.

(a) Comparison of Aerated and Non-aerated Homogenate:

See Table II., also Figures 12, 13 and 14.

In the non-aerated homogenates in physiological salt mixture and buffered with .017 M phosphate, pH 7.3 (Experiments 4, 5) 34% and 44% respectively of the protein nitrogen was turned into non-protein nitrogen in 45 hours. 17% and 19% of the initial protein nitrogen was in amino acid N form after 45 hours incubation. The rate of NPN formation seemed to increase after 10 hours incubation, presumably due to exhaustion of the buffer resulting in a more acid pH favourable for proteolysis. In Experiment 6 the homogenate was subjected to the same treatment except that it was aerated with 100% O_2. Here only 15% of the protein nitrogen was transformed into NPN, 3% of the initial protein nitrogen, was present as amino alpha acid N.
Although these results are based on only three experiments they seem to indicate that proteolysis is inhibited by the aeration procedure. This is best illustrated by the alpha amino nitrogen curves which indicate that either alpha amino nitrogen formation stops earlier in the aerated suspension or else the amino group is further catabolised (Figures 12, 13 and 14). The same trend appears also when the homogenate is buffered with .025 molar bicarbonate and aerated with a 95% O_2, 5% CO_2 mixture. (Figure:15: Experiments 7 and 9, also Figure 16: Experiments 10 and 11: in the latter experiments the liver homogenates were buffered with bicarbonate, but rats were subjected to cold for 22 hours and fasted prior to killing.) The cessation of alpha amino nitrogen and inhibition of NPN formation was not correlated with the loss of total nitrogen that occurred in the aerated homogenates due to loss of solid matter with foaming before the Russian oil coating was introduced. In Experiment 9

TABLE II

NPN and Alpha Amino Nitrogen Formation in Aerated and Non-aerated Rat Liver Homogenate During 45 Hours Incubation
mg/gm fresh liver

Exp. No.	Condition of Homogenate	Zero Incubation Time				45 Hours Incubation Time			
		Total N *	NPN		Protein N	NPN		% protein nitrogen converted	
			Total	Alpha amino		Total	Alpha amino	to NPN	to alpha amino N.
4.	Krebs salt mixture 0.017 M phosphate buffer; not aerated.	33	1.6	.71	31.4	12.1	5.9	34	17
5.	"	31.4	2.1	.78	29.3	15.1	6.4	44	19
6.	Same medium; aerated with 100% O_2	34.8	3.4	1.05	31.4	8.0	1.9	15	3

* The total nitrogen was the same at 45 hours as a zero time in experiments 4 and 5: in experiment 6 the total nitrogen dropped to 27.6 gm.

(Figure 15) and also in Experiments 10 and 11 (Figure 16) the decrease in NPN and alpha amino nitrogen "formation" occurred although Russian oil had been used and the total nitrogen level was maintained. It should be mentioned that in Experiment 6 the homogenate was accidentally diluted to contain 0.145 gm fresh liver tissue per ml, instead of 0.17 gm. This was not the case in Experiments 7 - 11 however, where similar results were obtained.

Among the factors responsible for the different results in aerated and non-aerated homogenates may be the inhibitory effect of oxygen on proteolytic enzymes. As already mentioned the layer of homogenate in the non-aerated experiments was too thick to assure sufficient oxygen diffusion without shaking, and anaerobic conditions must have prevailed in the deeper layers.

The pH, due to an increased rate of glycolysis, may have been more favourable for proteolysis in the partially anaerobic experiments. Experiment 6 however, shows a low pH of 5.5 in the homogenate after 27 hours of incubation when the alpha amino-nitrogen concentration remained stationary.

The mechanical ~~effects of brought about by~~ the vigorous bubbling may have caused denaturation and inactivation of enzyme proteins.

(b) Distribution of Nitrogen Fractions: The concentration of total nitrogen contained in the whole homogenate in the final experiments averaged 33 mg/g fresh liver (see table III), the concentration of protein (total nitrogen minus NPN), averaged 30 mg. The concentration of proteins contained in the sediment free part of the homogenate averaged 19.3 mg/gm liver; i.e., it

TABLE III

Distribution of Initial Nitrogen Fractions
mg/gm Fresh liver

Exp. No.	Total N		Total NPN		Amino N		Protein N		% Protein N. extra cell.
	Entire homog.	Extra cell.	Entire homog.	Extra cell.	Entire homog.	Extra cell.	Entire homog.	Extra cell.	
3	36.0	29.2							
4	33.0	24.4	1.55	2.20	.71	.44	31.4	22.2	71
5	31.4	20.6	2.10	1.87	.78	.71	29.3	18.7	64
6	34.8	20.2	3.38	2.55	1.05	.66	31.4	17.6	56
7	32.8	20.7	2.46	2.10			30.3	18.6	61
9	30.0	19.1	1.65	1.55	.53	.51	28.3	17.5	62
10&	29.2	21.0	2.60	2.58	.50	.62	26.6	18.4	69
11&	34.5	23.0	1.44	2.22			32.6	20.8	66
Av.	33.0	22.4	2.23	2.06	.77	.58	30.1	19.3	64

& Liver homogenates from rats subjected to cold treatment.

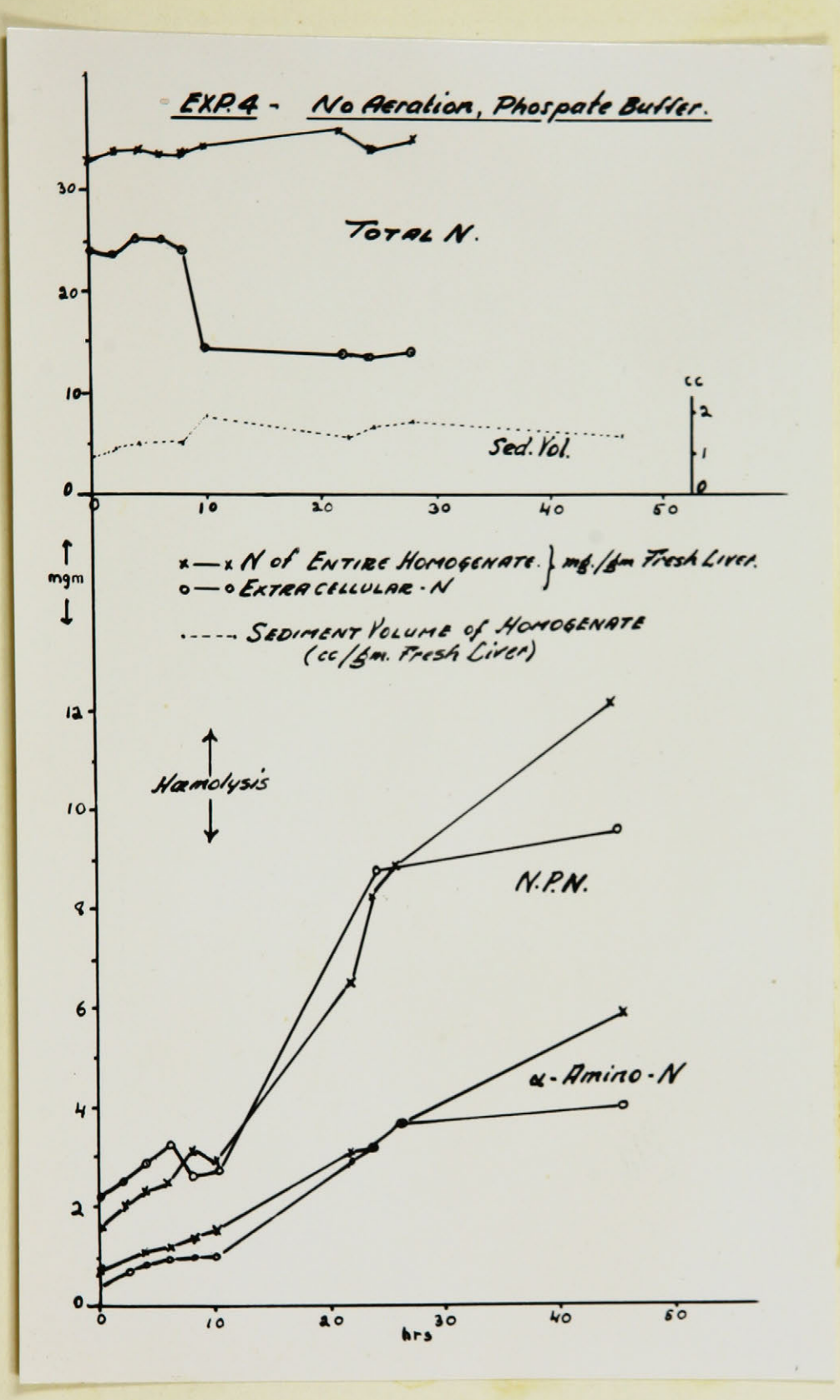


Fig. 12 Exp. 4 Changes with incubation time in nitrogen fractions of unaerated rat liver homogenate. (Incubation at 37.5°C, initial pH 7.3, Krebs' phosphate medium).

