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by

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ON PHOSPHOCREATINE IN THE DOG'S BRAIN

ON PHOSPHOCREATINE IN THE DOG'S BRAIN

In a review on "The Metabolism of Brain and Nerve" (1), Gerard states the following:

"Creatine phosphate content of dog and cat brain (about 11 mg. per cent) is alike in cerebrum and cerebellum; the substance decomposes with great rapidity unless this tissue is carefully frozen in situ (Kerr) (2). It has been obtained, however, from rabbit brain without such precautions and in even larger amounts (Gerard and Tupikova, unpublished data)."

Actually, Kerr (2) reported 11 mg. per cent of labile phosphorus (i.e., phosphocreatine phosphorus)* which is equivalent to about 76 mg. of phosphocreatine, or 46 mg. of creatine per 100 gm. of brain. But Gerard and Tupikova (4) found 40 - 45 mg.% of bound creatine (i.e. creatine precipitated by barium hydroxide in 66% alcohol, supposed by them to be bound as phosphocreatine) in dog brain - an amount which is equivalent to the labile phosphorus found by Kerr (2) - without freezing the brain in situ. Accordingly, an attempt was made in this laboratory to determine both the labile phosphorus and bound creatine on the same brain extract in order to see if any

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* Phosphocreatine phosphorus was designated by Fiske and Subarrow (3) as "labile" phosphorus because it decomposes within 30 minutes in acid molybdate solutions at room temperature.

excess of bound creatine could be found over what the labile phosphorus can account for.

M e t h o d: The brain was prepared for analysis by making a protein-free extract with 10% trichloracetic acid, according to the directions of Kerr (2) and Kerr and Blish (5), but without freezing the brain in situ; and the labile phosphorus was determined in 8 c.c. portions of the filtrate by the procedure of Fiske and Subarrow (3), after removal of inorganic phosphorus by precipitation with calcium hydroxide.

For the bound creatine determination, a 20 c.c. aliquot of the trichloracetic acid filtrate was saturated with barium hydroxide in a centrifuge tube, 45.5 c.c. of 95% alcohol was added to give a concentration of 66% alcohol, and the tube was left overnight. After centrifuging for ten minutes and decanting off the filtrate, the precipitate (containing the "bound" creatine) was washed with 66% alcohol saturated with barium hydroxide and centrifuged again. Ten c.c. of 3N H_2SO_4 were added to the precipitate, and the mixture autoclaved at 120°C. for one hour (instead of refluxing for three hours*) in order to convert the creatine to creatinine. Then the barium sulfate was removed by centrifuging and the precipitate washed in the centrifuge tube with H_2SO_4.

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* Controls showed that both methods could be used interchangeably.

The filtrate was neutralized and its creatinine content determined by Folin's Method (6)*. This modification of the method for bound creatine determination was the result of a study of the previous work of Gerard and Tupikova (7) (8), Baumann and Hines (9) (10), Harding and Eagles (11), Dulière (12) and Ochoa (13). We had to devise this modification because the method used by Gerard and Tupikova (4) was not available at the time of this work. Since this publication became available, we found that our modification is substantially the same as that of Gerard and Tupikova. The differences are: (a) Gerard and Tupikova's modification was adapted for smaller quantities. (b) They added the alcohol before neutralizing with $Ba(OH)_2$. (c) They did not saturate but only neutralized with $Ba(OH)_2$. (d) They did not let the Ba precipitate stand overnight, but centrifuged after 5 minutes for fear of splitting the phosphocreatine#. (e) They converted their creatine to creatinine by keeping in boiling water bath for 3 hours instead of autoclaving.

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* In most cases it was found convenient to add to the last filtrate an amount of the creatinine standard reagent equivalent to 1 mg. of creatine before proceeding with the determination in a 100 c.c. volumetric flask, and then subtracting this amount from the results.

\# Although we let stand overnight, thus giving a chance for an extensive lowering of our bound creatine content (4), yet we still had results that were too high to be explained by the labile phosphorus.

R e s u l t s:

Sample	Time taken to remove brain from dog to acid.	Labile phosphorus in mg. per 100 gm. of bfain.	Bound creatine as calculated from labile P	Bound creatine found, in mg. per 100 gm.
1.	5 sec.	2.58	10.9	24.0
2.	4 - 5 sec.	2.855	12.0	27.5
3.	3 sec.	3.69	15.59	31.0
4.	10 sec.	2.26	9.96	13.75*

D i s c u s s i o n: These results show that there is definitely more bound creatine in the dog's brain than can be accounted for by the labile phosphorus. The explanation for this discrepancy may be that either two molecules of creatine are bound to the phosphate radical (for the bound creatine is about twice that expected), or that the excess of creatine is bound to some compound other than phosphate. Another possible explanation is that this excess is not creatine at all, but some other chromogenic substance which gives color with picrate. Which of these three explanations is correct must be decided by further experiments.

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* This determination suffered considerable loss accidentally during the experiment.

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ON ADENOSINE IN HUMAN URINE

F O R E W O R D .

The pharmacological effect of adenosine and its derivatives has been ~~the~~ *a* subject of interest for the last twenty years.

In 1940, Kellaway and Trethewie (1) gave evidence to show that the effect of cobra venum was partly due to a liberation of adenyl compounds from the liver.

A discussion of the rôle of adenosine as a vaso depressor is given by Rigler (2). And it has been debated in this laboratory whether or not it plays a roll in traumatic shock. If traumatic shock is due to the liberation of adenosine from injured tissues, then it should be found in the blood plasma and urine of shocked animals. Thus, our interest in the question resulted in the attempt to detect small amounts of adenosine added to plasma or urine.

ON ADENOSINE IN HUMAN URINE.

