

THE AMINO-ACID NITROGEN OF THE BLOOD.

PRELIMINARY EXPERIMENTS ON PROTEIN ASSIMILATION.

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Two opposing views are held concerning the manner in which the organism assimilates the amino-acids formed in the intestine from digested proteins.

Abderhalden¹ believes that in passing the intestinal wall the amino-acids are synthesized into a blood protein. This enters the circulation, from which the cells of the tissues take it, break it down again into amino-acids and from these rebuild their own characteristic proteins. The reason for this view, that the amino-acids are resynthesized into protein while passing the intestinal wall, is, that although amino-acids are abundant in the intestinal contents, attempts to demonstrate them in normal blood have met with absolute failure. Against this view the objection is valid, that it is based on negative results and that more sensitive methods than those hitherto applied might reveal amino-acids in the blood.

Buglia² offers evidence indicating that the body is capable of utilizing amino-acids which have entered the blood stream. He injected intravenously into dogs amounts of amino-acid mixtures comparable to those formed from the food, and found that, when the injection was performed slowly, no serious ill effects followed and only a small part of the amino-acid nitrogen was excreted. Folin and Denis³ injected large amounts of amino-acids directly into the small intestines of cats and noted a subsequent increase

¹ *Synthese der Zellbausteine*, 1912.

² *Zeitschr. f. Biol.*, lviii, p. 162, 1912. Buglia also gives earlier literature.

³ *This Journal*, xi, p. 87, 1912.

in the fraction of blood nitrogen left after subtracting the protein and urea. Neither the results of Buglia nor those of Folin and Denis, however, demonstrate the presence of amino-acids in the normal blood during periods of either digestive activity or rest. So long as this demonstration remains unperformed, the contention of Abderhalden cannot be refuted, that during normal digestion, with the chyme gradually passing in small portions from the stomach to the intestine and from the intestine to the blood, the latter passage is accompanied by synthesis of food amino-acids into blood protein.

It was evident that for the solution of the problem a method was necessary sufficiently delicate to ascertain decisively whether amino-acids are present in or absent from the blood, and sufficiently accurate to detect the fluctuations normally occurring in amino-acid nitrogen if it is present. We have found that the nitrous acid method for determination of amino nitrogen can be easily used for the blood and answers the above requirements. We have consequently applied it to the problem of protein assimilation.

METHOD FOR DETERMINATION OF AMINO-ACID NITROGEN IN BLOOD.

Thirty to fifty cubic centimeters of freshly drawn blood are mixed with 9 or 10 volumes of 95 per cent alcohol to precipitate the proteins. The volume of the alcohol-blood mixture must be known; but in case it is not convenient to use a graduated cylinder for the mixture, its volume can be taken as the sum of the volumes of alcohol and blood without essentially affecting the results. The alcohol and blood are thoroughly mixed, the vessel containing them is closed, and twenty-four hours are allowed for precipitation of the proteins to become complete. The solution is filtered through a dry folded filter into a measuring cylinder, without washing the precipitate. The volume of filtrate obtained is noted, and is taken for analysis as an aliquot part of the total blood-alcohol mixture. The filtrate is then concentrated to a volume of 3-5 cc. and used for determination of amino nitrogen by Van Slyke's nitrous acid method.⁴ The use of a few drops of acrylic alcohol to prevent foaming is advisable.

⁴ This *Journal*, ix, p. 185; xii, p. 275.

The precipitation with alcohol is a simple and effective means of removing the proteins. The filtrate, even when concentrated, gives no biuret test. The completeness of the removal of proteins is further evidenced by the fact that when in control tests the alcohol was driven off from the filtrate and the residue was hydrolyzed with hydrochloric acid, the amino nitrogen set free by the hydrolysis amounted to only 2 mgm. per 100 cc. of blood.

Whether the alcoholic filtrate is concentrated on the water bath or under diminished pressure appears to make little difference with the results. We have, however, as a regular thing, concentrated under diminished pressure in a double-necked flask, transferring the solution towards the end of the distillation to a small flask, from which it can be removed with a minimum amount of wash water. This method of concentration is the most rapid, and the lipoids which separate when the alcohol is driven off take the form of a fine emulsion, which does not interfere mechanically or otherwise with the amino determination. They can be removed with ether, but this is unnecessary.

For the amino determination one can either wash the concentrated filtrate into a 10 cc. graduated flask, measuring off 9.7 or 9.8 cc. of this in the burette of the amino apparatus; or one can transfer the filtrate directly from the small distilling flask into the 10 cc. burette of the amino apparatus, using several portions of 2-3 cc. of water each to clean out the flask and wash the solution completely into the deaminizing bulb. We have usually used the latter method.