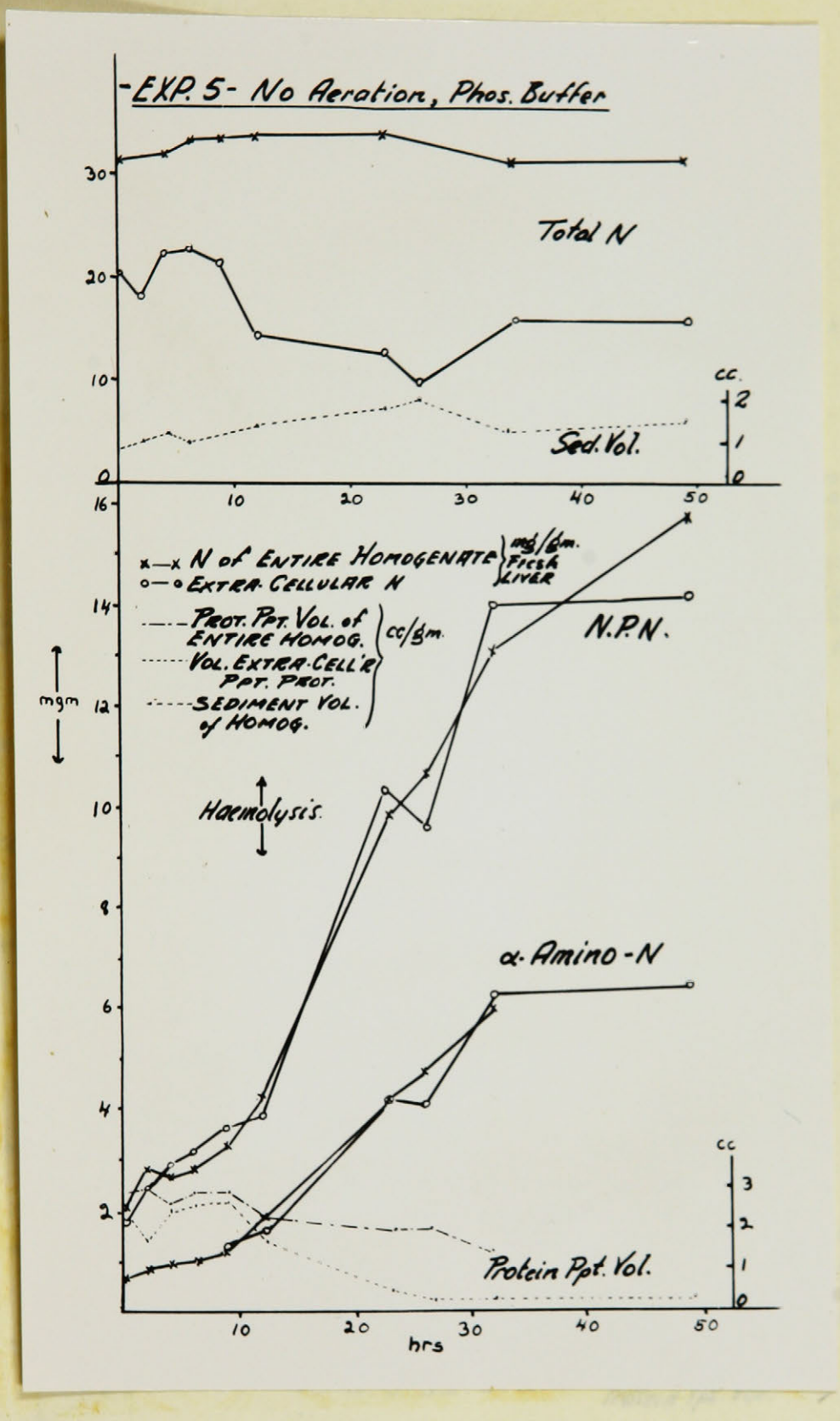


Fig. 13 Exp. 5 Changes with incubation time in nitrogen fractions of unaerated rat liver homogenate. (Experimental conditions as in Exp. 4).

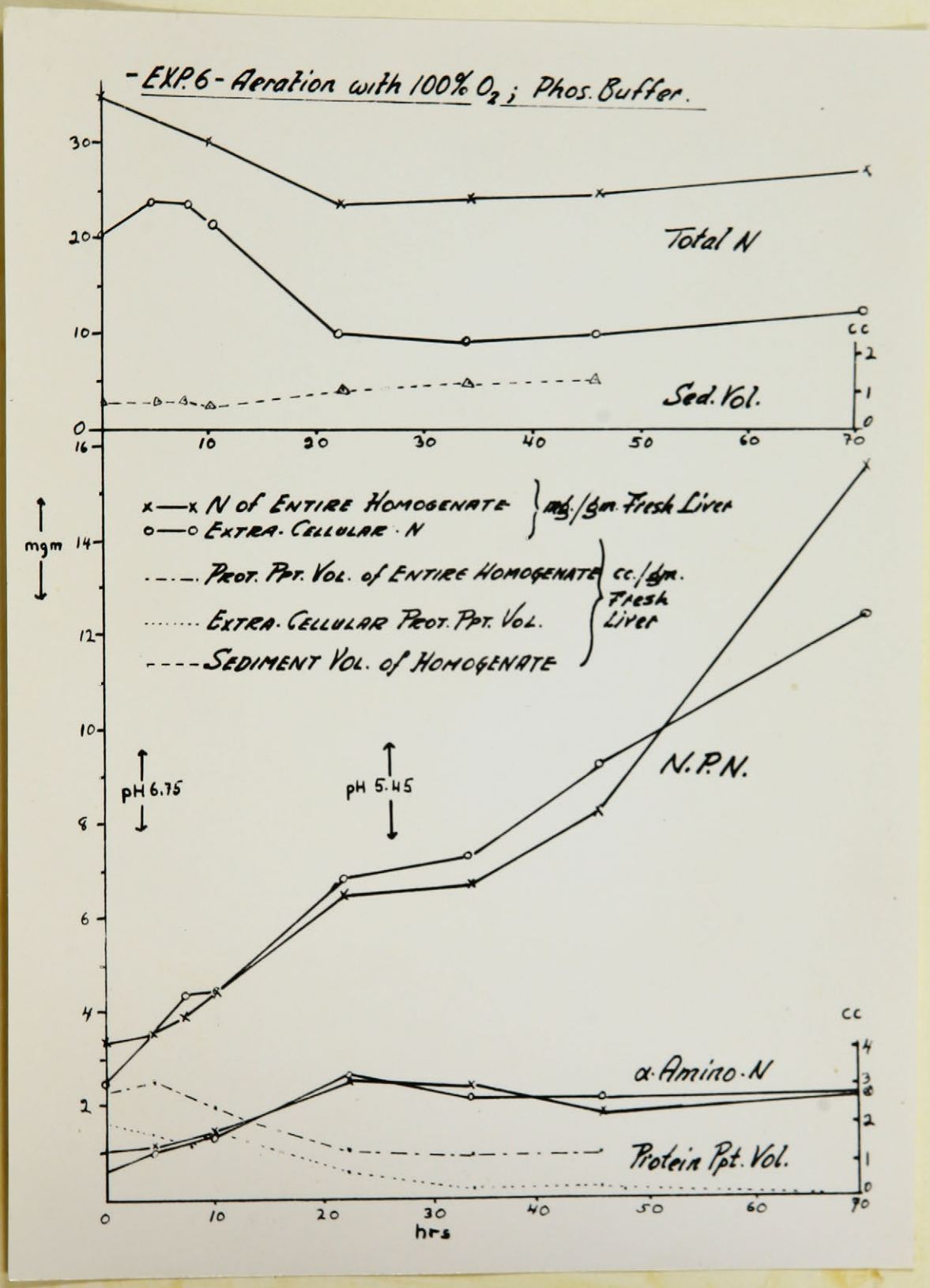


Fig. 14 Exp. 6. Changes with incubation time in nitrogen fractions of rat liver homogenate, aerated with 100% O_2 (Temperature, initial pH and medium as in Expts. 4 and 5).