In 1930, Calvery (3), following the technique of Embden and Zimmermann(4), isolated adenosine from human urine (300 mg. of adenosine picrate from 80 liters). Teitge (5), on the other hand, using a modification of the Salkowsky precipitation (6)(7) with ammoniacal silver nitrate, failed to detect adenine in any form in human urine. In our present work, it is shown that

although the Salkowsky precipitation as applied by Teitge precipitates free adenine (being a purine base), yet it fails to precipitate adenosine from urine; hence Teitge's finding cannot be considered a valid denial of Calvery's results.

A new method devised by Kerr and Seredarian (8)(9) for separate nucleotide, nucleoside, and free purine determinations in tissue extracts was applied to urine in an attempt to confirm or deny Calvery's findings; but it was found to be inapplicable for quantitative studies on large quantities of urine. It was possible, however, to demonstrate by Kerr's method the absence of adenine, either free or combined, from healthy human urine. We also have been unable to confirm Calvery's work even on applying his own method (3) to normal albumin-free urine. This, together with the qualitative result obtained with Kerr's method, leads us to the conclusion that the adenosine obtained by Calvery may have had its origin in the urine of some abnormal individuals and is not of general occurrence - a possibility which is acknowledged by Calvery himself.

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Section I

We had reason to believe that adenosine might be precipitated by ammoniacal silver nitrate (i.e. the Salkowsky method for purines) since adenosine is precipitated by sodium hydroxide and silver nitrate (9). Seven experiments were made with this method on different samples of urine (35 c.c. or 2 liters). In five of them no adenosine could be detected, and in two the results were doubtful. We find, however, that adenosine added to urine

cannot be recovered by this method.

M e t h o d : Phosphates were removed either by precipitation with 8% uranium acetate at pH 6-7(x) using ferrocyanide on a spot-plate as indicator (10); or with CaO suspension (3), or with magnesia mixture (6). In some experiments the phosphates were only partially removed as calcium phosphate by making the urine alkaline with ammonium hydroxide. In place of washing, the precipitate, separated by centrifugation, was dissolved in sulfuric acid and reprecipitated by neutralising with NaOH, and the wash liquid was added to the main supernatant fluid. To this, molar silver nitrate was added and the silver chloride dissolved by adding excess of concentrated ammonia. The centrifuged precipitate (supposed to contain free purines plus nucleosides) was washed once with dilute ammonium hydroxide. Small precipitates (from 25 c.c. of urine) were suspended in dilute sulfuric acid (final pH, 1.5-2) for 30 minutes in order to dissolve the nucleosides, leaving the acid-insoluble fraction containing the silver-purine compounds (9). Larger precipitates, on the other hand(from 2liters of urine) were suspended in water, made slightly acidic with sulfuric acid, and decomposed with

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(x) Sodium hydroxide and acetic acid were used for adjusting the acidity, and a calomel electrode for determining the pH. This method would also precipitate nucleotides if present (8)(9), but Calvery (3) reports absence of nucleotides from human urine, this being confirmed in our experiments as described later.

hydrogen sulfide, and the silver sulfide removed by centrifugation.(x) The supernatant fluid was aerated, made to pH 2 with sulfuric acid, and molar silver nitrate added again (2 c.c. per 100 c.c. of urine used) to precipitate the free purines, leaving nucleosides in solution (9). In either case, the resulting acid-insoluble fraction was washed three times with 0.05N sulfuric acid. To the supernatant fluid plus washings (i.e. the sulfuric acid-soluble portion of the silver precipitate) were added a few drops of molar siver nitrate solution and sufficient NaOH to precipitate some silver oxide. The precipitate (containing then nucleosides if present (9)) was centrifuged and washed once or twice with water. Each precipitate was then hydrolysed and extracted with hot 0.5N HCl, filtered through asbestos, and ribose determined on an aliquot of the filtrate (9). The amount of ribose found would be a measure of the amount of nucleosides in the precipitate examined.

Results: The results of these experiments were the following: (a) We could not detect any adenosine in human urine by using this method. (b) Adenosine added to urine either before or after the removal of phosphates could not be recovered either in the acid-silver precipitate or in the silver oxide precipitate, as shown by three experiments.

Conclusion: The ammoniacal silver nitrate method for purines

(x) The silver sulfide precipitate was shown to contain no nucleosides by the same analytical procedures which were applied to the succeeding precipitates.

does not precipitate adenosine. And as this was the method applied by Teitge (5), the latter's findings cannot be considered a valid denial of Calvery who isolated adenosine from human urine(3).

Appendix: It is important to note here that, while the uranium phosphate precipitate always gave the orcinol test for pentose in considerable amounts, yet there was no increase in the pentose content of that precipitate when adenosine was added to urine. Therefore the pentose it contained was not adenosine. It may be the urinary pentose isolated by Neuberg (11), Elliot and Raper (12), Levene and La Forge (13, 14), and identified by Hiller (15) and by Greenwald (16, 17) as d-xyloketose, the l-form of which was later shown by Greenwald (18) to be definitely capable of being further metabolised in the body.

Furthermore, the hydrolysed HCl solution of the uranium phosphate precipitate, when neutralised with NaOH and centrifuged (to precipitate the uranium), gave no precipitate with sodium bisulfite and copper sulfate (19), and therefore contained no purines. This confirms Calvery's finding that there are no nucleotides in human urine.

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Section II

In anticipation of interference with the purine and nucleoside separation (9) by the larger AgCl precipitate,

our first experiments were made on the recovery of adenosine added to small volumes of urine (10-25 c.c.).

M e t h o d : The phosphates are removed by precipitation with uranium acetate as described in section I. The supernatant fluid (plus washings) is brought to pH 2 with 10N sulfuric acid, and enough molar silver nitrate added to precipitate all the chlorides and an excess of 8 c.c. per 100 c.c. of urine to precipitate the free purines.(x) After 30 minutes, the precipitate is removed by centrifuging and washed two or three times with 0.05N sulfuric acid solution. The supernatant fluid and washings are made slightly alkaline with NaOH to precipitate some Silver oxide and nucleosides(9). The two precipitates (acid-silver precipitate and silver oxide precipitate) are treatd as described in section I, and pentose determined in the HCl extract of each by the method of Kerr and Soraidarian (9). The ribose determination could not, as a rule, be carried accurately due to the development of a persistent brown color interfering with the green.

