Thesis

Presented by C. P. Stewart

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I. On the aerobic and anaerobic oxidation of Xanthine and Hypoxanthine by Tissues and by Milk

by E.J. Morgan, C.P. Stewart, and F.G. Hopkins, F.R.S.

II. On Some compounds related to Histidine

by C.P. Stewart.

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Section I.—INTRODUCTORY.

Since the pioneer work of Burian, W. Jones and Schittenhelm, the conversion of the purin bases into uric acid by animal tissues has become a familiar case of biological oxidation. Apart from its physiological importance, the phenomenon must always be one of considerable chemical interest, because of the contrast between the remarkable ease with which it occurs under the influence of tissue catalysts, and the difficulty with which it is induced by ordinary laboratory oxidising agents. The literature of the subject seems throughout to emphasise the importance of free oxygen in the process. We have found, however, that certain animal tissues can induce the oxidation under conditions which are strictly anaerobic. This fact is significant in its bearing upon the nature of the catalysts concerned.

Our inquiry began with the observation that in milk containing methylene blue, as a hydrogen acceptor, the bases are rapidly oxidised to uric acid in the absence of free oxygen.

Schardinger, in 1902, showed that milk, when quite fresh, does not reduce methylene blue, but reduces it when formaldehyde is also present. When, in the absence of aldehyde, reduction occurs, it is due to the influence of bacteria. For some years after Schardinger's original publication, there was, it is true, much discussion as to the precise significance of the direct and indirect reduction respectively. The work of Schmidt, of Burri and Kursteiner, and especially of Trommsdorff, in 1909, made it clear, however, that the reduction of methylene blue in the presence of the aldehyde occurs under the influence of a specific catalyst, which is now commonly known as Schardinger's enzyme.

Bach showed that animal tissues contain a substance, never properly identified, which, when added to milk, can take the place of the aldehyde in the Schardinger test, and found also that the tissues themselves contain a catalyst identical with, or similar to, the enzyme in milk. He ultimately brought the action into the now familiar category of hydrolytic oxidation-reduction reactions (Bach, 1911). In the Schardinger system the aldehyde

acts as oxygen acceptor and the methylene blue as a hydrogen acceptor. In the tissues the reducing mechanism is complete; in milk the catalyst is divorced from efficient oxygen acceptors.

During the fractionation of yeast and tissue extracts preparatory to the separation of glutathione (Hopkins, 1921), it was observed that a fraction precipitated by phosphotungstic acid contained a substance which induced rapid reduction of methylene blue in fresh milk. Later, one of us (E. J. M.) found that this substance was not an unknown base but hypoxanthin.

If to a specimen of cows' milk free from any direct reducing power hypoxanthin be added, a reducing system is at once established, and the milk will actively decolorise methylene blue with a velocity which increases with the temperature up to 70° C. Xanthin acts similarly. If the phenomenon be viewed from Bach's standpoint, it is clear that hypoxanthin and xanthin must be looked upon as providing a highly efficient form of oxygen acceptor, releasing the activity of the "perhydridase" in milk.

It is easy, indeed, as we have repeatedly found, to show that these two purin bases are smoothly oxidised to uric acid by milk containing methylene blue; this under strictly anaerobic conditions. The catalyst controlling the processes of oxidation and reduction, which are simultaneously involved, shows marked specificity. Guanine and adenine as such do not act as oxygen acceptors. Cows' milk, however, usually contains some adenase, so that adenine may suffer slow oxidation as the result of a preliminary conversion into hypoxanthin. On the other hand, we have never found guanase in milk, and guanine is unaffected. Quite inert as oxygen acceptors are the methyl-purins caffein and theobromine; also the pyrimidin bases uracil, thymin and cytosin. Histidin (tested as a substance containing the iminazol ring) is also inactive. Uric acid is not further oxidised, nor is allantoin affected.

It is unlikely that the catalyst concerned in the oxidation of xanthin and hypoxanthin (assuming it to be the same in each case) is identical with the Schardinger enzyme, which works with aldehydes as oxygen acceptors. The high specificity of the acceptors, as displayed within the purin group itself, seems to exclude the likelihood that the catalyst would extend its influence to aldehydes. There is other evidence against its being identical with Schardinger's enzyme. The molar concentration of hypoxanthin necessary to reduce methylene blue with a given velocity is in some samples of milk 100 times smaller than the concentration necessary to induce the same velocity in the case of acetaldehyde. We have found, moreover, that the power to oxidise aldehydes and purins respectively varies in a relative sense very widely in different samples of milk.

A catalyst oxidising xanthin and hypoxanthin anaerobically in the presence of methylene blue exists in animal tissues. Preparations can easily be made from various organs which, while failing to reduce methylene blue alone (or reducing it very slowly), decolorise the dyestuff promptly on the addition of either of the purin bases. The oxidation of the base to uric acid is then carried out anaerobically. The question as to whether the catalyst concerned is identical with that familiarly known as xanthin oxidase will be raised later.

Proof that a catalyst oxidising anaerobically exists in the tissues is most easily obtained by allowing the organs to remain for 24 hours after removal before preparations are made from them. As is well known the tissues when first removed from the body reduce methylene blue with high intensity. On standing their reducing power diminishes nearly to zero. The events which occur during its disappearance are, doubtless, diverse. One is the exhaustion during survival processes of oxygen acceptors (or hydrogen donators in Thunberg's sense), and among these are xanthin and hypoxanthin. Only when the pre-existing concentration of these bases is reduced can the effect of fresh additions to the system be satisfactorily demonstrated.

Such tissue preparations are conveniently made by Bach's method. The fresh organs are ground up with ten volumes of 2 per cent. sodium fluoride solution and squeezed through linen. The colloidal suspensions which pass through the mesh of the linen lose, after standing for 24 hours, the greater part of their power to reduce methylene blue. If, however, xanthin or hypoxanthin be added to them a high reducing power is restored. A tissue preparation taking, say, from 1 to 2 hours to reduce a given quantity of the dye will, after the addition of a suitable amount of either base, reduce it in from 5 to 10 minutes. The bases have, needless to say, no direct reducing power in the absence of the tissue catalyst.

The above statements are based mainly upon a study of the tissues of the ox and the rat. We have found them true, for instance, of the liver, kidney, spleen and lungs of these animals, but they do not apply to muscle.

Milk, as we have found in very numerous experiments, is not only capable of oxidising hypoxanthin and xanthin anaerobically with the aid of hydrogen acceptors, it also oxidises them aerobically. We have added the bases to cows' milk and, after prolonged aeration of the fluid, have isolated uric acid in substance. No such oxidation occurs if the milk be first boiled. With the aid of Barcroft's differential apparatus we have followed the uptake of oxygen by milk to which the bases have been added. It is easy to show that the uptake in each case corresponds to the amount necessary for the conversion of the base into uric acid as an end product.

Certain aspects of the kinetics of the action and the general significance of our experimental results will be discussed after a description has been given of the experiments.

Section II.—Experimental.

The hypoxanthin used in the following experiments was prepared by ourselves from yeast and from commercial meat extracts. At the final stage the silver compound was twice recrystallised from hot nitric acid and the product decomposed with SH_2, the base was then twice recrystallised from water.

The product which served for most of the observations gave, by Kjeldahl, N = 41·05 per cent.; $C_5H_4N_4O$ requires 41·17.

The xanthin was prepared by deaminating guanine, a pure specimen of the chloride, made from yeast nucleic acid, being employed. Nitrogen, by Kjeldahl = 36·52 per cent.; $C_5H_4N_4O_2$ requires 36·85.

When not added to the milk or tissue preparation in the solid form the bases were dissolved by the aid of 0·2 N. NaHO, and in some experiments the solvent was then buffered with 0·2 M. KH_2PO_4. The *p*H was finally adjusted to 7·8. At this low grade of alkalinity, xanthin is but slightly soluble in the fluid at ordinary temperatures. In other experiments, therefore, the bases were simply dissolved in a minimum of NaHO. The strength of solution usually taken was 0·01 molecular in respect of each base.

Experiments involving the use of methylene blue as hydrogen acceptor will be first described. A few of these with milk were carried out in open test-tubes at 70° C., following the technique usually employed for the demonstration of the action of Schardinger's enzyme. In such cases the velocity of reduction is, of course, balanced against the velocity with which the decolorised methylene blue is reoxidised. When, however, the reduction time is short the observed end points are consistent.

The greater number of the observations, however, including all those concerned with tissue preparations, were made under anaerobic conditions in test-tubes with side tubes and hollow glass stoppers. When charged with the materials under study, these were evacuated and then closed vacuum-tight by turning the well-greased stopper so that a perforation in its wall no longer corresponded with the opening of the side tube.

Many of the anaerobic experiments were made at 35° C., the tubes being then placed for observation in a thermostat with glass sides: but it was frequently found convenient to work at room temperature. When the action is rapid the reduction may proceed somewhat far during the evacuation before the tubes are transferred to the bath. It is clearly more accurate to make

the whole observation at one temperature (*cf.* Experiment type III). In the greater number of the experiments the evacuation was carried out with a good water pump. Whenever comparisons were being made all of the tubes in a series were simultaneously connected with the same pump by a branched connecting tube, and thus evacuated to the same degree. In certain cases complete freedom from oxygen was secured by the use of a Geryck pump, the tubes being washed out twice or thrice with nitrogen and re-exhausted.