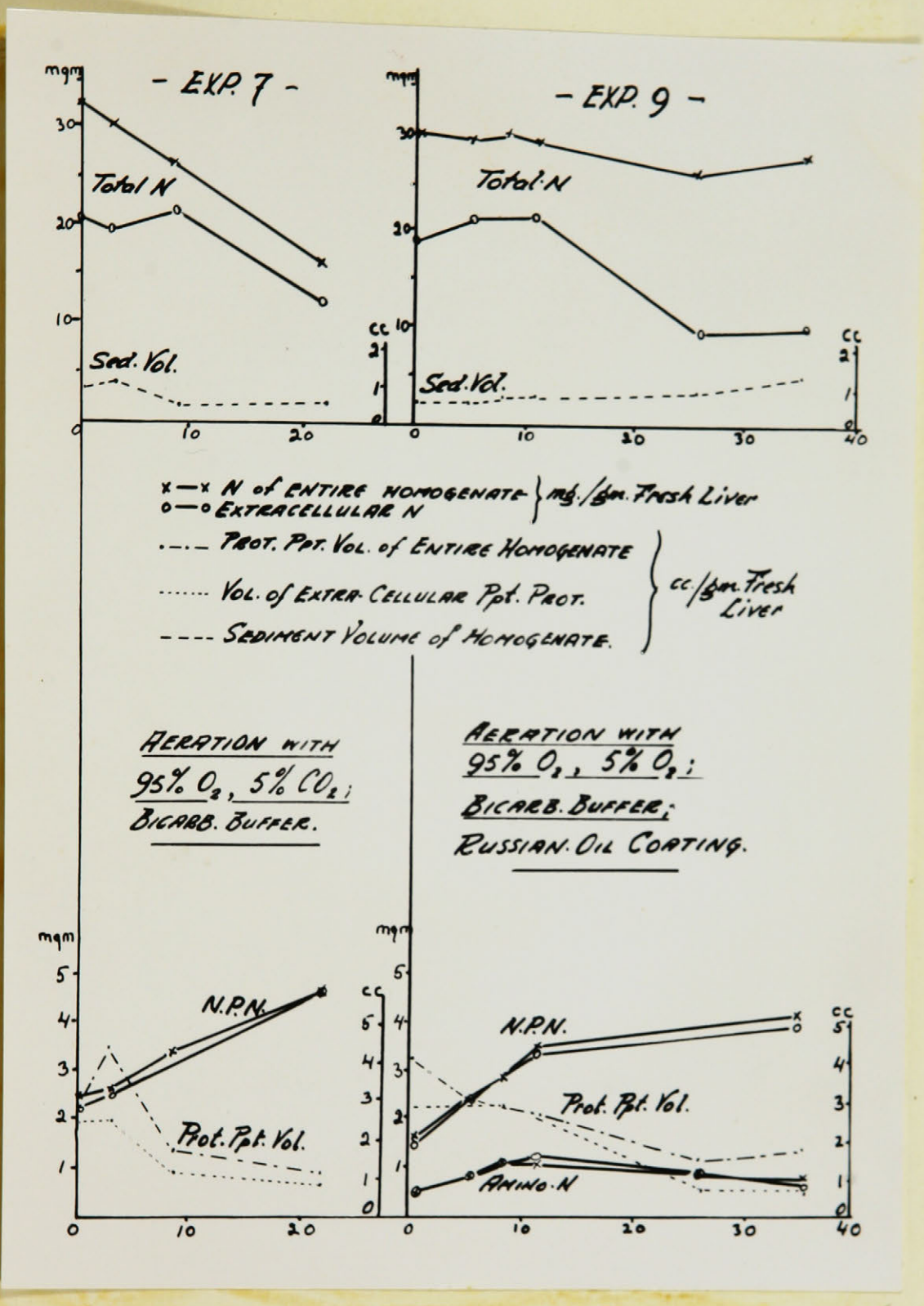


Fig. 15 Expts. 7 and 9. Changes with incubation time in nitrogen fractions of rat liver homogenate, aerated with 95% O_2, 5% CO_2. (Krebs' Bicarbonate medium, pH 7.3; temp. 37.5°C). In Exp. 9 glass walls in contact with the homogenate were coated with Russian Oil.

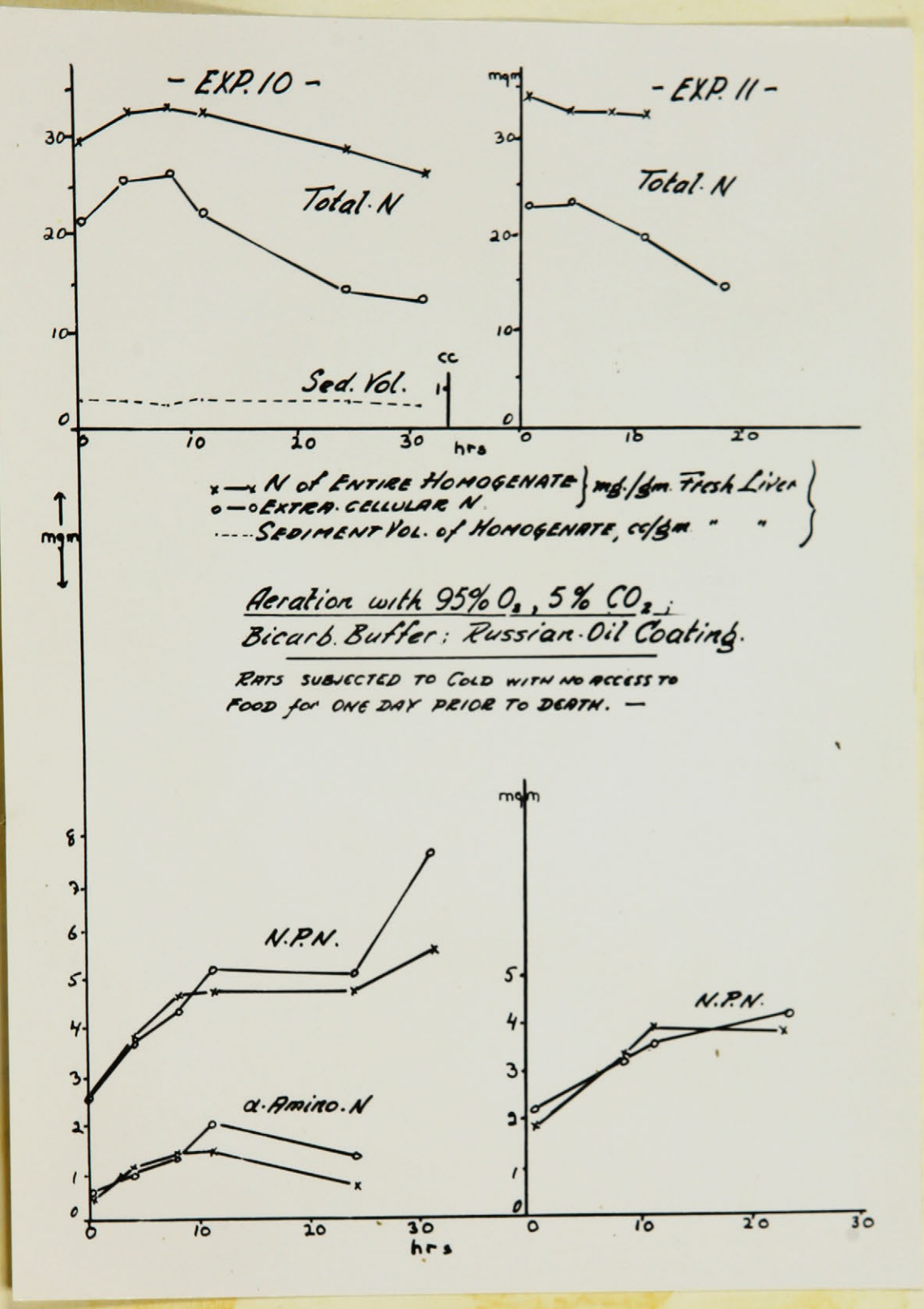


Fig. 16 Expts. 10 and 11: Changes with incubation time in nitrogen fractions of aerated rat liver homogenate. Experimental conditions as in Exp. 9 but livers were taken from rats subjected to cold for 22 hours prior to death.

contained 64% of all the protein. This high figure must be explained by the disintregation of cells due to homogenisation and possibly by discharge of intracellular protein. The N.P.N. content of the entire homogenate averaged 2.23 mg/gm liver, that of the sediment-free part 2.06 mg/gm liver. 0.77 mg and 0.58 mg respectively consisted of alpha-amino N.