After determining pentose, the remainder of the HCl extract was neutralised to phenolphthalein, centrifuged to remove any uranium, and the purines precipitated with sodium bisulfite and copper sulfate according to Hitchings and Fiske (19) and determined separately by the methods of the same authors (20, 21, 22).

(x) The reason for this relatively large excess of silver nitrate used (as compared with the experience of Kerr and Soraidarian(9)) is indicated in an appendix to this paper, p. 14 .

Results: Seven experiments were performed with small volumes of urine (10-25 c.c.), the acid precipitation being carried out at an acidity of 0.02-0.04N with respect to sulfuric acid. In these experiments it was impossible to obtain accurate ~~accurate~~ pentose determination due to the interference of the brown color mentioned above. But as judged by the naked eye, the added adenosine was recovered almost completely in the acid precipitate. In experiment 1. (Table I), however, a quantitative determination was attained by repeated washings of the precipitate; and it showed that adenosine comes down (84-96%) in the acid-silver precipitate where only the free purines are expected according to the experience of Kerr and Seraidarian with tissue extracts (9).

However, on raising the acidity to about 0.2N with sulfuric acid (pH 1.5-2.0), most of the adenosine remained in solution, and 77% was later recovered in the Ag_2O precipitate - expt. 2. In four other experiments carried out under the same conditions, it was impossible to have accurate ribose determinations, but as judged by the naked eye, they showed the same general result as experiment 2 indicated in the table.

On applying this method to urine samples big enough to permit purine determinations, there was marked interference by the bulky AgCl precipitate, so that added amounts of adenosine could not be recovered in the alkali precipitate, but were lost, 60 % being recovered in the ~~alkali~~ silver precipitate, as indicated by the ribose and purine determinations - expts. 3 and 4, (Table II.

Table I: The application of Kerr's Method to urine with and without added amounts of adenosine.

Expt. no.	Urine sample	Adenosine added	Ribose equiv. of added adenosine	Adenine N equiv. of added adenosine	Acidity for Ag precipitation
	c.c.	mg.	mg.	mg.	
1. a)	10	0	0		0.04N H_2SO_4
b)	10	0.406	0.228		0.04N H_2SO_4
c)	10	0.406	0.228		0.04N H_2SO_4
2. a)	10	0	0		0.2N H_2SO_4, pH 1.7
b)	10	0.394	0.22		0.2N H_2SO_4, pH 1.7
3. a)	500	0	0	0	pH 1.8
b)	500	5.2	2.92	1.36	pH 1.8
4. a)	500	0	0	0	pH 1.8
b)	500	9.1	5.1	2.39	pH 1.8

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* Here, the presence of a brown color prevented accurate pentose determination. Judging by the naked eye, however, there was considerable recovery of added adenosine.

ntose found		Recovery of added adenosine		Adenine N found	
n acid-Ag precipitate mg.	In Ag_2O precipitate mg.	In acid-Ag precip. %	In Ag_2O precip. %	In acid-Ag precip. mg.	In Ag_2O precip. mg.
0.692	trace				
1.016	trace	84.3	none		
1.08	trace	95.7	none		
trace	0.163				
trace +	0.333	10% estimated	77		
present	trace				0
large excess*	trace	considerable*	none		0
present	trace			0	0
large excess*	trace	considerable*	none	1.43	0

ecovery of added adenosine	
acid-Ag. precip. %	In Ag_2O precip. %
0	
0	

In known solutions of guanosine, the presence of such amounts of chlorides showed equally marked interference also, (not indicated in the table).

No adenine was found in any of the urine samples (either in the acid-silver precipitate or in the silver oxide precipitate), except in the ones to which adenosine had been added.

C o n c l u s i o n : Kerr's procedure for separating nucleosides from free purines may be successfully applied to small amounts of urine with added adenosine (pH 2) ; but in handling big amounts, the nucleoside (adenosine) is adsorbed (presumably by AgCl) in the fraction precipitated by silver from the acid solution.

The fact that no adenine was found in any of the urine samples (except those to which adenosine had been added) either in the acid-silver precipitate or in the silver oxide precipitate, together with the fact that no purines at all were to be found in the nucleotide-uranium phosphate precipitate, as indicated in section I, proves that there is no adenine in any form (either free or combined) in the urine examined. This confirms Teitge's results(x) (5) but not those of Calvery (2) who actually isolated adenosine from human urine.

(x) It may be mentioned here that we incidentally found in experiment 4, 2.175 mg. of guanine nitrogen per liter of urine (or the corresponding amount of xanthine nitrogen) by the method of Hitchings which does not distinguish between guanine and xanthine (22). This harmonises with Teitge's having found xanthine in the urine (5), and in roughly the same amounts. But we did not find any hypoxanthine - a fact which confirms Teitge's having found no carnine or hypoxanthine in healthy urine, carnine being found only in leucaemic urine (5).

Section III

In an attempt to duplicate Calvery's work, eighty liters of urine were collected from students. Phosphates were removed with CaO suspension*, and the purines were precipitated with copper sulfate in alkaline medium#. The precipitate was then decomposed with hydrogen sulfide, and the copper hydroxide precipitation repeated on the aerated solution; and the copper again removed. The aerated filtrate was treated with lead acetate in acidic medium and allowed to stand overnight to precipitate any nucleotide present. After centrifuging, the filtrate was made alkaline with ammonia to precipitate nucleosides. The precipitate was decomposed with H_2S, aerated, and the lead-ammonium-hydroxide precipitation repeated.