The complete experimental records would occupy much space. In the following only a selection of typical experiments is given, and in the case of those which involved a large number of observations (*e.g.*, in a series of varied concentrations) the records are shortened.

A. *Experiments with Milk* (*Anaerobic Technique*).

In every case the material was used as soon as possible after the milking of the animal, and the reducing power of the milk by itself was determined. This was generally *nil.*

Experiment of Type I.—Open tubes; bath at 70° C. In each tube 5 c.c. milk with 0·3 c.c. methylene blue (1 in 5000). The milk without addition of base showed no reducing power. RT = time for complete reduction.

Hypoxanthin M/100. (c.c.)	RT.	Xanthin M/100. (c.c.)	RT.
	Cows' Milk.		
0·01	∞	0·01	∞
0·02	45 secs.	0·02	∞
0·04	45 „	0·04	92 secs.
0·06	45 „	0·06	91 „
0·10	45 „	0·10	90 „
0·20	44 „	0·20	90 „
0·50	44 „	0·50	90 „
	Goats' Milk.		
0·09	∞	0·09	∞
0·10	5 mins. 10 secs.	0·10	∞
0·20	5 „ 10 „	0·20	9 mins. 10 secs.
0·30	4 „ 58 „	0·30	8 „
0·40	4 „ 58 „	0·40	9 „
0·50	4 „ 58 „	0·50	9 „ 20 „

Stœchiometric relations are seen to hold in that twice as much xanthin as hypoxanthin is required to reduce completely a given amount of methylene blue. Increase in concentration of base is seen to have no effect on the velocity of the action throughout the range studied. Goats' milk always shows less activity than cows' milk.

Experiment of Type II.—In evacuated tubes; bath at 35·7° C. Perfectly fresh milk showing no reduction alone. In each case the sample was heated to 70° C. for ten minutes and cooled before use. The solutions of the bases

were buffered at *p*H 7·8 and the volume in each tube was made the same by suitable additions of phosphate buffer at same *p*H.

Cows' Milk.

Hypoxanthin M/100. (c.c.)	RT.	Xanthin M/100. (c.c.)	RT.
0·20	3 mins. 10 secs.	0·20	6 mins. 15 secs.
0·40	3 „ 10 „	0·40	6 „ 10 „
0·60	3 „	0·60	6 „ 10 „
0·80	3 „	0·80	6 „ 10 „
	Goats' Milk.		
0·20	9 mins.	0·20	18 mins.
0·40	9 „	0·40	18 „
0·60	9 „	0·60	19 „
0·80	9 „	0·80	20 mins. 30 secs.

This experiment illustrates the fact that when hypoxanthin is the oxygen acceptor, the velocity of reduction (and therefore of oxidation) is double that observed when xanthin is the acceptor. A fourfold increase in the concentration of the base has little or no effect on the velocity.

Experiment of Type III.—For this experiment samples of milk were obtained from four separate cows of known pedigree and history. The milk was cooled immediately after milking and was used within three hours. The observations were made in evacuated tubes at room temperature. The solution of the base was added to the milk and the methylene blue run on to the surface without admixture. The tubes were then rapidly evacuated, and at the moment the mercury in the manometer reached its highest point the tubes were shaken and the reduction time measured from the same moment. It is clear that this method is more satisfactory than when the tubes are evacuated at room temperature, and subsequently transferred to a bath at higher temperature. In each tube 5 c.c. milk, 0·5 c.c. M/100 solution of base, 0·5 c.c. methylene blue (1 in 1000). Temperature of room 18° C.

Date of milking.	Animal No.	RT Hypoxanthin.	RT Xanthin.	Remarks.
8.V.22	1	17 mins.	34 mins.	Original milk.
„	2	9 „	18 „	„ „
„	3	11 „	20 „	„ „
„	4	5½ „	12 „	„ „
13.V. 22	1	21 „	39 „	„ „
„	2	30 „	61 „	„ „
„	3	14 „	27 „	„ „
„	4	9½ „	20 „	„ „
„	4	35 „	68 „	Milk heated to 70° C. for 15 mins. before use.

The remarkable constancy of the relation in reduction times is seen in this experiment. When hypoxanthin is the oxygen acceptor, reduction is twice as fast as when xanthin is used. This result is obtained consistently with

samples of fresh milk. The ratio is seen above (Cow No. 4) to remain undisturbed when the activity of the milk has been reduced by heating. We have found the velocities tend to come nearer together (though hypoxanthin gives always the greater velocity) in stale milk, even in cases when the sample has shown little or no reduction in the absence of the base.

It will be observed that in the case of Cows 1, 3, and 4 above there was little change in the activity of the milk during an interval of five days between successive milkings. In the case of Cow 2, however, the change was considerable. We could trace no relation between the activity of the catalyst and the period of lactation, nor any relation with the previous history of the animal. Cow 1 was six years old, had had four calves, and at the time of the experiment was in the ninth week of lactation. Cow 2 was seven years old, had had five calves, and was in the fifth week of lactation. No. 3, four years old, with two calves, and in the thirty-first week of lactation. No. 4, four years old, with two calves and in the fourteenth week of lactation. The most variable sample came from the animal (No. 2) which had been milked during the shortest period. No chemical analysis of the samples was made.

Experiment of Type IV.—This experiment was carried out to determine if under anaerobic conditions the oxidation of the base proceeds to completion. A measured quantity of a standard solution of hypoxanthin, containing 5 mgrms. of the base, was added to 5 c.c. of cows' milk. The milk was in a large test-tube; the cork closing the tube was provided with inlet and outlet tubes and was pierced with a third hole to admit the glass nozzle of a burette. The tube was placed in a bath at 60° C. and methylene blue (1 in 500) was run in in small quantities, very slowly towards the end. 13·1 c.c. were fully decolorised; 13·2 c.c. remained blue for a long period. The methylene blue used contained three molecules of water. Taking the molecular weight of this as 374, the hypoxanthin should have reduced 13·7 c.c. of the solution during its oxidation to uric acid. Other experiments gave consistent results. The oxidation under such conditions is, to judge from the figures, nearly complete. The actual solubility of uric acid in milk is undetermined, however. If it be not considerably higher than in water the greater part of the acid produced in the above experiment would have separated from solution. We have in other experiments found that the presence of a soluble urate appreciably slows the reduction of methylene blue by the milk-hypoxanthin system.

Isolation of the Uric Acid Produced by the Oxidation of Hypoxanthin and Xanthin by Milk.

Experiment V.—Fifty milligrammes of hypoxanthin and a similar amount of xanthin were in each case dissolved in 2 c.c. 0·2 N. NaHO and 5 c.c. of water. Each solution was added to 50 c.c. of fresh cows' milk mixed with a few cubic centimetres of methylene blue (1 in 500). The flasks used were placed in a bath at 37° C. and 1 c.c. of chloroform added to each. Air was bubbled through the solution during 24 hours (a much longer time than was actually necessary, as we have since found). The milks were then diluted with an equal bulk of water and dilute acetic acid cautiously added to precipitate the caseinogen. The filtrates from the caseinogen were boiled and again filtered. The filtrates and washings were evaporated to 150 c.c.; 5 c.c. of strong hydrochloric acid were added to each and the solutions heated over the water-bath for 1 hour, the original bulk being kept constant. (This treatment breaks down traces of colloidal material which otherwise interferes with the ease of separation.) The cooled solutions were first made alkaline with ammonia and then saturated with ammonium chloride. After standing, the precipitated ammonium urate was filtered off and decomposed with warm HCl. From the 50 mgrm. of xanthin 44 mgrm. of uric acid were obtained; from the hypoxanthin 27 mgrm.

It is a difficult matter to separate quantitatively small quantities of uric acid from milk. Fortunately, other experiments prove the quantitative course of the oxidation of the bases to uric acid as an end product (see later). The product isolated, as above, showed typical crystalline forms; it gave the murexide, Schiff's and Folin's tests. Dissolved in strong H_2SO_4 it separated on dilution in crystals identical in form with those obtained by treating a sample of pure uric acid in a similar manner. In the experiment described methylene blue was present as an oxygen carrier. We have obtained similar results, however, in the absence of the dye, that is, by direct aerobic oxidation. No uric acid could be isolated in the case of control experiments in which the milk used was first boiled.

Experiments of Type VI.—Early in this research many comparisons were made between aldehydes and the purin bases as oxygen acceptors in the reduction of methylene blue by milk. One or two of these may be briefly recorded. In the case of a sample of mixed dairy milk (5 c.c.), the addition of 0·1 c.c. M/100 hypoxanthin was sufficient to induce reduction of 0·3 c.c. methylene blue in 1 minute, whereas it required 1·0 c.c. of M/10 acetaldehyde (100 times as much) to bring about the same velocity of reduction. Somewhat similar, but not identical, results were obtained in other cases. In goats'

milk, at any rate when the observations are carried out in open tubes at 70° C., that is, with the usual technique used for Schardinger's action, the difference is still greater. It has been stated, indeed, by several authors that goats' milk does not contain the Schardinger enzyme. Using anaerobic methods, however, we have found acetaldehyde and formaldehyde efficient as oxygen acceptors, but to produce a given velocity of reduction very much higher concentrations of aldehyde are required than of the purin bases.