It was found that after a certain length of incubation, approximately 10 hours, the extra-cellular total nitrogen (i.e., the nitrogen contained in the supernatant of a centrifuged sample of homogenate (before protein precipitation) dropped to about one-half its initial value, (Figures 12-16.) This was probably due to the change of pH to the isoelectric point of some proteins. They were thus rendered insoluble and carried down with centrifugation. This was reflected in the increase in sediment carried down from the homogenate in all experiments where the sediment volume was recorded, with two exceptions: In Experiment 7 the volume decreased probably due to the excessive loss of solid matter on the walls from foaming as reflected in the decrease in the total nitrogen of the entire homogenate which was the largest of all experiments. In experiment 10 the volume remained constant.

Coincident with the decrease in extra-cellular nitrogen there was a change in the appearance of the centrifuged homogenate. The red blood cells which till then were seen as a distinct layer in the sediment had disappeared, and the supernatant previously slightly opaque (even if filtered repeatedly) and straw coloured, was clear and light red due to

haemolysis of the red blood cells contained in the homogenate. (Fig 5.6) The red colour decreased in intensity in the samples taken during the later stages of incubation presumably because of the breakdown of haemoglobin.

The recordings of protein precipitate volumes both from the entire homogenate and from the cell free supernatant reflect the decrease in the amount of protein as incubation proceeds. In Experiment 6 all the proteins precipitable by the sulfate tungstate mixture in the cell free supernatant had been broken down after 72 hours incubation, since no precipitate was formed.

II. COMPARISON OF NORMAL AND DAMAGED ANIMALS:

1. Methods: The aim in the following experiments was to compare the in vitro liver metabolism of rats subjected to widely different types of damaging stimuli with that of normal rats. The experiments fall into the following groups.

(Group I): Normal control group. This group, had free access to food (purina fox chow) and water prior to killing.

(Group II): Fracture of left femur 3 to 4 days prior to death. Rats were anaesthesized with .125 ml of a nembutal (1gr/ml) solution and operated on by the method of Cuthbertson et al, 1939. An incision was made over the site of the fracture. The muscles on the lateral side of the left leg were separated and the femur cut completely with a blunt pair of scissors. The skin edges were then sutured. Very little bleeding accompanied the operation. The wound always healed well. Animals had free access to purina fox chow and water until death but usually ate little the first days after operation. The food and water

intake was not recorded. Animals were killed 3 to 4 days after the operation because at this time the rats are at the height of negative nitrogen balance after this type of operation according to Cuthbertson, McGirr and Robertson (1939) and Cohen (1945). Two animals each were also killed 1 to 2 days (Group IIa) and 5 to 6 days (Group IIb) after operation. The number of animals in the last two groups was too small to warrant any definite conclusions from the results obtained.

(Group III): Exposure to cold and fasting for 1 day prior to death. Rats were placed in an icebox at a temperature of 2^{o} to 8^{o} C for 21 to 27 hours prior to killing. They had no access to food or drink during this period.

(Group IV): Exposure to cold for 1 day prior to death without fasting. Rats were placed in an icebox as in the previous group but were given purina fox chow and a 30% glucose solution at libitum. During this period they ate 12 to 30 grams fox chow and drank 4 to 30 ml of glucose solution each.

(Group V): Starvation at room temperature for 1 day prior to killing. Rats were deprived of food and drink for 22 to 26 hours before killing.

The method of killing and the experimental procedures after sacrificing the animals have already been described in the section dealing with the standardization of methods. Conditions were now satisfactorily adjusted so that as far as is known the only variable was the treatment of the animals prior to death. The experimental data include:-

Physical:

1. Initial body weight determination.
2. Liver weight determination.
3. Adrenal weight determination. Adrenals were removed within 1 hour after death and, after fixing in Haydenhain's Suza solution, freed of non-glandular tissue and weighed on a microbalance.

Chemical:

Analyses were made on whole homogenate only (in Krebs bicarbonate medium in equilibrium with 95% O_2 5% CO_2) with incubation time limited to 9 hours.

1. Total nitrogen at 0 and at 9 hours incubation time, to check loss of solid material.
2. NPN at 0, 1½, 3, 6, and 9 hours incubation time.
3. Alpha amino nitrogen at 0, 1½, 3, 6, and 9 hours incubation time.
4. pH determinations over the period of incubation. (see Table I). No difference in the maintenance of pH between the homogenates of the different groups were found.

Histological:

Smears were made from a normal control homogenate at 0, 3, 6, and 9 hours incubation time, stained with haematoxylin and eosin and photographed, (see Figures 1 - 8.)

All pertinent data were statistically analysed after Snedecor (1940). Reference tables were taken from Fisher (1944). The following procedure adapted to compare experimental data on two groups of different sizes was chosen: First, the mean value for one group of observations is computed ($\bar{x}$).

The difference of each observation from the mean is then obtained (d_1), and squared (d_1^2). The sum of the squares (ξd_1^2) is then added to the sum of the squares obtained in the same manner from the group that is to be compared with the first one (ξd_2^2). To obtain the variance the pooled sum of squares is divided by the degrees of freedom; i.e., the number of observations (n) in both groups minus 2: $V = \frac{\xi d_1^2 + \xi d_2^2}{n_1 + n_2 - 2}$

The variances of the two <u>means</u> are V/n_1 and V/n_2: the variance of the <u>difference</u> between the means is the sum of these: $V/n_1 + V/n_2$. The square root thereof finally gives the standard error of the difference between the means, $S_{\bar{d}} = \sqrt{V/n_1 + V/n_2}$

The standard error of the difference between the means is then divided into the actual difference between the means of the two groups to be compared. $\frac{\bar{X}_2 - \bar{X}_1}{S_{\bar{d}}} = t.$

The bigger t, the more significant are the results. A table is consulted that gives the probability (P) of the results being due to chance for each value of t (depending on the degrees of freedom). If P was less than 0.01, i.e., the probability that results were due to chance was 1 in a hundred, the data were initialled "hs" (highly significant): if P was between 0.01 and 0.02 data were marked "s+" (significant plus): if P was between 0.02 and 0.05 data were marked "s"; if P was between 0.05 and 0.1, data were marked "d" (doubtful significence.) If P was larger than 0.1 i.e., the probability was greater than 1 in 10 that results were due to chance, data were marked with a dash. (not significant).

EXAMPLE

Purpose: to determine the significance of the difference between means of two groups of data on liver protein content.

Protein Content of Livers from Untreated Rats (micrograms/total liver/gm rat)			
Exp. No.		d_1	d_1^2
18	940	50	2500
19	850	40	1600
24	1020	130	16900
27	850	40	1600
29	910	20	400
32	760	130	16900
40	970	80	6400
46	840	50	2500
	$\bar{x}_1 = 890$		$\Sigma d_1^2 = 48800$

$n_1 = 8$

Protein Content of Livers from Rats Starved 24 hours at Room Temperature (micrograms/total liver/gm rat)			
Exp. No.		d_2	d_2^2
49	720	10	100
50	610	100	10000
51	680	30	900
54	810	100	10000
	$\bar{x}_2 = 710$		$\Sigma d_2^2 = 21000$

$n_2 = 4$

$$V = \frac{\Sigma d_1^2 + \Sigma d_2^2}{n_1 + n_2 - 2} = \frac{48800 + 21000}{10} = 6980$$

$$S_{\bar{d}} = \sqrt{\frac{V}{n_1} + \frac{V}{n_2}} = \sqrt{\frac{6980}{8} + \frac{6980}{4}} = 51$$

$$t = \frac{\bar{x}_1 - \bar{x}_2}{S_{\bar{d}}} = \frac{890 - 710}{51} = 3.5$$

Degrees of freedom = $n_1 + n_2 - 2 = 10$

$P < 0.01$

Result: The difference between the means of the two groups is highly significant.

2. Results:

a. In vivo observations: This section deals with observations on organ weights, and the concentration of the different nitrogen fractions in the livers of the different groups 35 minutes after death of the animals.

(a) Liver Weights: The data on liver weights are presented in Table IV. Liver weights were expressed in percentage body weight to rule out the effect of the animal's size. (It was assumed that organ weight was proportional to body weight.) In groups II to V liver weights were expressed as percent of the animal's body weight before initiation of treatment. The body weight varied between 245 and 265 grams. The absolute liver weights ranged from 3.87 grams in a rat subjected to cold and starvation to 9.25 grams in an untreated rat.

It will be seen that the highest liver weights are those of the untreated group, averaging 3.11% body weight. All the treated groups showed a lower average. The decrease in liver weight was statistically significant in Group II (Rats with fractured femurs) with a 14% decrease, in Group III (Rats subjected to cold and fasted) with a 32% decrease, and in Group V (Rats fasted at room temperature) with a 29% decrease. The 10% decrease in liver weight of Group IV (Rats subjected to cold but fed) was statistically not significant.