So far Calvery's directions were followed exactly as described, except that in removing the sulfides of heavy metals, we filtered hot in order to minimize the danger of loss by adsorption.

The final aerated filtrate, supposed to contain nucleosides, if any, amounted to 420 cc., and was slightly yellow. Before decolorizing it with Norrit, as described by Calvery,

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* This probably precipitates any pathological leucocytes (which may be a foreign source of adenosine) too. But it may be that on standing, the adenosine had diffused out of the leucocytes to contaminate the urine, before Calvery started the precipitation.

\# Though this precipitates all the purine bases, yet it precipitates only half of the nucleosides (Kerr, unpublished data).

a 25 c.c. aliquot was examined to see if it contained purines or pentose. It was made 0.5 N with respect to HCl and hydrolyzed on a boiling water bath for 30 min., for subsequent ribose and purine determination, by the methods previously described. Ribose, adenine and hypoxanthine were found to be absent. The uric acid content of the entire 420 c.c. batch was 120 mg. - Folin's method (23). Hitchings' colorimetric method (21), which does not distinguish between guanine and xanthine, indicated the presence of about 366 mg. of guanine N, or 293 mg. of xanthine N, in the total 420 c.c. batch.

In spite of the failure to find adenine in the 25 c.c. aliquot, Calvery's procedure was continued. The remaining 395 c.c. were decolorized with Norrit*, concentrated in vacuum to a thin syrup (40 c.c.), and a saturated alcoholic solution of picric acid was added in excess. This did not give the immediate precipitation described by Calvery. On standing an hour, however, a precipitate was obtained (135 mg.), which on double recrystallization gave needle-like crystals which melted with decomposition at 275 - 279°C. (uncorrected), preceded by a slight orange color at 260°C. (uncorrected). (The melting point of adenosine picrate is 185°C.)

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* Norrit was used cold for fear of adsorbing the purines. Even this is probably not safe, for Fiske (24) used Norrit for quantitative adsorption of purines.

The nitrogen content, as determined by the Micro-Kjeldahl* procedure (8), was 13.33%, while that of adenosine picrate should be 13.62%. The orcinol test for pentose was negative, while equivalent samples of adenosine picrate from yeast nucleic acid (prepared by Dr. G. Fawaz) gave positive ribose tests, though considerably masked by the color of the picric acid.

The picrate precipitate was dissolved and acidified, then shaken with ether, to extract the picric acid. The remaining aqueous solution gave no test for ribose or adenine by the methods previously described.

The compound has not been identified, but it is certainly not adenosine picrate.

Therefore, basing our judgement on our inability to demonstrate any adenosine by Kerr's method, and also on our failure to confirm Calvery even by his own method of isolation from a large volume of urine, we can safely conclude that there is no adenosine in healthy human urine. That found by Calvery may have been due to some abnormal urine in the batch he investigated.

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* It should be remembered here that by this procedure the nitrogen of the nitro groups of the picrate is not determined.

Summary

1. The Salkowsky method of precipitating purines with ammoniacal silver nitrate fails to precipitate adenosine.

2. There are no nucleotides in normal human urine.

3. Uric acid in the urine precipitates almost quantitatively with silver nitrate at pH 3 only if enough silver nitrate is added to precipitate all the chlorides, plus an excess of 5-10 c.c. of molar silver nitrate per 100 c.c. of urine. A curve is described showing the peculiar behavior of uric acid in urine before that point is reached (Appendix).

4. With this taken into consideration, Kerr's procedure for separating nucleosides from free purines may be successfully applied to small amounts of urine with added adenosine, but in handling big amounts the nucleoside (adenosine) is mostly adsorbed (presumably by AgCl) in the fraction precipitated by Ag from acid solution.

5. Kerr's quantitative method for determining ribose cannot be applied with great accuracy to any of the fractions from urine, because of the development of a brown color which interferes with the colorimetric determination.

6. A considerable amount of pentose is found in the urine, most of it being found in the uranium phosphate precipitate,

but no adenine or hypoxanthine nitrogen is found in any of the urine fractions; hence the pentose is believed to be the d-xyloketose described by previous authors. Some guanine (or xanthine) was found and determined in both the nucleoside and the free purine fractions.

7. Calvery's isolation of adenosine from 80 liters of urine (3) could not be confirmed by repeating the procedure he described.

8. It is concluded that adenosine does not occur in normal human urine.

Appendix

In a study of Kerr's method applied to urine, account had to be taken of the large excess of uric acid present there, since it later interferes with the separate purine determinations (21) (22). The amounts of silver nitrate used for precipitating purines and chloride at pH 2 in tissue extracts (9) were naturally not enough to precipitate all the uric acid, much less the other purines, due to the presence of so much chloride in the urine.

But in our study of the amount of silver nitrate needed, the urinary uric acid exhibited a very peculiar behavior towards silver nitrate at pH 2. For, at first, silver urate precipitates rather fast together with AgCl, but a point is reached (fig. 1) where, on further addition of $AgNO_3$, we find an increase of uric acid in the supernatent fluid. This point corresponds roughly to about one third the amount of $AgNO_3$ needed to precipitate all the chlorides.* However, when all the chlorides are precipitated, an excess of 5 - 10 c.c. of molar silver nitrate per 100 c.c. of urine is sufficient for complete precipitation of uric acid#. This excess is 2-5

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* It may be, as suggested by Prof. W. A. West, that the increase in nitrate concentration in the supernatent fluid makes silver urate relatively more soluble until a large excess of the silver ions is afforded. But this is only a hypothesis, and needs further testing under controlled conditions.

\# The small amount of uric acid left can later be destroyed with HNO_3 as described by Hitchings (21) (22) just preceding the purine determinations in which it interferes.

times the amount that is found sufficient for purine precipitation in tissue extracts (5).