B. *Experiments with Milk* (*Aerobic Technique*).

To follow the uptake of oxygen of the bases when dissolved in milk Barcroft's well known apparatus with a differential manometer was employed. The apparatus was shaken in the usual manner by a motor, the flasks being immersed in a water-bath. When the uptake of one base was to be compared with that of the other simultaneously, each therefore being in a separate apparatus (our usual custom), we found it very important, apart from care in calibration, that the behaviour of each individual set of apparatus should be carefully tested. A relatively small difference, for instance, in the shape of the flasks may, by affecting the surface movements during shaking, appreciably affect the velocity of oxygen uptake. We have been able, however, to work with strictly comparable sets. Milk in one respect is an unsatisfactory fluid in a process involving shaking, because of the tendency for the fat to separate. This, nevertheless, seldom seemed to interfere with the smoothness of the curves of uptake. Variations in the rate of shaking produce, of course, a large effect in this respect.

Most, if not all, milks show a small but definite uptake of oxygen when aerated without any addition. The effect of this could be eliminated by balancing milk containing the added base against an equal quantity of the original sample contained in the second flask of the apparatus. We usually, however, placed water in the second flask; the uptake of the milk alone being determined in a separate apparatus and its amount deducted at each corresponding point on the curve of uptake by the sample containing the base. Potash for the absorption of CO_2 was in every case placed in the receptacle provided. It is necessary, of course, to allow a few minutes for the attainment of equilibrium in the bath before the flasks are connected with the manometer and the readings begun. As our apparatus did not permit us to add the base after equilibrium was attained, in most experiments the oxidation had proceeded to a certain degree before the readings were commenced. This circumstance is of small importance, except in so far as it affects the estimation of the total uptake of oxygen, which under the circumstances must of course, be short of theory. By suitable precautions it proved possible to

eliminate the error, and in several experiments, even with no more than a milligramme or so of base to be oxidised, we have obtained—especially with hypoxanthin—a very exact correspondence between the final readings and the theoretical amount of oxygen required for the conversion of the base into uric acid. It is indeed of interest to find how satisfactory the experimental results may be, though the method has to be made a micro-method owing to the small amounts of milk (best 2 c.c. only) which can be safely shaken in an apparatus of standard size, and the necessity of obtaining the final result within a reasonable time.

Experiments of Type VII.—In these the oxygen taken up by 0·5 c.c. M/75 solution of the base in 2 c.c. of cows' milk was determined on the lines and with the precautions just discussed. We give a few curves representative of the many obtained. Details are supplied in connection with each curve (figs. 1 and 2).

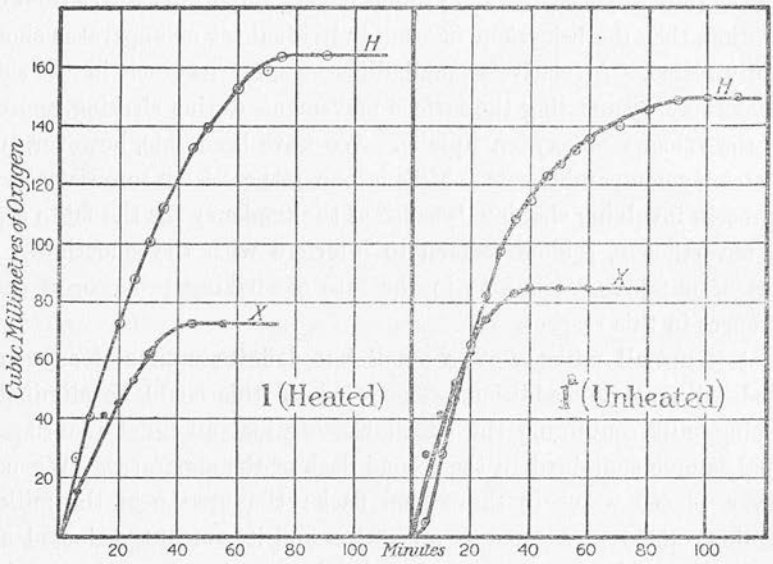


FIG. 1.—Purin bases in milk. Curves of oxygen uptake by hypoxanthin (H) and xanthin (X). Corrected for uptake of control. In the fresh unheated milk (Ia) the initial velocity is the same in each case. In the case of milk (not the same sample) which had been heated for 10 minutes at 70° C. before the experiment (I), the hypoxanthin uptake is the faster, though the completion of oxidation occurs later than in the case of xanthin. Theoretical uptake for conversion into uric acid: hypoxanthin, 149·3 c.mm.; xanthin, 74·6 c.mm.

Experiments of Type VIII.—It will be seen from the curves that with fresh unheated milk the velocity of oxygen uptake on aeration is, in contrast with the results of the anaerobic studies, identical in the case of each base.

In order to control this result we carried out certain experiments on the lines of a null method. Milk (2 c.c.) was placed in each flask of the apparatus. To one flask hypoxanthin solution was added and to the other an equimolecular proportion of xanthin. Under these circumstances, the manometric

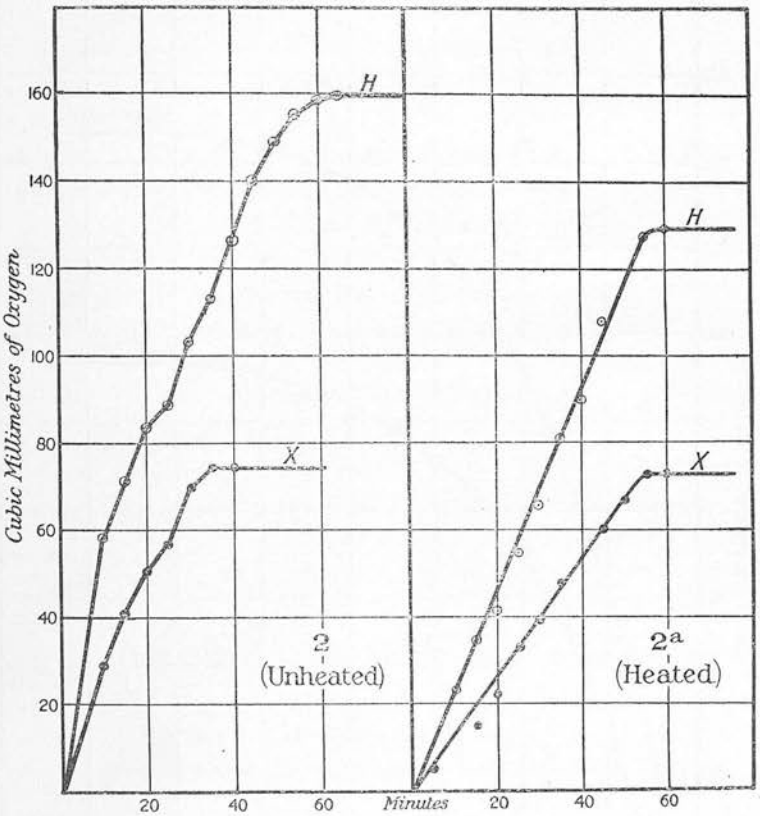


FIG. 2.—Condition similar to fig. 1, with different milks. In case of unheated milk the time taken for maximum uptake by hypoxanthin is half that occupied by xanthin. In milk previously heated to 70° C. for half an hour, the maximum is reached at about the same time. In the latter case the uptake is less than theory, because 15 minutes were allowed to elapse for the attainment of equilibrium before readings were taken. In these curves the data are not corrected for temperature. Theoretical uptake : hypoxanthin, 168 c.mm. ; xanthin, 84 c.mm.

fluid did not move (showing identity of oxygen uptake) during periods which represented about two-thirds of the time required for the oxidation of the xanthin. After this the manometer fluid began to move towards the flask containing the hypoxanthin. Each sample of milk was then tested with Folin's agent and the formation of uric acid demonstrated in both.

C. *Experiments with Tissues (Anaerobic Technique).*

As stated in the introductory section the tissue preparations were made according to a method described by Bach. The perfectly fresh organs