(b) Adrenal Weights:

The adrenal weights were determined on 7 animals in the normal group, 8 in Group II, 3 each in Groups III and IV, and 5 in Group V. No significant difference in adrenal weights between the normal and the treated groups was found (See Table V). This is somewhat surprising since adrenal hypertrophy

TABLE IV

Liver Weights of Normal Rats and Rats Subjected to Damaging Agents.

	Liver Weight as % Body Weight	Percent Change from Normal
I Controls	xi 3.11	
II Fractured killed day 3-4	hs vii 2.68	-14
IIa " " 1-2	- ii 2.88	-7
IIb " " 5-6	- ii 2.85	-8
III Cold, 5°C. for 24 hrs. (fasted)	hs vii 2.10	-32
IV Cold (fed)	- iv 2.81	-10
V Room temp., fasted 24 hrs.	hs v 2.21	-29

hs highly significant ($P < 0.01$).
- not significant ($P > 0.1$).
Roman numerals- numbers of animals.

TABLE V

Adrenal Weights of Normal Rats and Rats Subjected to Damaging Stimuli

	Adrenal Weight as % Body Weight	Percent Change from Normal
I Controls	vii .0143	
II Fractured, killed day 3-4	- viii .0166	+16
IIa " " 1-2	- ii .0168	+17
IIb " " 5-6	- i .0164	+15
III Cold, 5°C for 24 hrs. (fasted)	- iii .0148	+3
IV Cold (fed)	d iii .0173	+21
V Room temp., fasted 24 hrs.	- v .0140	-2

d doubtful significance (P: 0.05-0.01).
- not significant (P > 0.1).

Roman numerals- numbers of animals.

in response to the damaging stimuli was expected. Probably time was not sufficient to allow for adrenal hyperplasia to become so marked as to be detectable in adrenal weights. In any case these results do not exclude increased adrenal cortical secretion in response to the damaging stimuli.

(c) Liver Proteins:

The data on liver nitrogen fractions are recorded in two ways (1) as mg per gram liver to compare the concentration, (2) as micrograms per total liver weight per gram body weight to compare the actual amounts of protein. From Table VI and VII it may be seen that the protein contents of the liver in all treated groups showed lower averages than the normal group, whereas the average protein concentrations were higher in all treated groups. The increase in protein concentration was statistically significant in the rats subjected to cold and fasting (Group III) averaging a 15% increase, and in the rats subjected to fasting at room temperature (Group V) averaging a 10% increase. The decrease in total amount of protein present was significant in all treated groups except in the group subjected to cold for 1 day but with food available. The liver proteins dropped 20% in the group fasted 1 day at room temperature, 17% in the group subjected to cold and fasting for 1 day, 11% in the group killed 3 to 4 days after femur fracture.

(d) Non-protein nitrogen:

The non-protein nitrogen concentrations of the livers 35 minutes after death showed no significant difference among any two groups (Table VI). The non-protein nitrogen

TABLE VI

Rat Liver Protein Nitrogen, Non-protein Nitrogen, and Alpha Amino Nitrogen Concentration, (mgm/gm fresh liver)

	Protein N	NPN	Amino N
I Controls	viii 28.6	viii 2.52	vii .65
II Fractured, killed day 3-4.	- v 30.4	- vi 2.52	- vi .61
IIa " " 1-2	- ii 30.4	- ii 2.74	- ii .70
IIb " " 5-6	s ii 32.9	- ii 2.44	- ii .53
III Cold, 5°C for 24 hrs. (fasted)	hs iii 32.9	- iii 2.51	- vi .58
IV Cold (fed)	d iv 31.2	- iv 2.72	- iv .55
V Room temp., fasted 24 hrs.	s iv 31.6	- iv 2.72	- iv .57

hs highly significant ($P < 0.01$).
s significant (P: 0.02-0.05).
d doubtful significance (P 0.05-0.1).
\- not significant ($P > 0.1$).
Roman numerals- numbers of animals.

TABLE VII

Rat Liver Protein Nitrogen, Non-protein Nitrogen, and Alpha Amino Nitrogen Content 35 min. after Death. (micrograms/total liver/gm rat).

	Protein N	NPN	Amino N
I Controls	viii 890	viii 79	vii 20.5
II Fractured, killed day 3-4.	s v 790	- vi 67	d vi 16.2
IIa " " 1-2	- ii 870	- ii 79	- ii 20.2
IIb " " 5-6	- II 930	- ii 69	- ii 14.9
III Cold, 5°C for 24 hrs. (fasted)	s+ iii 740	s iii 52	hs vi 11.6
IV Cold (fed)	- iv 830	- iv 76	s iv 15.3
V Room temp., fasted 24 hrs.	hs iv 710	s iv 60	hs iv 12.6

hs highly significant ($P < 0.01$).
s+ significant plus (P: 0.01-0.02).
s significant (P: 0.02-0.05).
d doubtful significance (P 0.05-0.1).
- not significant ($P > 0.1$).
Roman numerals- numbers of animals.

content of the whole livers however, was again significantly less in the group subjected to cold and fasting, with a decrease of 35%, and in the group fasted at room temperature with a decrease of 25%. The 15% decrease in the group killed 3 to 4 days after femur fracture was statistically not significant. (Table VII).

(e) Alpha amino nitrogen:

The alpha amino nitrogen concentration also did not vary significantly from the normal in any treated group, (Table VI). The alpha amino nitrogen content of the livers, however, was significantly decreased in all treated groups except the group with fractured femurs where the decrease was of doubtful significance (P between .05 and .10 Table VII). The average amino nitrogen decrease was 43% in the group subjected for 1 day to cold and fasting, 39% in the group fasted for 1 day at room temperature, 25% in the group subjected to cold for 1 day but with food available, and 21% in the group killed 3 to 4 days after femur fracture.

From Table VIII it may be seen that the alpha amino nitrogen fraction of the treated groups showed a greater percentage decrease than the remaining non-protein nitrogen (and also the total NPN).

In summary one may say that the procedures involving fasting of the animals resulted in the most striking changes in liver weight as well as in the concentrations and absolute amounts of the nitrogen fractions analysed. Thus, Group III (Rats subjected to cold and fasted, and Group V (Rats fasted at room temperature) show the greatest decrease in liver weight,

TABLE VIII

Percent Decrease of Nitrogen Fractions
in Damaged Groups Compared to Control Group
(% calculated from values in Table VII.)

	Total NPN	NPN Other Than Alpha Amino N.	Alpha Amino N.
II	15	13	21
III	35	32	43
IV	4	4 (increase)	25
V	25	20	39

the greatest increase in protein nitrogen concentration, and the greatest decrease in absolute amounts of liver protein - non protein -, and alpha amino nitrogen. The combined stimuli of cold plus fasting (Group III) did not produce any significantly greater effect than fasting at room temperature (Group V). The data on the remaining groups show a similar trend although the results are not always significant.

b. In vitro observations: This section deals with the in vitro formation of non-protein nitrogen and alpha amino nitrogen by the homogenized livers from the various groups of animals. Increases in concentration (mg per gram liver), as well as in amounts (microgram per total liver weight per gram body weight) over 1½, 3, 6, and 9 hours incubation were recorded taking the value at the outset of incubation as 0, i.e. subtracting that value from the absolute values obtained at the above mentioned incubation times.