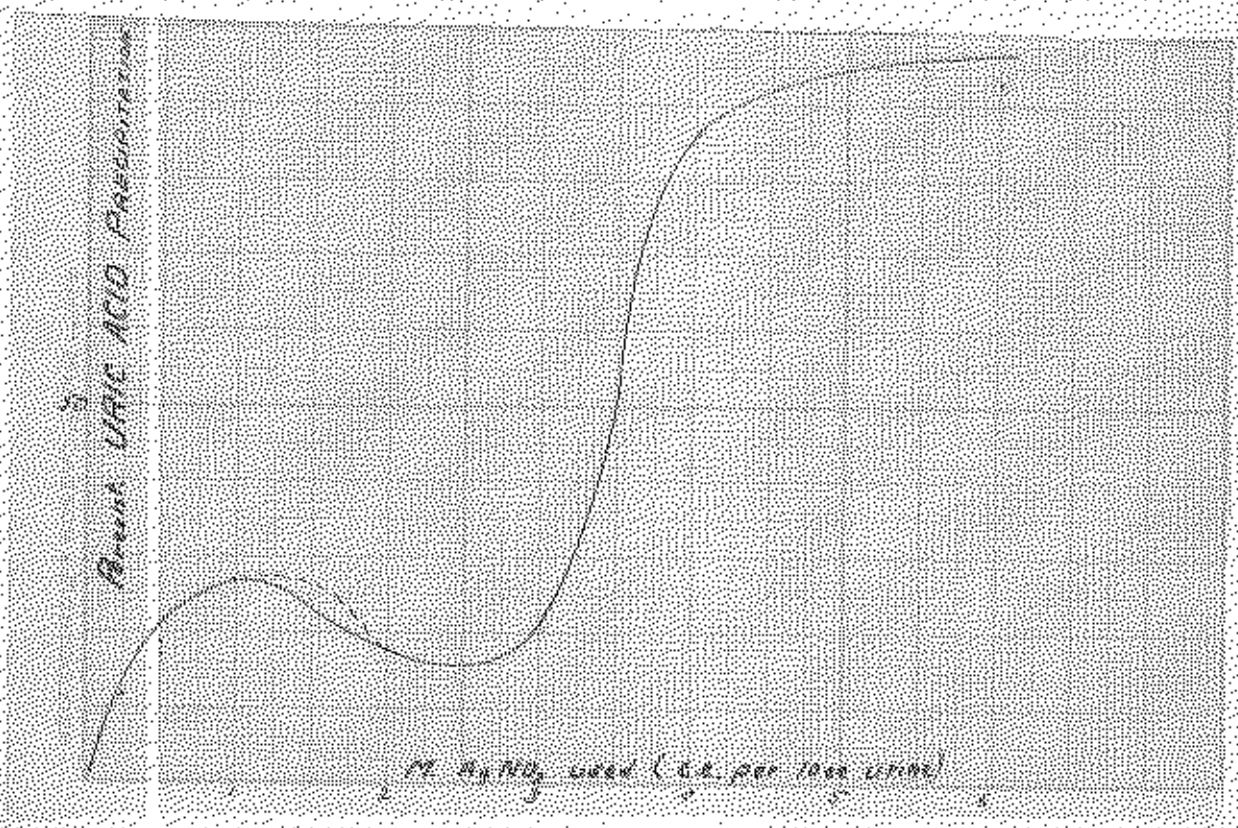

Fig. 1. The behavior of urinary uric acid with molar silver nitrate at pH 8. The chlorides were completely precipitated by 3.5 c.c. molar silver nitrate. Uric acid determinations were carried on the supernatant fluid by Folin's method (23). There is some evidence (not yet certain) that the curve actually follows the dotted path.

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THE PATHWAY OF AUTOLYSIS OF ADENINE NUCLEOTIDE IN HEMOLYSED AND UNHEMOLYSED PIG BLOOD

THE PATHWAY OF AUTOLYSIS OF ADENINE NUCLEOTIDE IN HEMOLYSED AND UNHEMOLYSED PIG BLOOD

The presence of adenylic acid in blood was indicated in 1923 by Jackson (1, 2), and the compound was isolated in 1925 by Hoffman (3). It was further showed by Kerr and Daoud (4) that the adenylic acid of blood was actually present in combination with the pyrophosphate radical first recognized by Zucker and Gutman (5), as adenosine triphosphate. This compound was isolated in the same year by Fiske (6) from rabbit blood.

The first study on the course of autolysis of this compound in blood was made in 1929 by Mozolowski (7). Using the Parnass method (8) for adenine and hypoxanthine he found that when hemolyzed pig blood is allowed to stand for 48 hours, the adenine nucleotide is gradually deaminised to inosinic acid (thus accounting for part of NH_3 liberated) and the latter converted to inosine or hypoxanthine. This was supported in 1937 by Kerr and Antaki (9) whose results showed that nearly all the hydrolysable phosphorus and about one fifth of the nucleotide nitrogen were lost over a 1 1/2 hours incubation of hemolysed pig blood. Longer periods of autolysis resulted in more complete disintegration of the nucleotide. This lead them to believe that adenosine triphosphate loses two phosphate groups and is

deaminised within 1 1/2 hours to inosinic acid, and then further broken down to inosine and hypoxanthine. Fiske (6), in isolating adenosine triphosphate, found that about 8% of the nucleotide was deaminised even in unhemolysed pig blood by the time the blood had reached the laboratory.

Heller and Klisiecki (10), on the other hand, using the method of Ostern and Parnas (11) for adenine nucleotide, found that in sheep blood deamination of purine nucleotide occurs only after hemolysis. They attributed the results of Mozolowski to the fact that he had worked with hemolysed blood.