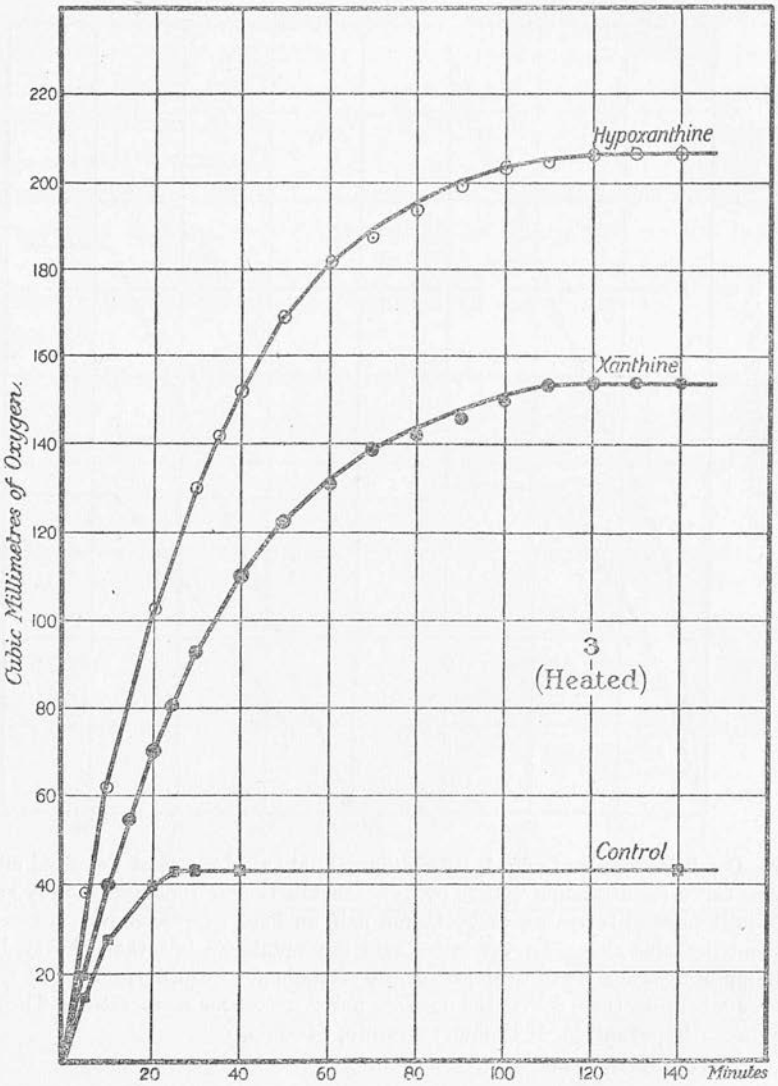


FIG. 3.—Shows curves of uptake not corrected for control. In this sample of milk the uptake by xanthin was in excess of theory. (See text.)

were ground up with a little sand and ten times their volume of 2 per cent. sodium fluoride solution. The mass was then squeezed through linen and the suspension so obtained allowed to stand (usually for 24 hours at room

temperature but longer if necessary) until the original reducing power of the solution had almost disappeared. The fluoride present was sufficient to prevent bacterial growth.

To such a preparation the bases were added in known concentration with a measured quantity of methylene blue and the reduction time determined in evacuated tubes similar to those used in the case of milk.

The following protocols give only a small proportion of the data obtained, but are representative of the whole.

Experiments of Type IX.—In the case of each tissue 5 c.c. of the preparation were measured into each of three tubes, of which one served for the control while to the others 0·2 c.c. of M/100 solution of either base was added. The solutions of the bases were buffered with phosphates to *p*H 7·8, and to the control 0·2 c.c. of a buffer solution at *p*H 7·8 was added. Methylene blue 0·5 c.c. (1 in 5000). Readings to nearest ½ minute.

Tissue.	RT control.	RT Hypoxanthin.	RT Xanthin.
Ox liver.....	1 hour 45 mins.	5 mins.	10 mins.
Ox spleen	1 „ 29 „	4 „	8 „
Rat liver	2 „ 18 „	8 „	17·5 „
Rat liver	1 „ 5 „	3·5 „	7 „
Rat kidney	1 „ 17 „	16·5 „	32 „
Rat spleen.....	1 „ 55 „	8·5 „	17·5 „
Rat spleen.....	2 „ 29 „	9·5 „	17·0 „
Rat lung	2 „ 36 „	17 „	33·5 „
Rat muscle	28 „	28 „	43 „

It will be seen that in the case of all the tissue preparations except those from muscle added hypoxanthin induces a reduction velocity which is double that induced by xanthin. The residual reducing power of the preparations is mostly too slow to affect the comparison.

D. *Experiments with Tissues (Aerobic Technique).*

These were undertaken only to decide whether the tissues of the rat (see discussion in the next section) can oxidise the bases aerobically.

Preparations of the liver of the rat made as above were shaken in a Barcroft apparatus after receiving the addition of known amounts of hypoxanthin or xanthin. Any uptake of oxygen due to the preparation alone was separately determined. The concentration of the catalyst in such preparations is considerably less than in milk and the uptake of oxygen proportionately slower. We obtained, however, perfectly satisfactory results. There was in all experiments a steady uptake of oxygen which ceased at a point exactly corresponding with that required for the complete conversion of either base into allantoin. It is of interest indeed to find how accurate the result may

be when very small quantities of material are employed in this way. The results of one experiment will suffice for illustration.

In one apparatus 2 c.c. liver preparation, 0·5 c.c. phosphate buffer solution of *p*H 7·6; in a second apparatus 2 c.c. preparation, 0·5 c.c. hypoxanthin solution (containing 0·76 mgrm.) buffered to *p*H 7·6; in a third 2 c.c. preparation, 0·5 c.c. xanthin solution (containing 0·68 mgrm.) similarly buffered. Bath at 35° C. The mixtures remained fully aseptic throughout. There was at first a steady uptake of oxygen in each apparatus. In the control this ceased absolutely when 57 c.mm. had been absorbed. In the apparatus containing hypoxanthin the uptake ceased at 235 c.mm.; in that containing xanthin at 178·7 c.mm. The uptake of the former base was, therefore, 178 c.mm. (theory for allantoin 180 c.mm.) and of the latter 121·7 c.mm. (theory 120 c.mm.).

Section III.—Discussion of Results.

The mammary gland is known to transmit with the milk certain of the hydrolytic enzymes normally produced in the body. The activity of these in the milk is for the most part very small however, yet the data of the last section show that cows' milk contains the catalyst, or catalysts, involved in the oxidation of the purin bases in highly effective concentrations. This is no less true of goats' milk, though the concentration is always lower. Human milk, to judge from our examination of a single specimen, also contains the catalyst, though the amount would seem to be relatively small. Other milks we have not examined. It is remarkable that the mammary gland should eliminate this particular catalyst (assuming for the moment that only one is concerned), together with a little adenase, while apparently failing to secrete in any ascertainable quantity the enzymes concerned with other stages of nucleic acid metabolism. The physiological significance of the facts would seem to be quite obscure and we shall make no attempt to construct a theory here.

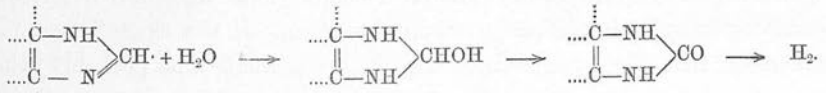
There can be little doubt that the catalyst in the milk which we have shown to be capable of producing uric acid anaerobically is identical with that which we have found to act similarly in tissues. It is highly probable that intact tissues may contain hydrogen acceptors from which in milk the catalyst is divorced, and that in the former the complete active system may therefore be present. In such tissue preparations as those employed by us, in which pre-existing xanthin and hypoxanthin have become almost completely oxidised, such acceptors would have been to a large extent simultaneously reduced by the survival processes.

It is clear that the chief new fact established by our observations is the

existence of a mechanism which can induce the biological oxidation of the purin bases under strictly anaerobic conditions. The question arises as to whether the catalyst then concerned is identical with that which (studied under aerobic conditions) is ordinarily recognised under the name of xanthin-oxidase. This question is part of the wider one as to whether any or all of the biological observations usually ascribed to "oxidase" systems may be in reality controlled by catalysts identical with those which—so long as efficient hydrogen acceptors are present—induce oxidations anaerobically. If this be so, the more familiar aerobic oxidation of the purin bases may represent a special case of the action of the enzyme which has been under discussion; the case, namely, in which oxygen itself becomes the hydrogen acceptor.

It must be admitted that at the present time it is not easy to decide whether this or that case of oxidation by the tissues can be ascribed to an oxidase system as ordinarily understood, to a hydrolytic oxidation-reduction process in the sense defined by Bach, or to the activation and transport of hydrogen as pictured by Wieland and Thunberg.

The anaerobic oxidation of xanthin or hypoxanthin in the presence of methylene blue, etc., may be easily viewed as a typical case of simultaneous oxidation and reduction by the elements of water. On the other hand, it fits with equal ease into Wieland's conception, in so far as this is to be thought of as really distinct from Bach's. For it is more than probable that the oxidation which occurs (in the iminazol ring in the case of xanthin and in this as well as in the pyrimidin ring when hypoxanthin is concerned) involves the preliminary addition of water:—



The final stage is then represented by the transport of activated hydrogen, either to a reducible substance in the case of the anaerobic reactions, or, under aerobic conditions, to oxygen itself functioning as the hydrogen acceptor. The catalyst may well then be the same in each case. Batelli and Stern, it should be noticed, point out that "activation," whether of hydrogen or of oxygen, remains experimentally unproven. They suggest that it is more justifiable to look upon an oxidising-reducing catalyst simply as an agent controlling the distribution of the water ions. It is clear that, from the chemical standpoint, it would be a normal occurrence if two hydroxyl groups became first attached at the ethylene linkages which (in the structure of the bases) unite nitrogen to carbon. The elimination of a

molecule of water would then establish the uric acid structure. This view involves a scheme somewhat different from that given above.

Whatever importance such distinctions may prove to possess, it is clear that the trend of opinion at the moment is in favour of the belief that many oxidations supposed to depend upon the presence of "oxidases," as defined by Engler and Bach and Bach and Chodat, can in reality be induced by catalysts, which also act anaerobically.