(a) Total non-protein nitrogen: (Tables IX, X, XI)

It will be seen from Tables IX and X that the average increase in non-protein nitrogen concentration as well as in absolute amounts was greater than normal in all treated groups throughout the period of incubation. The significance of the deviation of the average values at the different stages of incubation from that of the normal group at the corresponding stages is indicated by the initials over the figures. The increase in amounts as well as concentration over the 3 hour period is significant in the

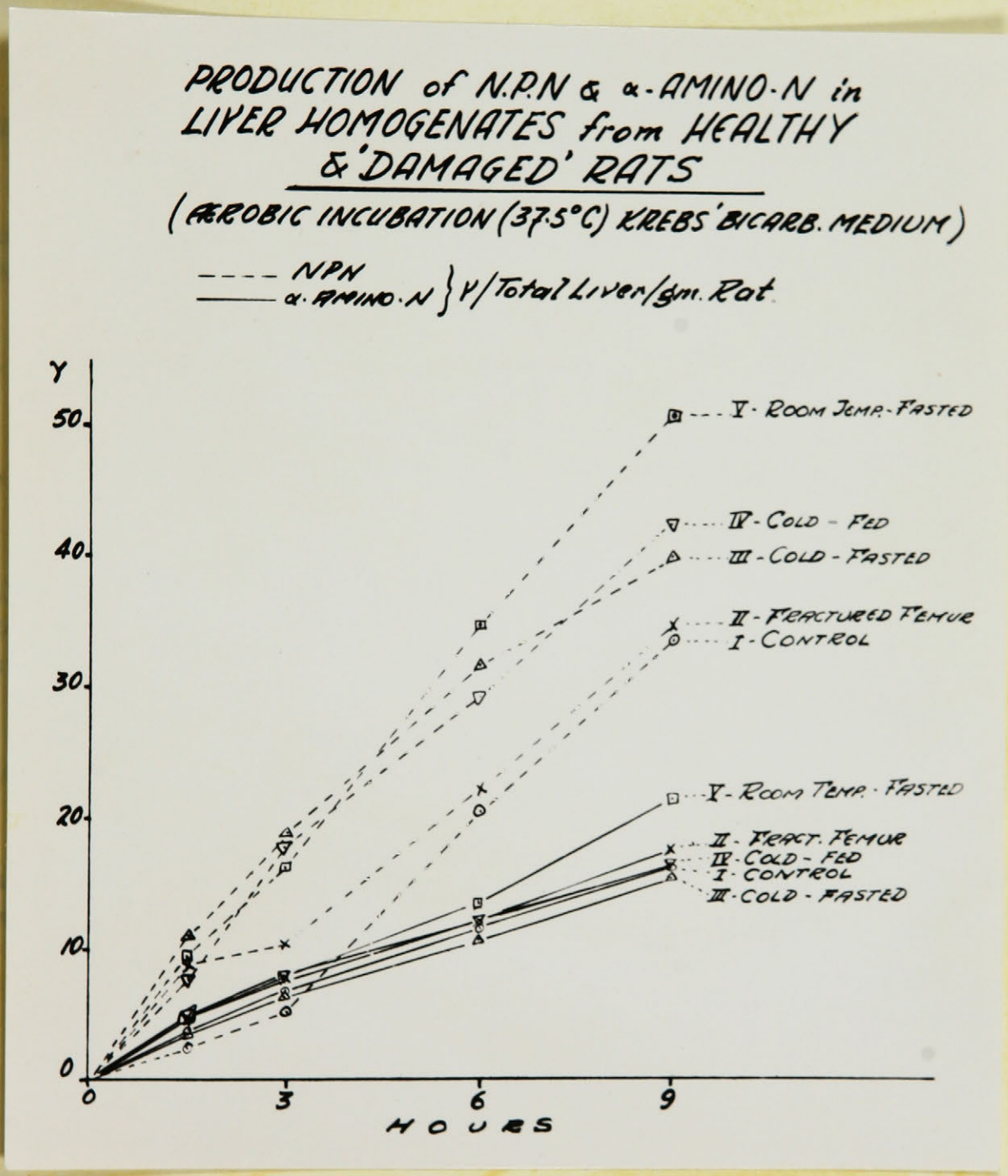


Fig. 17 Production of Non Protein Nitrogen and Alpha Amino Nitrogen in liver homogenates from healthy and damaged rats. (Aeration with 95% O_2 5% CO_2, Krebs' bicarbonate medium pH 7.3, 37.5°C)

TABLE IX

Increments in NPN with Time
mg/gm fresh liver

Incubation times	0-1½	0-3	0-6	0-9 hours
I Controls	v .07	vi .15	vi .65	v 1.08
II Fractured, killed day 3-4	d vi .33	d v .39	- vi .80	- vi 1.33
IIa " " 1-2	- ii .24	- ii .46	- ii .90	- ii 1.49
IIb " " 5-6	- ii .32	- ii .45	- ii .90	- ii 1.29
III Cold, 5 C for 24 hrs. (fasted)	hs ii .64	hs ii 1.07	s iii 1.69	- iii 2.17
IV Cold (fed)	d iv .30	hs iv .64	s+ iv 1.05	- iii 1.49
V Room temp., fasted 24 hrs.	s+ iv .42	hs iv .74	hs iv 1.59	hs iii 2.33

Symbols as in Table VII

TABLE X

Increments in NPN
micrograms/total liver/gm rat

Incubation times	0-1½	0-3	0-6	0-9 hours
I Controls	v 2.4	vi 5.2	vi 20.4	v 33.9
II Fractured, killed day 3-4	- vi 8.7	- v 10.2	- vi 22.3	- vi 35.0
IIa " " 1-2	- ii 6.9	- ii 13.7	- ii 26.8	- ii 42.9
IIb " " 5-6	- ii 9.1	- ii 12.7	- ii 25.6	- ii 36.8
III Cold, 5 C for 24 hrs. (fasted)	d ii 11.5	s ii 18.8	d iii 31.8	- iii 40.1
IV Cold (fed)	- iv 7.8	hs iv 17.8	d iv 29.2	- iii 42.8
V Room temp., fasted 24 hrs.	d iv 9.5	hs iv 16.1	hs iv 34.9	s iii 51.5

Symbols as in Table VII

TABLE XI

Rate of NPN Appearance in Rat Liver Homogenate
γ/total liver wt/gm rat/hr

Rate between	0-1½	1½-3	3-6	6-9 hours
I Controls	v 1.59	v 1.13	vi 5.12	v 5.00
II Fractured killed day 3-4	- vi 5.76	- v 2.15	- v 3.74	- vi 4.23
III Cold, 5°C for 24 hrs. (fasted)	s ii 7.66	s ii 4.87	- ii 5.58	- iii 2.75
IV Cold (fed)	- iv 5.20	s iv 6.66	- iv 3.78	- iii 4.53
V Room temp., fasted 24 hrs.	d iv 6.34	d iv 4.40	- iv 6.27	- iii 6.33

Symbols as in Table VII.

homogenates of all treated animals except those with fractured femurs. It will be noted that the increase in concentration over normal was more marked than the increase in amounts due to the liver shrinkage in the treated rats. Figure 17 contains the graphical representation of the increase in non-protein nitrogen of the different groups calculated per total liver weight per gram body weight. The average rates of NPN increase are summarized in Table XI (average rates were computed over the 0 to 1½ hours incubation period, over the 1½ to 3 hour, over the 3 to 6 hour, and over the 6 to 9 hour incubation period.) It is evident that over the first 3 hours of incubation the rate of NPN increase is much lower in the normal than in the treated groups. After 3 hours, however, the rate in the normal homogenates increases markedly and is not significantly different from the treated groups. The treated groups, with the possible exception of Group II, (rats with fractured femurs), do not have this initially lower rate. The change in rate within each group over the period of incubation was analysed statistically. The only significant change was the increase in rate in the normal group after 3 hours incubation.

(b) The alpha amino nitrogen fraction of the non-protein nitrogen. (Tables XII, XIII, XIV)

In contrast to the results obtained from the total non-protein nitrogen analyses, no significant difference was found between the increase in absolute amount of alpha amino nitrogen in the homogenates from the normal group throughout incubation as compared to the

homogenates from the treated groups with one exception: the average 9 hour value of the homogenates from the group fasted at room temperature was significantly higher than the average value of the normal group. However, the values at earlier stages of incubation are not significantly different from those of the normal group. This high 9 hour value must have been caused, therefore, by an increased rate of alpha amino nitrogen appearance during the last 3 hours of incubation compared to the normal rate at that time. From Table XIV which lists the rates of alpha-amino N appearance at different incubation times it is seen that the only significant difference from normal among all groups at all stages of incubation was the average rate of increase in alpha amino nitrogen amounts over the 6 to 9 hour period in the homogenates of the group fasted at room temperature. (The increase in rate of alpha amino nitrogen appearance from the second 3 hour period to the third 3 hour period within this group was of doubtful significance (P between .05 and .10)).