This was confirmed in 1938 by Euler and Allen (12). By incubating unhemolysed rabbit blood for 15 hours at 37°C., they found that: (a) During the period of glycolysis (the first 6-9 hours) the adenosine triphosphate suffered little or no decomposition. (b) After the cessation of glycolysis (when the reactions which normally rephosphorylate adenylic acid no longer took place) there was a gradual fall of nucleotide nitrogen and a corresponding rise in nucleoside plus free purine nitrogen*. (c) The sum of nucleotide, nucleoside and free purine nitrogen* was constant throughout; so there could have been no deamination of adenine nucleotide during the period studied. Hence they concluded that the autolysis of adenine nucleotide in

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* The procedure used was that of Kerr and Blish (13).

unhemolysed rabbit blood proceeds by way of complete dephosphorylation to adenosine and not by deamination to inosinic acid.

If that is the case, all the adenine lost from the nucleotide fraction should be recovered as adenine in the nucleoside plus free purine fraction, and little or no hypoxanthine (besides the initial content previous to autolysis) should be found in any of the above-mentioned fractions. But the method of Kerr and Blish (13) which Eiler and Allen used (12) is not meant for individual purine determinations and can therefore give only indirect evidence about the purine derivatives concerned, from the nitrogen determinations. Hence we decided to test the conclusion of Eiler and Allen and the previous work just reviewed, by applying the more specific procedure of Kerr and Seraidarian (14) supplemented by the methods of Hitchings and Fiske (15, 16) and of Hitchings (17) for the separate determinations of the individual purines.

M e t h o d: Pig blood was defibrinated at the slaughter house and the control (unautolysed) specimen was measured at once into 4 vols. of ice-cold 10% trichloracetic acid. The remaining portion of the blood was either brought as such to the laboratory and incubated at 37°C. without hemolysis, or it was hemolysed at once with two volumes of water. After the desired period of incubation, the

proteins were precipitated by adding enough trichloracetic acid to make a final 1:5 dilution of blood in 8% trichloracetic acid, and kept on ice for 30 minutes before filtering (13, 9)

250 c.c. of filtrate (= 50 c.c. whole blood) were neutralized in a 300 c.c. centrifuge bottle and the nucleotides precipitated with uranium acetate as described by Kerr and Seraidarian (18). The precipitate was washed with 0.8% uranium acetate solution into a 50 c.c. centrifuge tube, centrifuged, the precipitation dissolved in 2 c.c. of 10 N H_2SO_4 and diluted with water to 15 c.c. The analysis of this nucleotide fraction was then carried out as described in (18), using the Hitchings and Fiske methods (15, 16, 17) for individual purine determinations.

The filtrate was treated as described by Kerr and Seraidarian (14) for separate determinations of nucleosides and free purines, except that the precipitation of free purines was carried out with 2.5 times the amount of $AgNO_3$ used for tissue extracts because of the larger amount of chlorides in blood.

As there was no adenine found either in the acid-silver precipitate or in the silver oxide precipitate, but only hypoxanthine, and since the copper bisulfite-purine precipitate from the nucleoside fraction was too small for analysis, in some cases, we combined the copper bisulfite-purine precipitates from both fractions and designated them as the nucleoside plus free purine fraction.

Results: The data for unhemolysed blood are presented in Table I. On the basis of this table the calculated percentage decrease in adenine nucleotide content, and the corresponding changes in hypoxanthine nucleotide, inosine, and free hypoxanthine are shown graphically in fig. 1.

These data show the following: (a) The adenine nucleotide content decreases to zero after 11 1/2 hours. (b) The hypoxanthine nucleotide content rises slightly for the first seven hours, and then gradually falls to zero. (c) No adenine at all was found either in the nucleoside or in the free purine fraction. (d) The free hypoxanthine accounts for 100% of the total purine after 14 hours. (e) The inosine content rises slightly at first, and then falls to zero. (f) At any period during the autolysis, the fall of adenylic acid content is fully compensated by corresponding changes in the content of inosinic acid, inosine, and free hypoxanthine (chiefly the latter), so that the total number of millimols of adenine and hypoxanthine derivatives remains constant throughout.

The data for hemolysed pig blood are presented in Table II. Here it was found convenient, for reasons previously mentioned, to combine the purine bisulfite precipitates from the nucleoside with those from the free purine fractions, and express them as millimols of nucleoside plus free purine. The results, however, are substantially similar to those presented for unhemolysed blood, except that the process of autolysis is generally faster than in unhemolysed

Table I: Adenine nucleotide, hypoxanthine nucleotide, inosine, and free hypoxanthine in unhemolysed blood after various periods of autolysis. Adenosine and free adenine are absent throughout.

Expt. number.	Hematocrit %	Period of autolysis. hrs.	Milli Moles per liter of whole blood.						
			Nucleotide		Nucleoside		Free purines		Sum-total
			Adenine	Hypoxanthine	Adenine	Hypoxanthine	Adenine	Hypoxanthine	
1.	?	0	0.848	0.074	0	0.03	0	0.104	1.056
		3	0.82	0.096	0	0.044	0	0.1	1.06
		9	0.61	0.156	0	0	0	0.32	1.086
		14	0	0	0	0	0	1.066	1.066
2.	47	8	0.784	0.144	0	0	0	0.46	1.388
		10	0.126	0.11	0	0	0	1.23	1.466
		11 1/2	0	0.04	0	0	0	1.36	1.4
		13	0	0.04	0	0	0	1.36	1.4

T a b l e II: Adenine nucleotide, hypoxanthine nucleotide, and inosine plus free hypoxanthine in hemolysed pig blood after various periods of autolysis. Adenosine and free adenine are absent throughout.