In this connection it is significant to remember that our own experiments have shown conclusively that milk can oxidise the purin bases, either anaerobically with a hydrogen acceptor, or directly in the presence of molecular oxygen. In the case of such a fluid it is easier than with tissue preparations to compare the activity in the two cases. Our quantitative observations, in which Barcroft's differential apparatus was employed for measuring oxygen uptake, leave little doubt in our own minds that the same catalyst is concerned in each case. We have found at least, from a number of observations, that the anaerobic and aerobic oxidising capacities vary together. A sample of milk, which in the presence of xanthin or hypoxanthin reduces methylene blue with special rapidity, shows under similar circumstances a specially rapid uptake of oxygen; another sample, if less active in the one sense, will be proportionately less active in the other. Heating to, say, 70° C. during graded periods, seems to produce proportionate reductions of the activity in each case. As we have already pointed out, the oxidation in milk proceeds completely to the stage of uric acid. On the other hand, we have never found a sample of milk which could oxidise uric acid itself.

The case of the tissues seems (in connection with the particular oxidation in question) to require some further consideration. A number of our observations have been carried out upon the tissues of the rat. They show conclusively that the liver and spleen of this animal (and probably other organs, though not the muscles), can freely oxidise xanthin and hypoxanthin under anaerobic conditions in the presence of methylene blue. Some years ago, however, Rohde and Jones (1909), studying the matter under ordinary aerobic conditions, came to the conclusion that no organ of the rat contains an oxidase capable of oxidising these bases. They were therefore led to suppose that the uric acid excreted by this animal must arise along special and unusual lines. Now if we conclude that in the rat the bases are oxidised under anaerobic conditions alone, we must assume that its tissues contain hydrogen acceptors acting as equivalents to the methylene blue used in our experimental studies. In this assumption there is little difficulty; but if there were no oxidation aerobically it would be also necessary to assume that a special enzyme is present, differing from the familiar catalyst always spoken

of as an "oxidase," which produces uric acid from its precursors in many organs of other animals. For this latter assumption there is, however, no need. The liver of the rat (other organs we have not yet tested) oxidises hypoxanthin and xanthin quite readily under aerobic conditions in the absence of all hydrogen acceptors other than molecular oxygen. Several observations, such as that reported under the head of Experiments, pp. 121–122, have made this clear. In proof of the fact we have used micro-methods, but the results are quite unequivocal. Not only does the addition of either base to a liver preparation increase its oxygen uptake when it is shaken in Barcroft's apparatus, but the additional uptake finally corresponds exactly with that necessary for the conversion of the added base into, not uric acid (as in the case of milk which contains no uricase) but allantoin. The formation of allantoin can scarcely fail to involve the intermediate formation of uric acid. It is easy, moreover, to obtain direct and reliable proof of this without departing from micro-methods. If a suitable preparation be made from the liver of the rat, freshly excised, it will give a strong colour reaction with the sodium tungstate reagent of Folin. After standing for 36 hours with an efficient antiseptic it no longer gives the reaction. If at this stage a milligramme or two of xanthin or hypoxanthin be added to one-half of the preparation, the other half serving as a control, both fractions being then aerated for 2 hours, it will be found that the portion to which the base had been added will give, once more, an intense reaction; the control will show none. Later on, the oxidation of uric acid to allantoin is completed, and no reaction will be obtained in either portion.

Our results fail, therefore, to confirm those of Rohde and Jones, and the case of the rat seems to offer no evidence to compel a belief in the existence of a catalyst acting under anaerobic conditions alone. With regard to the discrepancy just mentioned, we feel that an explanation may be found on some simple lines. The American authors sought for the oxidase in strained-off aqueous extracts of the tissues, a procedure fully justified by their large experience with the organs of other animals. It is just possible, however, that in the rat the catalyst is more firmly attached to the tissue residue than is usual. Our own tissue preparations were essentially cell suspensions.

The point just discussed is not altogether a minor one. Apart from its bearing upon the general nature of the oxidising catalysts in animal tissues, it is clear that it raises the question whether various organs in other animals hitherto stated to contain no xanthin oxidase may not after all be capable of oxidising xanthin and hypoxanthin under suitable conditions.

While discussing the question whether the anaerobic and aerobic catalysts are identical or otherwise we have hitherto assumed that, in any case, the

same catalyst deals with both the purin bases. In connexion with the aerobic oxidation by tissues, the same assumption has been made, tacitly or otherwise, by most investigators. Gideon Wells, on the other hand, has claimed, though without giving experimental details, to have found evidence for the existence of an oxidase specially related to each base individually. We can best discuss this point in connection with what we are able to say generally concerning the kinetics of the oxidation.

The catalytic mechanism in milk shows marked thermostability, though the actual rate of destruction at a given temperature varies a good deal in different samples of milk, probably as the result of variations in pH or in fat-content. We have frequently employed milk for estimations of oxygen uptake after the original sample had been heated for 10 minutes to 70° C. One sample of cow's milk, after exposure to this temperature for 30 minutes, lost less than 30 per cent. of its activity. Rapid destruction occurs, however, on boiling.*

The relative stability of the oxidising mechanism in fresh milk may to a great extent depend upon the physico-chemical characters of the fluid itself. In tissue preparations which, unlike milk, yield, of course, a heavy coagulum when heated, the mechanism is much less stable towards heat.

When milk is heated its power to oxidise hypoxanthin anaerobically falls off at the same rate as its activity towards xanthin, a fact which suggests at least that one catalyst only is concerned. So also does the circumstance that while different samples of fresh milk vary widely in actual oxidising power, the variation seems always to affect the oxidation of hypoxanthin and xanthin to the same degree.

The velocity with which the oxidation occurs is, throughout a wide range, independent of the concentration of the base. This represents a circumstance now familiar enough in connection with a great number of actions controlled by colloidal catalysts. The data which illustrate it in the present case have been chiefly obtained anaerobically with milk. (Experiments I and II.) It can readily be shown, however, to hold in the case of tissue preparations.

The relation which obtains between the velocities with which hypoxanthin and xanthin are severally oxidised introduces considerations of considerable interest.

Xanthin of course requires only one atom of oxygen to convert it into uric acid, and this becomes associated with the carbon atom at the 8-position in

* In various samples of commercial dried milk examined we have found the catalyst destroyed or nearly destroyed. It is of course extremely unlikely that this fact has any bearing upon the nutritive value of such preparations.

the iminazol ring. Hypoxanthin requires two atoms, one at the 2-position in the pyrimidin ring and the second as in the case of xanthin. If the former of these is first taken up separately xanthin must of course be an intermediate stage in the oxidation of hypoxanthin. There is no strict justification for assuming this to be the case, nor, on the other hand, for the assumption that both rings in the purin structure are oxidised with equal ease.

The following considerations hold:—

If uric acid be produced from hypoxanthin by two consecutive actions, then, if the two rings are oxidised with equal ease, the velocity of oxygen uptake by this base will be the same as in the case of xanthin. From the former base, only half as much uric acid will be formed in a given time.

If, on the other hand, owing to a difference in the iminazol and pyrimidin rings, the (assumed) consecutive reactions have different velocities, then the rate at which hypoxanthin is oxidised will be controlled by the slower of these actions.

If, however, we can assume that once the catalyst is in relation with the purin structure, the two oxygen atoms required by hypoxanthin are added simultaneously, then this base will take up oxygen twice as fast as xanthin. The molecule of either base will then yield uric acid with the same velocity.

All the evidence we have obtained goes to show that the third and last of these alternatives is the one which, under anaerobic conditions, fits the facts.

If the protocols of our anaerobic experiments be consulted, it will be seen that, when the bases are added in equimolecular proportion to milk containing measured quantities of methylene blue, hypoxanthin consistently induces a velocity of reduction, which is exactly twice as great as that induced by xanthin. We have found this relation to hold whenever perfectly fresh milk is employed. The protocols record but a small proportion of the observations actually made. The same result has been obtained in the case of milks from a number of different individual cows, and also with different samples of goats' milk. It is still observed when the samples of milk are heated. The reduction velocity is then reduced, but to the same extent in the case of each base. The relation holds also in a striking way in the case of tissue preparations (Experiments I, II, III, and IX).

It indicates, of course, that the molecule of hypoxanthin (requiring two atoms of oxygen) and the molecule of xanthin (requiring only one) are, under anaerobic conditions, and with methylene blue as hydrogen acceptor, oxidised at the same rate. It means either that the former base is oxidised to uric acid directly without the intervention of xanthin as an intermediary, or, what is much less likely, that the time taken for the

attachment of oxygen to the pyrimidin ring is negligible compared with that required for oxidation in the iminazol ring.

If two different enzymes were concerned, the explanation of the facts would, of course, be different and simpler. The difference in the velocities might depend solely or mainly on a difference in the relative concentrations of the catalysts. It is, however, difficult to believe that, even if two enzymes are present in the tissues of various animals in an exact ratio of two to one, this ratio would be undisturbed in preparations artificially made, and equally undisturbed during the secretion of milk. It is also unlikely that they would respond identically to the effect of heat. All our results appear indeed to favour the belief that one enzyme is concerned with the oxidation of both bases.

In addition to the observations made with methylene blue, we have followed in milk and in tissue preparations the uptake of oxygen due to the aerobic oxidation of hypoxanthin and xanthin when present in known amounts. For this purpose we used, as already stated, Barcroft's differential apparatus. The method employed, though a micro-method, has yielded results which in many respects are satisfactory. Certain minor technical difficulties which are inherent in it, and other experimental details, were discussed in the previous section. A few typical examples of the curves obtained showing the velocity of oxygen uptake are reproduced on pp. 118–120.