The alpha amino nitrogen concentration, however, increased more rapidly in the homogenates from the treated groups than in the controls (Table XII), despite the fact that the absolute amount of alpha amino nitrogen did not increase more rapidly in the treated groups. The greater increase in alpha amino nitrogen concentration in the livers from the treated groups is most likely related to the loss of liver glycogen from these animals. Since the livers had considerably decreased in weight the same amount of

TABLE XI I

Increments in Amino Nitrogen with Time
mg/gm fresh liver

Incubation times	0-1½	0-3	0-6	0-9 hours
I Controls	vii .114	vii .215	vii .370	vii .515
II Fractured killed day 3-4	s vi .185	d v .285	d vi .455	s vi .665
IIa " " 1-2	- ii .130	- ii .230	- ii .330	- ii .510
IIb " " 5-6	- i .140	- ii .190	- ii .360	- ii .470
III Cold, 5° C for 24 hrs. (fasted)	d iii .175	s- vi .320	hs vi .540	hs vi .785
IV Cold (fed)	- iv .170	- iv .260	d iv .430	d iv .595
V Room temp., fasted 24 hrs.	s+ iv .215	hs iv .360	hs iv .600	hs iii .970

Symbols as in Table VII

TABLE XIII

Increments in Amino Nitrogen
micrograms/total liver/gm rat

Incubation times	0-1½	0-3	0-6	0-9 hours
I Controls	vii 3.7	vii 6.8	vii 11.7	vii 16.3
II Fractured killed day 3-4	- vi 4.8	- v 7.5	- vi 12.1	- vi 17.6
IIa " " 1-2	- ii 3.7	- ii 6.6	- ii 9.5	- ii 15.3
IIb " " 5-6	- i 4.0	- ii 5.4	- ii 7.4	- ii 13.3
III Cold, 5° C for 24 hrs. (fasted)	- iii 3.4	- vi 6.3	- vi 10.7	- vi 15.5
IV Cold (fed)	- iv 4.8	- iv 8.0	- iv 12.1	- iv 16.5
V Room temp., fasted 24 hrs.	- iv 4.8	- iv 7.9	- iv 13.5	hs iii 21.5

Symbols as in Table VII

TABLE XIV

Rate of Amino Nitrogen Appearance in Rat Liver Homogenate
γ/total liver wt/gm rat/hr.

Rate between	0-1½	1½-3	3-6	6-9 hrs
I Controls	vii 2.74	vii 2.10	vii 1.63	vii 1.52
II Fractured, killed day 3-4	- vi 3.23	- v 2.07	- v 1.57	- vi 1.82
III Cold, 5°C for 24 hrs. (fasted)	- iii 2.28	- iii 1.60	- vi .145	- vi 1.60
IV Cold (fed)	- iv 3.23	- iv 2.10	- iv 1.36	- iv 1.47
V Room temp., fasted 24 hrs.	- iv 3.17	- iv 2.12	- iv 1.85	s iii 2.50

Symbols as in Table VII.

proteolytic enzyme activity would bring about an increased quantity of alpha amino nitrogen formation per gram liver although the same absolute amount would be formed in the entire liver. The greatest increase in alpha amino nitrogen concentration was observed in Groups III and V, the same groups that showed the greatest loss in liver weight. Failure to consider this fact may lead to erroneous conclusions as has been pointed out by Fuhrman and Field (1945), who determined the effect of liver glycogen content on oxygen consumption. They found the Q_{O_2} (i.e. the mm^3 of oxygen consumed <u>per mg</u> dry weight per hour) of livers from rats starved 12 to 24 hours higher than that of normal rats, due to the fact that in the normal rat the active liver tissue was diluted with glycogen and its water of deposition. During fasting the glycogen and the water associated with it was largely lost resulting in an increased concentration of respiring tissue (this increased concentration was not due to water loss since the water concentration remained constant i.e. the liver glycogen had the same amount of water associated with it as the other liver solids.)

The increases in alpha amino nitrogen are plotted on Figure 17. The curves of all 5 groups showed a decrease in rate of alpha amino nitrogen appearance with incubation time. The average rate over the 3 to 6 hour period was significantly lower than the average rate over the first 1½ hours in Groups I, II and IV. In the other two groups the drop was of doubtful significance, (P between .05 and .1).

By comparing the increase of total non-protein nitrogen with that of the alpha amino nitrogen fraction (Figure 17) in the various groups it is seen that in the liver homogenate from the normal group the increase in NPN over the first 3 hours can be accounted for entirely by the increase in the alpha amino nitrogen fraction. (The NPN values are actually lower than the alpha amino nitrogen values but this is not statistically significant and must be due to inaccuracies involved in the analyses.) In the homogenates from all the treated groups the increase in alpha amino nitrogen after 1½ and 3 hours incubation is much less than the total non-protein nitrogen increase.

In summary it may be said that the homogenates of all the treated groups vary from the homogenates of the normal groups by a higher rate of NPN appearance over the first 3 hours, which is not accompanied by an increased rate of alpha amino nitrogen appearance. All damaging stimuli seemed to be equally effective with exception of the femur fracturing where the in vitro results are least clear cut.

The rate of alpha-amino N appearance dropped during the course of incubation in these homogenates from all groups including the normal control group.

DISCUSSION

In interpreting the results obtained it must be kept in mind that the increases in amounts of the various substances during autolysis are not necessarily due to an increased rate of their formation (that expression was carefully avoided) but may represent an overall picture of a variety of chemical reactions. Similarly, changes from normal in the initial nitrogen fractions may be the result of various underlying causes.

With regard to changes observed immediately after death it has already been stated that fasting seemed to be the most effective stimulus in evoking such changes. One wonders whether even in the other groups, insufficient food intake may not have been an important contributing factor in eliciting the changes from the normal picture. Thus the rats whose femur had been fractured certainly ate less than normal after the operation. It might also be argued that the rats subjected to cold may not have eaten enough to satisfy their increased energy requirements.

The liver atrophy and increase in initial protein concentration was most likely due to a reduction in glycogen stores. Fenn in 1939 observed that during a forty hour fast the glycogen content in rats dropped from an initial average value of 3.8 gm/100 gm fresh liver to 0.2 gm. Conant et al. found an average value of 0.12 gm per 100 gm fresh liver after a 24 hour fast. (Conant, Cramer, Hastings, Klemperer, Solomon, and Vennesland, 1941)

The glycogen lost during fasting depends upon the previous dietary intake. Mirski, Rosenbaum, Stein and Wertheimer (1938) noted that the glycogen stores in animals fed a high protein diet were maintained or even rose from an initial level of 1% to a starvation level of 1-2.2% while in animals on a previous high carbohydrate diet, the liver glycogen dropped from an initial 4% to below 0.1% in 24 hours. The maintenance and rise in liver glycogen in protein rich livers was ascribed to glyconeogenesis. In adrenalectomized animals this protein effect was absent.

Protein loss was a contributing factor to liver shrinkage. The writer's findings with respect to fasting confirm those of other investigators. Harrison and Long (1945) observed a 32% decrease in liver protein nitrogen (from 120 mgm to 82 mgm/100 gm rat) after a 48 hour fast on a 20% casein diet. Addis, Poo and Lew (1936) noted a 20% decrease of original liver protein content during a 48 hour fast, all other organs and tissues only losing 4% of their original protein content. As has been mentioned, a 20% protein loss was observed in this series of experiments in Group II (rats fasted 24 hours at room temperature), a 12% loss in Group III (rats subjected to cold and fasted 24 hours) and a 11% loss in the fracture group. However, a protein loss alone could hardly account for the decrease in liver weight since the protein concentration actually increased in the treated animals.

Water loss also must have contributed to the decrease in liver weight but it was not likely to be responsible for the increase in protein concentration since Fuhrman and Field (1945) have shown that the wet weight/dry weight ratios of livers taken from rats after 24 hours fasting does not differ from those of non-fasted rats.

The lowered initial protein values in the livers of treated animals could be due either to a decreased rate of their formation in vivo or an increased rate of their removal or both.

In the light of the protein metabolism regulating function ascribed to the adrenal cortex due to its protein anabolic and protein catabolic principles one might interpret the lower protein content of the livers as a manifestation of the predominant effect of the protein catabolic, or gluconeogenic principle. Since fasting was the most effective stimulus in this series of experiments the observations of Cohen (1945), mentioned earlier on the necessity of the adrenal cortex for a protein catabolic response to food withdrawal are of interest.

The decreased non protein nitrogen content (total nitrogen as well as the alpha amino nitrogen fraction) of the livers also could be ascribed to decreased production or increased rate of disappearance of the non protein nitrogen containing substances. If one assumes that the protein loss is due to increased protein catabolism one would expect an increased production of NPN containing substances. The lowered NPN contents would then be explainable if the NPN containing substances were removed faster than they were formed. It will be remembered that the alpha amino nitrogen fraction showed a larger percentage decrease in the treated groups than the remaining non protein nitrogen (Table VIII). This might be due to an increased rate of deamination and urea formation in vivo in the treated rats.