Expt. number.	Hematocrit %	Period of autolysis hrs.	Milli Moles per liter of whole blood.				
			Nucleotide		Nucleoside+free purine		Sum-total
			Adenine	Hypoxanthine	Adenine	Hypoxanthine	
1.	38.8	0	0.866	0.09	0	0.05	1.006
		1/2	0.846	0.096	0	0.09	1.032
2.	40	0	1.193	0.09	0	0.07	1.352
		1 1/2	1.034	0.14	0	0.23	1.404
		2 1/2	0.806	0.12	0	0.448	1.474
		3 1/2	0.676	0.116	0	0.732	1.524
3.	46.5	0	1.48	0.146	0	0.1	1.726
		4 1/2	0.776	0.204	0	0.81	1.79
		5 1/2	0.64	0.21	0	1.01	1.86
		6 1/2	0.472	0.19	0	1.3	1.962

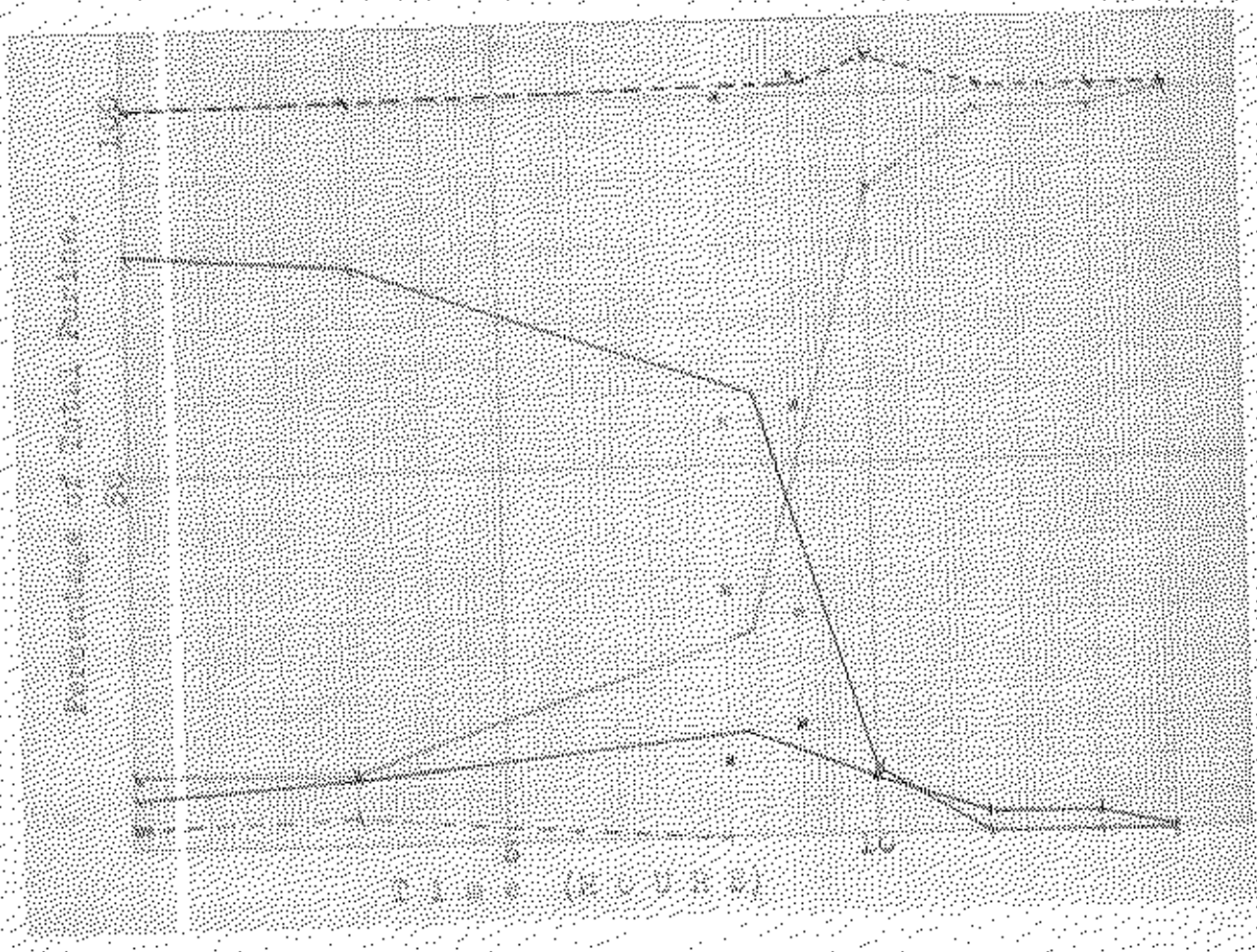

Fig. 1. The autolytic decomposition of purine nucleotides of unhemolyzed pig blood at 37°C. Red, adenine nucleotide; Black, hypoxanthine nucleotide; Dotted red, ribo; Black, hypoxanthine nucleotide; Dotted black, inosine; Green, free hypoxanthine; Dotted black, sum-total. All results are calculated as percent of the sum-total mill moles.