In the case of fresh milk, a notable difference was observed between the anaerobic results and those obtained by aeration. Under the latter conditions hypoxanthin fails to take up oxygen twice as fast as xanthin; almost invariably indeed the initial rate of uptake is the same with both bases. In fig. 1*a* the velocity is seen to be identical during the earlier stages of the action. Fig. 2, based upon another experiment, shows that the completion of the oxidation of xanthin occupied only half the time required for hypoxanthin. These two curves represent satisfactorily the numerous data obtained. The results thus obtained were satisfactorily confirmed by the use of a null method, in which hypoxanthin in one flask of the Barcroft apparatus was balanced against xanthin in the other (see previous section, Experiments of Type VIII).

This difference between the anaerobic and aerobic results might be expected. Under the conditions of the methylene blue studies the progress of the change is observed in a homogeneous system. It is not difficult to understand that once some association between the catalyst and the hydrogen and oxygen acceptors is established, the two atoms of oxygen required by hypoxanthin may be as readily available as the one required by xanthin, since the concentration of water, which is the source of oxygen, is indefinitely large. When, however, a solution is shaken with air in a closed vessel we are dealing

with a heterogeneous system. We, as it were, titrate the solution with oxygen, and the velocity of change depends upon the rate of its supply, which, under the conditions, is relatively slow. In such a case the hypoxanthin molecule, when compared with the xanthin molecule, represents twice as much material to be oxidised, and when oxygen is supplied at a steady rate its oxidation takes twice as long.

We have found, however, that when a sample of milk is heated before use, and the concentration of the catalyst thus reduced, the velocity of oxygen uptake by hypoxanthin usually tends to become relatively the faster, even with the aerobic technique (figs. 1 and 2*a*). If the concentration of the catalyst be low enough, this, and not the oxygen supply, becomes the limiting factor. With the correspondingly low concentration of the active system (catalyst plus base) the dissolved oxygen may be maintained at a concentration proportionately high enough to make the conditions of uptake by either base approximate to those of the anaerobic homogeneous system. Figs. 1 and 2*a* show curves obtained in experiments done with heated milk.

We have obtained certain results during our observations which are difficult to explain. Though we believe them to be of minor importance, they require mention. Not infrequently when stale milk is employed the rate with which it reduces methylene blue in the presence of hypoxanthin tends to approximate to the rate displayed in the presence of xanthin. This has been observed in the case of milks which when fresh gave an exact two to one relation, and the same relation after heating for various periods. When milk as the result of bacterial growth has developed a reducing power of its own, the phenomenon can be easily understood. But we have observed it in cases when no appreciable autoxidising power seemed to exist. Whatever the explanation of this we do not think it proves the existence of two enzymes of varying stability. If there were such the effect of heat would be expected to differentiate them, which it certainly does not.

Another fact we have found somewhat difficult to explain. In the case of oxidation by atmospheric oxygen hypoxanthin never shows an uptake beyond that required to produce uric acid. In certain milks xanthin seems to take up 10 to 20 per cent. more than this, although milk shows no tendency to oxidise uric acid itself. In the case of a micro-method it is, of course, possible that errors of measurement may account for some departure from theory, but we seem to have observed the phenomenon too often for it to be merely a coincidence.

In the case of tissues we have made fewer observations with the aerobic technique. We have been content to show that the tissue preparations when able to bring about oxidation anaerobically likewise induce oxidation by

atmospheric oxygen. This point has already been discussed. There seems to be no evidence to show that when the purin bases are oxidised to uric acid biologically, an "oxidase" system as ordinarily defined is concerned. It is noteworthy in this connection to remember that the oxidation of purin bases to uric acid by peroxides has never been observed in the laboratory. We, ourselves, have been unable to detect the production of any trace of uric acid when hypoxanthin or xanthin is treated with hydrogen peroxide either by itself or in association with iron salts, or with milk, as catalysts.

We have made no attempt to calculate velocity constants from the curves of oxygen uptake. The method by which they were obtained was such as to make a comparison between the behaviour of xanthin and hypoxanthin wholly legitimate. The two bases were always studied in each experiment side by side under precisely similar conditions. The exact form, however, of any curve would be affected by a factor which with our apparatus could not be maintained completely constant, namely, the rate by which the Barcroft apparatus was shaken and the consequent rate of oxygen supply. This matter seems to be of relatively small importance however, since there is no doubt that the really significant time relations are linear.* So long as a moderate concentration of base is present in the system the velocity of change is constant. This is doubtless the real biological relation. When an action catalytically influenced by an enzyme is studied *in vitro* the combination of factors leading to a modification of the essential linear velocity is often of so accidental a nature that an equation made to fit the results may apply only to the particular conditions of the experiment in question and may have little general significance.

Summary.

It is shown that, in the presence of methylene blue, certain animal tissues are able to bring about the oxidation of xanthin and hypoxanthin to uric acid under strictly anaerobic conditions. Evidence discussed makes it probable that the catalytic system responsible for the anaerobic oxidation is not distinct from that which controls oxidation in the presence of oxygen.

Milk contains the same, or a similar, catalytic system, and can bring about the rapid oxidation of the bases aerobically and anaerobically. It furnishes, therefore, a convenient medium for the study of the process. The catalyst in milk is highly specific; no purin derivative experimentally tested, other than adenine xanthin and hypoxanthin, showed any signs of being able to induce the reduction of methylene blue in milk. The first of these is not directly

* Compare H. E. Armstrong and E. F. Armstrong, 'Roy. Soc. Proc.,' B, vol. 86, p. 561 (1913); *ibid.*, pp. 328–343.

oxidised. Milk contains a little adenase, and adenine is therefore slowly converted into hypoxanthin.

When, however, either hypoxanthin or xanthin is added to fresh milk containing methylene blue the dyestuff is rapidly reduced. Quantitative observations in evacuated tubes show that, when the bases are in equimolecular concentration, hypoxanthin reduces twice as fast as xanthin. Uric acid is therefore produced at equal velocity in each case, the former base taking up two atoms of oxygen as fast as the latter takes up one atom. It seems probable that the same catalyst is concerned in each case.

In aerobic experiments with fresh milk, on the other hand, hypoxanthin was found to take twice as long as xanthin for complete conversion into uric acid. Probable reasons for this difference in the anaerobic and aerobic events are discussed.

The tissues of the rat have been stated to contain no "xanthin oxidase." We have found that, as a matter of fact, they oxidise both bases either anaerobically or aerobically. This result suggests that the special organs of other animals supposed to be free from oxidase may, in some cases at least, be able to oxidise the bases when the conditions are suitably defined.

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ON

SOME COMPOUNDS RELATED TO HISTIDINE.

by

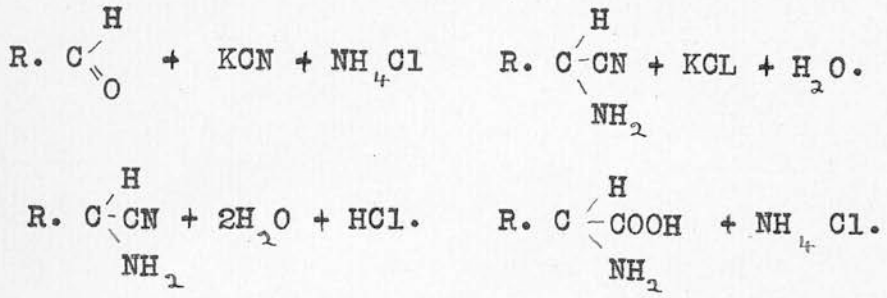
C. P. STEWART.

I. IMINAZOLYL-GLYCINE, THE LOWER HOMOLOGUE OF HISTIDINE.

In 1908 Engeland (Z. Physiol. Chem. 51, 49) isolated from human urine a small quantity of a picrolonate, melting at 244°C, to which he assigned the formula $C_5H_7O_2N_3 . C_{10}H_8O_5N_4$. From this, by decomposition with hydrochloric acid, he obtained a solution which gave a red coloration with sodium diazobenzenesulphonate. He concluded from these observations that the substance was the lower homologue of histidine.

It was believed that the synthesis of this amino-acid would be useful, partly as it might to some extent support Engeland's conclusion as to the identity of his substance, and partly because a knowledge of its properties might facilitate its isolation from other natural sources.

The only method available for the synthesis appeared to be that of Strecker (Annalen. 75 (1850) 27) in which, by the joint action of potassium cyanide and ammonium chloride, an aldehyde is converted into an amino-nitrile, from which the amino-acid is obtained by hydrolysis.



The iminazolyl - 4(or 5) formaldehyde (Pyman, J.C.S. 109 (1916) 186) was prepared by the oxidation of iminazolyl - 4(or 5) - methyl alcohol, and the latter substance was obtained from citric acid by the method used by Pyman (J.C.S. 99 (1911) 668) in his fundamental synthesis of histamine and histidine. The additional experimental details supplies by Koessler and Hanke (J. Amer. Chem. Soc. 40 (1918) 1716) were useful in securing good yields in the various stages of the synthesis.

The iminazolylglycine was obtained impure, in rather small yield, and had to be purified through the picrolonate. The highest melting point of this synthetic salt, - 243°C. (uncorr.) - is in close agreement with Engeland's value of 244°C. So far as this slender evidence goes, it tells in favour of Engeland's view as to the nature of the substance which he isolated from urine. As was to be expected, iminazolylglycine closely resembles histidine; it does not, however, give Knoop's reaction.