From the evidence of increased protein metabolism in vivo in response to damage it was hoped that the homogenized livers of the treated animals would show an increased rate of proteolysis. Actually a greater amount of NPN did appear in the homogenate from the treated groups during the first 3 hours of incubation, but no increased rate of alpha amino nitrogen appearance was observed. The question arises as to the source of the extra non protein nitrogen appearing in the livers from the groups subjected to damaging stimuli. Unfortunately the nature of that extra non protein nitrogen was not determined (Analysis on a sample of homogenate from a normal control rat before incubation showed that 85% of the non protein nitrogen/that was not alpha amino nitrogen consisted of urea N). Urea nitrogen, ammonia nitrogen and the amide nitrogen of glutamine are likely to be the major components. The guanidine group of arginine and the nitrogen of heterocyclic compounds (purine and pyrmidine derivatives and substances containing them such as nucleic acid) would also be included among the non protein nitrogen not composed of alpha amino acids. Amino groups other than those in the alpha position in amino acids would have been estimated as alpha amino nitrogen. This would also be the case for the free amino nitrogen in peptides if they should have escaped precipitation.

On the theory that the non protein nitrogen formed during incubation is derived from the deaminated alpha amino group of amino acids one has to assume that the rate of alpha amino acid formation was correspondingly increased in

the livers of the treated groups but that the rate of removal of the alpha amino groups would counterbalance their increased rate of formation so that the alpha amino nitrogen appearance in the homogenate of the treated groups would not differ significantly from normal. It is somewhat disconcerting that the rates as well as the changes in rate of alpha amino nitrogen appearance are so similar in the homogenates of the normal and the treated groups (See Figure 17). However, the fact that in vivo the percentage decrease among the NPN fractions was greatest in the alpha amino nitrogen components (Table VIII) which could be due to increased deamination with corresponding urea formation might speak for the assumption that in vitro also there was an increased rate of alpha amino nitrogen deamination with corresponding increased urea production.

Increased arginine hydrolysis could not account for the extra NPN formed since the guanidine group already appears as non protein nitrogen without being hydrolized. On the contrary for every arginine molecule hydrolized an additional free amino group is liberated (the delta amino group of ornithine) which would give a quantitative yield of amino nitrogen by the colorimetric method employed. One would therefore expect an increase in amino nitrogen not accompanied by an increase in NPN formation and not the opposite findings.

It is possible that in damaged animals a type of proteolysis predominates which results in the liberation of a higher percentage of amino acids containing guanidine and amide groups. This would explain an increased NPN formation

compared to normal not accompanied by an increased alpha amino nitrogen appearance. One might assume either a different amino acid composition of the proteins to be hydrolized or a different type of enzyme activity. In that case however the different type of proteolytic activity would have been inhibited to the same degree by prolonged incubation as is evident from the alpha amino nitrogen formation rates which were remarkably similar in the normal and in the damaged groups. This is possible but does not seem very likely.

A liberation of amide -, guanidine -, or secondary amino-nitrogen groups of amino acids still contained in the peptide chain might also contribute to the increased NPN values. That amino acids can be catabolized while still in peptide likage has already been discussed (e.g. lability of the amide nitrogen in glutamine containing peptides; dehydropeptidase activity). In this connection the fact that after 3 hours NPN formation in the normal group began to reach the same high rate as the treated group is of interest. One might expect an increased susceptibility of these nitrogenous groups to enzyme attack if the proteins were denatured and the peptide chains more or less unfolded, exposing these groups. Possibly such a denaturation might explain the high rate of NPN formation in the normal group and, assuming that it had already occurred in vivo in the damaged animal, also in the treated groups.

An increased liberation of nucleic acid from nucleoproteins may also contribute to the results obtained. The cause for this might be a greater cell fragility in the liver of the treated groups.

The high rate of NPN formation in the normal group after three hours' incubation might equally be attributed to increased cell fragility accompanied by nucleoprotein breakdown, due to a decrease in cellular vitality and resultant cell destruction which may justifiably be expected after a three hour incubation period.

The advantage of assuming other sources than alpha amino nitrogen for the increased non protein nitrogen values in the damaged animals, is that one does not have to presuppose an increased rate of proteolysis so delicately balanced by an increased rate of deamination that the resulting rate of alpha amino nitrogen appearance, and the eventual decrease in rate with incubation time are practically identical in all five groups. The rate of alpha amino nitrogen liberation would have to be approximately three times as great in the damaged groups during the first three hours to account for the extra non-protein nitrogen formed. After three hours, suddenly the rate has to drop to that of the normal group, since, from then on the NPN formation rate is the same in all groups. This is hard to picture, in the writer's opinion.

From a comparison of the damaged groups it is evident that the smallest in vitro effect was obtained by fracturing the femur. In this connection it may be well to quote Selye's discussion on alarming stimuli (Selye, 1940):

"By definition any agent capable of producing an alarm reaction is an "alarming stimulus." It is well to realize, however, that agents causing merely local damage which requires no general adaptive adjustment (e.g., amputation of limbs) are relatively mild alarming stimuli, while exposure to even moderate cold, solar radiation or muscular exercise, which evoke intense adaptive phenomena, produce very severe

alarm reaction symptoms.

Limb amputation is however not strictly comparable to femur fracture since a larger area of damaged tissue remains in contact with the circulation. The in vitro results obtained by the writer nevertheless seem to indicate that femur fracture too is a relatively mild alarming stimulus. In vivo, however, the total initial protein content in the livers from fractured animals (Group II) was significantly lowered, if only by a small amount (11%) and the liver weight was significantly decreased (14%), both effects being more marked than in the group subjected to cold but with access to food (Group IV). The latter group also showed a higher initial NPN content than the fracture group. However, in all other respects (in vivo as well as in vitro results), the fractured group varied least from normal. It may be possible that the time of sacrificing the animals after damage was not the most favourable one for revealing the effects of damage. The results in Group IIa (animals killed 1-2 days after fracture) and Group IIb (animals killed 5-6 days after fracture) while only based on two animals in each group, do not seem to warrant this assumption. (see Tables V-XIII).

There remains to be discussed the drop in rate of amino nitrogen formation noted in all groups, with the exception of the rise in rate between 6-9 hours in the group subjected to fasting at room temperature.

An increased rate of deamination as incubation proceeds may be one explanation. Although Krebs found

oxidative l-amino acid deamination dependent upon cellular integrity, and one would thus rather expect a decrease in deamination, Green isolated an l-amino acid oxidase from rat liver, obviously active extracellularly. Another possibility is a decreased in proteolytic enzyme activity due to some inhibiting factors. The observations mentioned earlier, indicating that proteolysis proceeds for 2-3 days are not necessarily contradictory to this assumption, since the pH was not kept constant at 7.3. If a slowing down of proteolytic activity is a cause, a gradual decrease in substrate concentration might have been a contributing factor, although the decrease is so little (only 4% after six hours when alpha-amino acid formation is markedly reduced), that it cannot play an important part.

It may be pointed out that many factors which may be instrumental in causing alterations in protein metabolism in vivo are necessarily eliminated by in vitro conditions. Among these are changes in oxygen supply, in pH, and in circulatory conditions, affecting the removal of endproducts. They may however alter in vitro metabolism provided they exerted irreversible effects in vivo. Further, since homogenization induced a considerable amount of cell breakage, one might argue that any in vivo effect on cell integrity would be obscured by the more drastic results of in vitro manipulation. However, cell breakage due to homogenization occurred presumably to the same extent in all groups, normal and damaged, and the majority or at least half the cells escaped such a destruction. If the remaining cells had

already been damaged previously in vivo, an alteration in their metabolism might well be reflected in vitro.

It is noteworthy that the alterations in in vitro metabolism elicited by damaging stimuli inflicted on the living animal, were also evoked in vitro by a three hour incubation period, in the homogenates from the untreated group. It is possible that the same mechanism was in play in both cases.

SUMMARY

The literature is discussed with regard to the mechanism of protein breakdown and factors affecting it.

A method for the study of in vitro protein metabolism in liver tissue is described.

In rats subjected to various damaging stimuli; femur fracture (animals sacrificed 3 - 4 days postoperatively); starvation for 24 hours at room temperature; cold ($\pm 5^{o}C$) for 24 hours; and starvation plus cold for 24 hours, the following observations were made: all stimuli evoked to a more or less marked degree a loss in liverweight, in liver protein content, in liver NPN and the alpha-amino N fraction. Most effective in evoking these changes were the procedures involving fasting. The smallest effect was induced by fracturing the femur.

The liver homogenates from rats subjected to damaging agents differed from normal by manifesting a higher rate of NPN production during the first three hours of aerobic incubation at $37^{o}C$ and pH 7.4. The high rate was maintained throughout the 9 hour incubation period. This effect was again least clear cut in the homogenates from the fracture group.

The same high rate of NPN production was also attained by the homogenates from the control group, but only after an incubation period of three hours.

The rate of alpha amino N appearance in the liver homogenates from damaged animals did not vary significantly from normal during the nine hour incubation period with one

exception; during the 6-9 hour period the rate was significantly increased in the homogenates from the group fasted at room temperature.

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