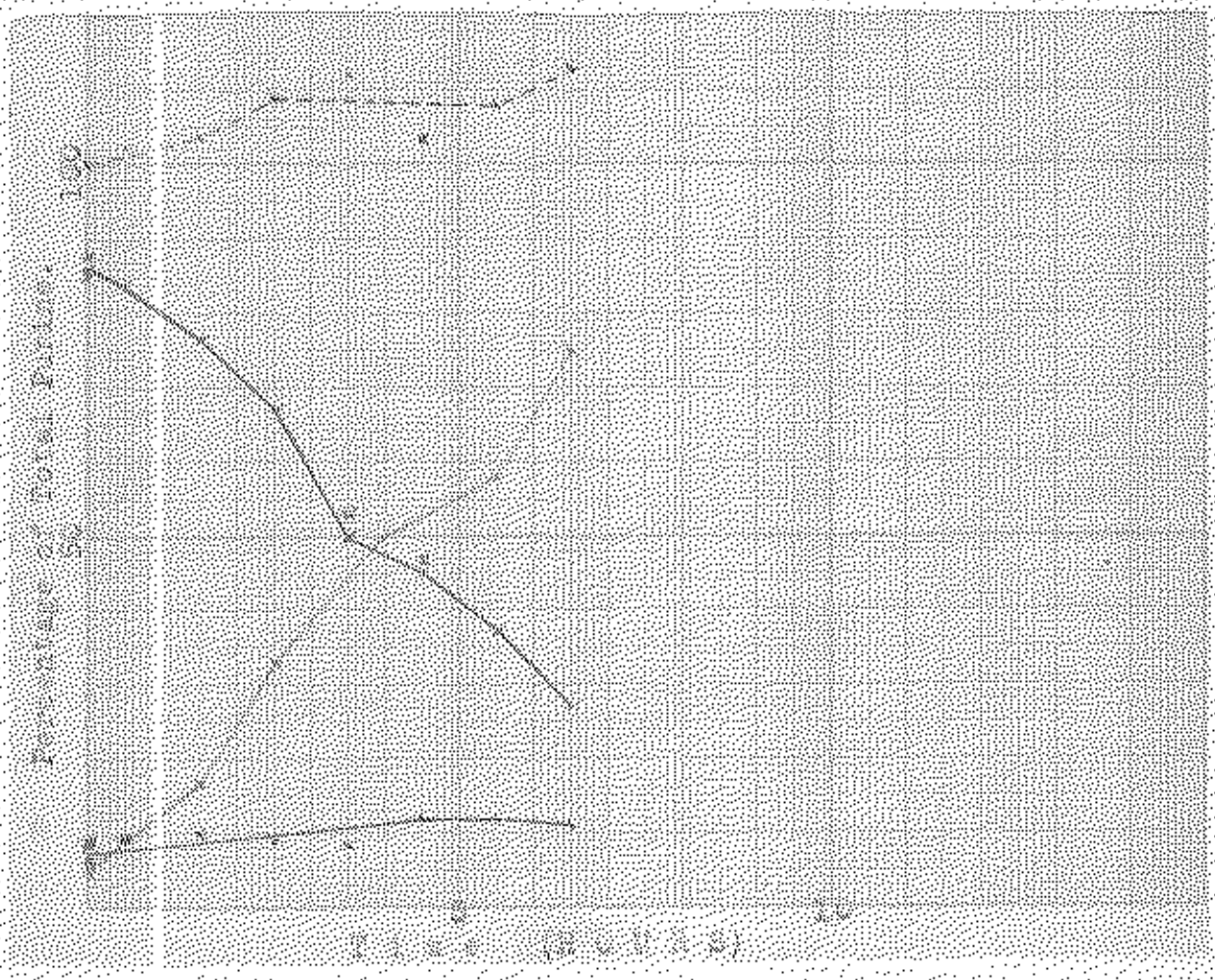

Fig. 2. The enzymic decomposition of purine nucleotides at hemolyzed pig blood at 37°. Red, adenine nucleotide; Black, hypoxanthine nucleotide; Green, inosine plus free hypoxanthine; Dotted black, sum-total. All results are calculated as percent of the sum-total still Moles.

blood. However, there is a gradual rise in the number of millimols of total adenine plus hypoxanthine derivatives. This increase reaches about 13 1/2 % of the original amount within 6 1/2 hours.

D i s c u s s i o n: As pointed out by Kiler and Allen (18), the fall in the nucleotide content is not so rapid during the first six hours in the case of unhemolysed blood. This was attributed by them to the rephosphorylation of the adenylic acid during the period of glycolysis.

As the adenine nucleotide breaks down, there is a slight rise in the inosinic acid content for the first 7 hours, after which it gradually falls to zero. This suggests that adenine nucleotide is first deaminised to hypoxanthine nucleotide,and the latter is then broken down to inosine and hypoxanthine. Besides the fact that no adenine at all was found either in the nucleoside or in the free purine fraction, while the hypoxanthine content of these fractions was constantly rising, is additional evidence that the pathway of autolysis is not through adenosine but through hydrolysis preceded by deamination. The fact that the inosine content rises slightly at first suggests that the inosinic acid (resulting from deamination) is first broken down to inosine, and then rapidly converted to free hypoxanthine. This point, however, is not to be regarded as established due to the difficulty encountered in the nucleoside free purine separation. The gradual rise in the number of millimols of total adenine plus

hypoxanthine derivatives in hemolysed blood has not been accounted for.

If allowance for the variations in hematocrit is made (20)(21), our results for the total nucleotide in the resting specimens are in good agreement with those of Kerr and Daoud (4) obtained on the basis of phosphorus determinations.

C o n c l u s i o n: Our results have established by direct individual purine determinations the previous indirect findings of Mozolowski (7), Fiske (6), and Kerr and Antaki (9) for pig blood, namely, that its adenine nucleotide is first deaminised during autolysis to hypoxanthine nucleotide, and then converted to inosine or hypoxanthine. This is found to be true for both hemolysed and unhemolysed pig blood; although different results were obtained by indirect methods for sheep and rabbit blood respectively by Heller and Klisiecki (10) and by Eiler and Allen (12). A possible explanation of the discrepancy between our results and those of Eiler and Allen is the contamination of the purine-bisulfite precipitate with nitrogenous products of autolysis in the old Kerr and Blish procedure (13) as pointed out by Peham (19), and confirmed by Kerr (unpublished data). Such a contamination would tend to counterbalance the loss of nitrogen by deamination. In our studies this source of error was avoided by direct determinations of adenine and hypoxanthine. Another explanation of the discrepancy between our results and those of Eiler and Allen may be in some difference in the course of autolysis between rabbit blood and pig blood.

S u m m a r y: On the basis of separate individual purine determinations on the nucleotide, nucleoside and free purine fractions of fresh and autolysed pig blood, we have shown that the pathway of autolysis of adenine nucleotide is through deamination to hypoxanthine nucleotide followed by hydrolysis to inosine and hypoxanthine. The same was found to be true for both hemolysed and unhemolysed pig blood.

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