EXPERIMENTAL PART.

PREPARATION OF IMINAZOLYL - 4(or 5)- GLYCINE.

Equimolecular proportions of iminazolyl-formaldehyde (1 gram), potassium cyanide (0.65 grams) and ammonium chloride (0.53 grams), were dissolved in the minimum of water (5 cc.) and allowed to stand at room temperature for 48 hours. The solution gradually darkened in colour, and after a few hours, became almost opaque. Attempts were made to prevent this by filling the reaction vessel with nitrogen and with hydrogen, but were unsuccessful. It is important that the concentration of the reacting substances be kept as high as possible; on one occasion, when about twice the usual volume of water was used, scarcely a trace of amino-acid was obtained.

The amino-nitrile was not isolated, but was straightway hydrolysed by adding to the reaction mixture an equal volume of concentrated hydrochloric acid and heating on the boiling water bath for three hours. The liquid was then evaporated to dryness, and the residue dissolved in water. Chlorides were precipitated by silver nitrate, in the presence of nitric acid. After filtration, silver nitrate was added in sufficient quantity to give an immediate brown precipitate with barium hydroxide, which was then/

then added in excess. The silver compound of the amino acid, mixed with silver oxide, was filtered off, washed, and decomposed by hydrogen sulphide.
The filtrate from silver sulphide was boiled to expel hydrogen sulphide, freed from barium by sulphuric acid (without the addition of any excess of sulphuric acid), and evaporated. In the early experiments the resulting crude amino-acid was purified through the picrolonate, but later, it was found possible to precipitate the silver salt fractionally, and so obtain the bulk of the amino-acid sufficiently pure to allow of simple recrystallisation (after boiling with a very small amount of animal charcoal) from dilute alcohol. After several recrystallisations it formed sheaves of microscopic, colourless plates, which darkened at 220°C, and melted with decomposition at 254°C. It gave Pauly's reaction for glyoxalines, the triketohydrindene hydrate reaction for amino acids, but not Knoop's test for histidine, neither in its original form, nor as modified by Hunter (Biochem. J. 16 (1922) 637).
It was readily soluble in water, slightly soluble in methyl alcohol, almost insoluble in ethyl alcohol.

Nitrogen by micro-Kjeldahl.

4.92 mg.(dried at 120°C) gave 1.275 mg N_2. N = 25.9%

Calculated for $C_5H_7N_3O_2$. N = 26.4%

The/

The yield was variable and somewhat low: from 1 gram of the aldehyde 0.25 to 0.50 gram of crude amino-acid was obtained.

The phosphotungstate crystallises from hot water in glistening rhombic plates.

PREPARATION OF THE PICROLONATE.

The monopicrolonate was prepared by adding a hot saturated aqueous solution of rather less than the theoretical quantity of picrolonic acid to a concentrated aqueous solution of the amino-acid (Levene & Van Slyke, J. Biol. Chem. 12 (1912) 127).
On cooling, the picrolonate separated in deep yellow crystals, which, after recrystallisation from water and from ethyl alcohol, melted and decomposed at 243°C - a figure in good agreement with that given by Engeland.

Nitrogen of Micro-Dumas.

4.14 mg. (dried at 100°C) gave 0.854 cc. N_2 at 11°C & 762 mm.

N = 24.66%

Calculated for $C_5H_4N_3O_2 \cdot C_{10}H_8N_4O_5$ N = 24.2%

ESTIMATION/

ESTIMATION OF PICROLONIC ACID.

Picrolonates are decomposed by nitron, and as in the case of nitrates and picrates, the nitron compound is practically insoluble in water. The reaction can therefore be used for the estimation of picrolonic acid, and gives satisfactory results even on the micro scale.

Thus 21.09 mg. pure picrolonic acid gave 45.92 mg. nitron picrolonate. % picrolonic acid = 99.80.

12.16 mg. iminazolyl-4(or 5) glycine picrolonate gave 17.43 mg. nitron picrolonate.

% picrolonic acid = 65.7

Calculated for $C_5H_4N_3O_3 \cdot C_{10}H_8N_4O_5$.

% picrolonic acid = 65.2

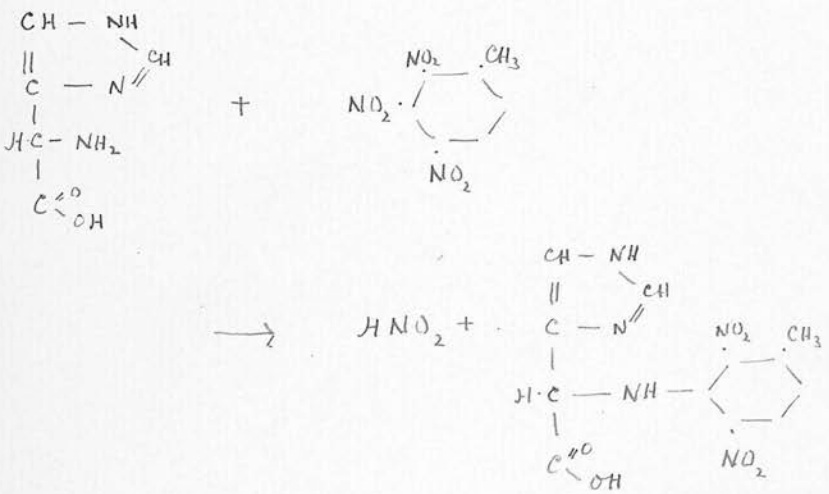
DECOMPOSITION OF THE PICROLONATE.

The picrolonate was dissolved in dulute hydrochloric acid and the free picrolonic acid thus formed was removed by extraction several times with benzene, and then with ether. The last traces were removed by boiling with a little animal charcoal, and filtering through a hardened filter. The solution thus obtained gave the red coloration with sodium diazo-benzene-sulphonate and (when neutralised) the violet colour with/

with triketohydrindene hydrate. On evaporation, however, the amino-acid hydrochloride was found to be hygroscopic, and recrystallisation from dilute alcohol did not improve matters. When attempts were made to dry it at 100°C, it decomposed. Similar results were obtained when the hydrochloride was prepared by dissolving the free amino-acid in hydrochloric acid. (histidine monohydrochloride melts at 117-119°C. (Pyman J.C.S. 99 (1911) 1396))

C. 4-(or 5) - IMINAZOLYL - N.2.4. DINITROTOLYL-3-GLYCINE.

β- and γ-trinitrotoluene condense with compounds containing a primary amine group, with elimination of nitrous acid (Barger & Tutin Biochem. J. 12 (1918) 402). In this case, 2·3·4· trinitrotoluene was used, the 3-nitro group disappearing in the process.



20 mg. of the amino acid and 60 mg. (a considerable excess) of 2·3·4·trinotrotoluene were dissolved in dilute alcohol and heated under a reflux condenser for three hours. The liquid gradually darkened to a deep yellow. It was evaporated to dryness, and the unchanged trinitrotoluene was removed by repeated extraction with hot benzene. The deep yellow, crystalline condensation product remained behind. It was very soluble in water, but insoluble in ethyl alcohol. Recrystallised from dilute alcohol, it decomposed violently at 270°C.

The nitrogen content was estimated by a modified micro-Kjeldahl. Eckert (Monats. fur Chem. 34 (1913) 1694) was able to use the Kjeldahl method in presence of aromatic nitro-groups by adding sulphur as a reducing agent. 0.2 - 0.5 g. of substance was mixed with 0.4 g of sulphur, and heated on the water bath for one hour with 20 cc. of 30-40% fuming sulphuric acid. The further procedure was as usual.

In adapting this method for use on the micro-scale, it was found advisable to increase the amount of sulphur to two or three times the weight of substance, and also to heat on the water bath for two hours. Shorter heating did not give satisfactory results with the substances tried, which, however, as they/

they contained two nitro-groups, afforded a fairly severe test of the method.

6.95 mg.of N-2.4.dinitrotolyl 3-histidine were found to contain 1.407 mg. nitrogen N = 20.3%

Calculated for $C_{13}H_{13}N_5O_6$. N = 20.9%

3.58 mg of C.4(or 5)-iminazolyl-N-2.4 dinitrotolyl-3-glycine contained .775 mg nitrogen. N = 21.65%

Calculated for $C_{12}H_{11}N_5O_6$. N = 21.8%.

This portion of the work has been published in the Biochemical Journal (17 (1923) 130).

II. β - IMINAZOLYL-4-(or 5)- PYRUVIC ACID.

Since the work of Neubauer on the oxidation of phenyl aminoacetic acid (Zeit. Physiol. Chem. 42 (1904) 81) it has more and more come to be recognised that α-Keto acids play an important part in the intermediate metabolism of amino-acids. The further work of Neubauer, Knoop (e.g. the work on the catabolism of y-phenyl amino butyric acid (Zeit. Physiol. Chem. 71 (1911) 252)), Flatow (Zeit. Physiol. Chem. 64 (1910) 367) and others, has demonstrated that the oxidation of an α-amino-acid to an α-Keto-acid is often the first stage in its breakdown in the aminal body. Dakin ("Oxidations and Reductions in the Animal Body" (1922) 71) concludes that "in general, the fate in the body of α-amino and α-ketonic acids is identical, whereas the α-hydroxy-acids, being presumably secondary reduction products, may behave differently. It is therefore assumed that α-ketonic acids are obligate products of the direct oxidation of α-amino-acids, whereas the hydroxy-acids are not directly derived from the amino acids."

The preparation of the hitherto unknown β-iminazolyl-4(or 5) - pyruvic acid was undertaken in readiness for a projected study of the metabolism of histidine, which, together with arginine, has been suggested/

suggested by Ackroyd and Hopkins (Biochem. J. 10 (1916) 551) as a possible precursor of purines.

The obvious method was to oxidise the corresponding hydroxy-acid, which is readily obtainable in good yield by the action of silver nitrite on histidine mono-hydrochloride (Frankel, Monats. f. Chem. 24 (1903) 229). The difficulty, however, was to find a suitable oxidising agent. Knoop found that barium permanganate (B. Ph. P. 10 (1907) 118) oxidised β-iminazolyl-4(or 5) - lactic acid to iminazolyl-4 (or 5) - acetic acid, and that with nitric acid as the oxidising agent (loc. cit. 116), iminazolyl-4(or 5) -glyoxylic acid was obtained. Frankel (B. Ph. P. 8 (1906) 159) oxidised histidine itself with bichromate and sulphuric acid, and obtained hydrocyanic acid and acetic acid.

In spite of the apparently complete breakdown of the histidine molecule observed by Frankel it was considered advisable to try the effect of chromic acid on β -iminazolyl-4(or 5) - lactic acid, as being the usual agent for the oxidation of secondary alcohols. To this end, the theoretical amounts of β -iminazolyl -4(or 5) - lactic acid and chromic acid were dissolved in water and the solution was warmed on the water bath at/

at 50-60°C. until the chromic acid was completely reduced - i.e. until a drop of the liquid no longer gave a coloration with ether and hydrogen peroxide. Apparently, a chromate of β -iminazolyl-4(or 5)-lactic acid was first formed, so that in testing for chromic acid, the drop of fluid under examination had to be acidified by the addition of sulphuric acid. At the end of the reaction, the liquid was filtered, and precipitated with phosphotungstic acid, excess being avoided. The phosphotungstate was soluble in hot water and was recrystallised before being decomposed by barium hydroxide. The substance obtained in this way was, however, identified by melting point, analysis, etc. as unchanged β -iminazolyl-4(or 5)-lactic acid.

Hydrogen peroxide was found to break up the iminazole ring, for a solution of β -iminazolyl-4(or 5) -lactic acid or of histidine, after warming with hydrogen peroxide, gave no coloration with sodium diazo-benzenesulphonate.

In these circumstances the idea of obtaining the Keto-acid by oxidation was abandoned, at any rate until a study of the synthetic compound should have shown the feasibility of such a method. A convenient method of synthesis presented itself in that used by Ellinger (Zeit. Physiol. Chem. 109 259) in the preparation of/

of β- indol-3-pyruvic acid. This consisted in the condensation of indole-3-aldehyde with hippuric acid, and hydrolysis of the resulting oxazolone by means of 40% caustic soda. The 2-Phenyl-4- (1-acetyl iminazolyl-4(or 5)- methylidine) -oxazolone required was prepared according to the directions given by Pyman (J.C.S. 99 (1916) 193).

EXPERIMENTAL PART.

PREPARATION OF -IMINAZOLYL-4(or 5)-PYRUVIC ACID HYDROCHLORIDE.

1.5 grams of 2- Phenyl-4-(1- acetyl glyoxaline -4(or 5)- methylidine)-oxazolone were added to 15 cc. of 40% caustic soda, and the solution was boiled under a reflux condenser until evolution of ammonia had practically ceased - 2½ hours. The clear, straw-coloured solution, on cooling, formed a pasty mass, owing to the separation of sodium benzoate. This was acidified with hydrochloric acid, and cooled in ice. The precipitated benzoic acid was filtered off, and, after washing with a little water, and drying, was found to weigh .63 grams - nearly the theoretical quantity. The filtrate from the benzoic acid was evaporated/

evaporated to dryness, and the residue was repeatedly extracted with absolute ethyl alcohol. The alcoholic extracts were combined and evaporated to dryness, and the residue, after washing with petroleum ether to remove traces of benzoic acid was re-crystallised from ethyl alcohol or a mixture of ethyl alcohol and acetone. Yield: .6 gm., about 60% of the theoretical.

The β- iminazolyl-4(or 5)- pyruvic acid hydrochloride thus obtained was very soluble in water, readily soluble in methyl and ethyl alcohols, but insoluble in other organic solvents. Heated in a capillary tube, it frothed at 108°C. and became pasty, darkened at about 220°C, and became liquid at 241°C. It gave a red coloration with sodium diazobenzenesulphonate, and (best in alcoholic solution) a deep bluish-green coloration with ferric chloride. It reduced alkaline potassium permanganate immediately and completely; it reduced an ammoniacal silver solution on warming; and slowly reduced Fehling's solution. It was precipitated in alkaline solution by mercuric chloride and by silver nitrate (the silver precipitate rapidly blackened, owing, doubtless, to the reduction of the silver oxide which was also present.).

Phosphotungstic acid gave a precipitate soluble

in/

in excess of phosphotungstic acid and in hot water.

Nitrogen by micro-Kjeldahl:

6.70 mg. contained 0.999 mg. nitrogen N = 14.9%

Calculated for C H N O . HCl. N = 14.7%

An attempt was made to prepare the nitro-phenyl hydrazone of β -iminazolyl 4(or 5)- pyruvic acid, but the substance proved difficult to isolate owing to its solubility. To a slight excess of p-nitro-phenylhydrazine dissolved in acetic acid, a concentrated aqueous solution of β- iminazolyl 4(or 5) - pyruvic acid hydrochloride was added, and the mixture was allowed to stand. The solution darkened somewhat, and after 24 hours a minute amount of precipitate had appeared. This was considerably increased on neutralising with sodium hydroxide, and was then separated by centrifuging. The precipitate was soluble in water, so that much of it was lost in washing, which was necessary to remove sodium acetate &c. It was soluble in alcohol and the alcoholic solution on spontaneous evaporation, left a yellowish crystalline residue. This substance was undoubtedly the nitro-phenyl hydrazone, but the amount available was too small for further investigation.

III. β -IMINAZOLYL-4(or 5) - PROPIONIC ACID.

This acid was required for the same purpose as the Keto-acid described in the preceding section.

Knoop & Windaus (B. Ph. P. VII (1906) 146) obtained β -iminazolyl-4(or 5)- propionic acid by reducing β -iminazolyl-4(or 5)- lactic acid with red phosphorus and hydriodic acid.

Frankel (B. Ph. P. VIII (1906) 159) prepared it by zinc dust reduction of β -iminazolyl-4(or 5)- α- chloropropionic acid.

Ackermann (Zeit. Physiol. Chem. 65 (1910) 504) isolated a minute quantity, as the platinichloride, from the products of the action of aerobic bacteria on histidine, the main product being histamine.

Hopkins & Cole (J. Physiol. 29 (1903) 458) obtained satisfactory yields of indole-propionic acid by growing anaerobic bacteria in a 1% solution of gelatine to which the necessary salts and 0.2% of tryptophane had been added. In the earlier experiments, the Rauchsbrand bacillus was used, in the later, B. coli communis. Recent attempts to repeat this work at Cambridge were unsuccessful (Ward, private communication to the author) until the pure culture of B. coli was replaced by a mixture of B. coli;/

B. coli; Chauvei; Sporagenes, and Oedematicini, when yields of 40 - 50% were obtained.

The yield of β -iminazolyl-4(or 5)- propionic acid by either Knoop's or Frankel's method being poor, it was decided to try to prepare it by an application of Ward's modification of Hopkins & Cole's method for indole propionic acid.

The apparatus and method of securing anaerobic conditions have been fully described by Hopkins & Cole. After Four weeks incubation at 37°C, the contents of the flask were acidified and filtered. Excess of mercuric sulphate was added, and the liquid was neutralised with barium hydroxide. The heavy white precipitate, which contained all the iminazole compounds, was filtered off, thoroughly washed, and decomposed by hydrogen sulphide. The filtrate from mercuric sulphide was evaporated to dryness, and the residue was repeatedly extracted with absolute alcohol, until the extract gave only a faint coloration with sodium diazobenzene sulphonate. The combined alcoholic extracts were evaporated to dryness; the residue was dissolved in water and precipitated with phosphotungstic acid. The phosphotungstate was extracted with hot water which, on cooling, deposited minute rectangular plates. This recrystallised phosphotungstate/

phosphotungstate was decomposed by barium hydroxide, and the filtrate from barium phosphotungstate, after being freed from barium by means of sulphuric acid, was evaporated to small bulk. On the addition of acetone, a white crystalline powder separated. This, after recrystallisation from dilute acetone, melted with decomposition at 205°C. and the melting point was not lowered by mixture with pure β-iminazolyl-4 (or 5)- propionic acid. It was soluble in water, with a strongly acid reaction, and was soluble in methyl and ethyl alcohols. It formed a copper salt, crystallising in small blue needles.

Nitrogen by micro-Kjeldahl.

5.43 mg. contained 1.080 mg. nitrogen N = 19.88%

Calculated for $C_6H_8N_2O_2$. N = 20.04%

The yield, however, was small, and for preparative purposes it appeared to be preferable to use the method of Knoop & Windaus.