The amino determination is much simpler than in the case of urine, because the amount of ammonia in the blood is negligible, and the urea content is so slight that the correction for the proportion of urea nitrogen (about 3 per cent), given off while the amino-acids are quantitatively decomposed, is scarcely sufficient to affect the significance of results if it were entirely neglected. We have, however, always determined and made this correction according to the principle utilized in the determination of amino nitrogen in the urine.⁵ The correction rests on the fact that in two to four minutes (according to the temperature) amino-acids give off 100 per cent of their nitrogen. Continuing

⁵ This *Journal*, xii, p. 275.

the reaction for an equal length of time thereafter results, when urea is present, in the evolution of a further amount of nitrogen from the urea, this amount being equal to that evolved from the *urea* while the amino-acid was being decomposed in the first period. With the small amounts of urea present in the blood it is unnecessary to make an entire extra analysis to determine the correction. The latter is ascertained as follows. For the decomposition of the amino-acids the reaction is run with constant rapid shaking for four minutes at a temperature below 20°, for three and a half minutes at 20–25° and for two to three minutes above 25°, the time being accurately measured. At the end of the reaction the nitrogen gas is purified and measured in the usual way, and then expelled from the apparatus through cock *c* (see figure 2, p. 278, preceding number of this *Journal*). The solution left in the deaminizing bulb *D* is now shaken and treated exactly as described on page 280 of the above article for determining whether the reaction is complete or not. The length of time between the end of the first reaction and that of the second should be accurately that of the first reaction. The gas formed during the second period is shaken out with permanganate and measured. This represents the urea correction and is to be deducted from the first reading. The latter, minus this correction, represents only amino-acid nitrogen, since other forms of amino nitrogen, such as fatty amines, amino-purines, amino-pyrimidines and ammonia, react slowly and would if present be corrected for with the urea. In case it is inconvenient to finish the second reaction in as short a period as the first, it may be allowed to run one or two minutes longer, the correction being calculated on the rate of urea nitrogen evolved per minute. It is essential that the solution should be shaken rapidly during the last minute to expel all the nitrogen formed. The fact that under these conditions very nearly the same amount of nitrogen is evolved in the first and second periods is indicated by the following four pairs of determinations, each of which was made with 10 cc. of 0.5 per cent urea.

	I cc.	II cc.	III cc.	IV cc.
N in first three minutes.....	2.0	2.2	2.1	2.4
N in second three minutes.....	2.2	2.2	2.2	2.6

The maximum difference, 0.2 cc., is equivalent to only 0.1 mgm. of amino nitrogen; and the corrections actually found in blood work are usually less than 2 cc.

To ascertain whether the technique above described involves losses of amino-acids, particularly whether they are precipitated with or adsorbed by the proteins, the following experiment was performed.

One hundred cc. of fresh defibrinated blood, drawn from a dog which had fasted twenty-four hours, was precipitated with 1 liter of alcohol. (Total volume = 1100 cc.) Of the filtrate, 800 cc., equivalent to 72.7 cc. of blood, were concentrated, freed from lipoids with ether and brought to 25 cc. Ten cc. portions, equivalent to 29.1 cc. of blood, were used for amino determinations.

I	cc.	II	cc.
N in first 4 minutes.....	2.95	N in first 3½ minutes.....	2.85
N in second 4 minutes.....	0.60	N in second 3½ minutes....	0.50
N from amino-acids.....	2.35		2.35

2.35 cc. of nitrogen at 26°, 754 mm., from 29.1 cc. of blood indicate 4.42 mgm. of amino-acid nitrogen per 100 cc.

To another 100 cc. of the same blood 1 gram of alanine, much more amino-acid than we have ever encountered in the blood in nature, was added. Other details of the analysis were identical with the above.

	I	II
N gas in three and one-half minutes.....	85.2 cc.	85.6 cc.
N from amino-acids and urea of blood...	2.9 cc.	2.9 cc.
N from added alanine.....	82.3 cc.	82.7 cc.
Temperature.....	26°	27°
Pressure.....	754 mm.	754 mm.
Alanine N per 100 cc. found.....	0.1554 gm.	0.1550 gm.
Alanine per 100 cc. found.....	0.9880 gm.	0.9860 gm.
Alanine per 100 cc. added.....	1.0000 gm.	1.0000 gm.

AMINO-ACID CONTENT OF BLOOD FROM NORMAL FASTING DOGS.

The animals used for the following experiments had fasted for twenty to twenty-four hours before the blood was drawn.* The results indicate that the amino-acid content varies within relatively narrow limits.

* For the samples from the carotid artery and the vena cava we thank Dr. Auer and Dr. Githens.

DOG NO.	SOURCE OF BLOOD	VOL. OF BLOOD EQUIVALENT TO FILTRATE USED	N (CORRECTED FOR UREA)	TEM- PERA- TURE	PRES- SURE	AMINO-ACID NITROGEN PER 100 CC. BLOOD
		cc.	cc.	degrees C.	mm.	mgm.
1	Femoral artery..	29.1	2.35	26	754	4.4
1	Femoral artery..	29.1	2.35	26	754	4.4
2	Femoral artery..	28.4	2.10	25	762	4.1
2	Femoral artery..	28.4	2.20	25	762	4.3
3	Vena cava.	21.5	2.10	24	760	5.4
4	Carotid artery...	59.0	5.80	25	760	5.4
5	Femoral artery..	51.0	4.20	25	760	3.7
6	Femoral artery..	35.5	3.30	25	760	5.2
7	Carotid artery...	37.5	2.20	31	758	3.1
8	Femoral artery..	40.0	3.00	30	758	4.0
8	Mesenteric vein..	40.0	2.90	30	758	3.9

Experiment I. Behavior of intravenously injected alanine.

A male dog of 14 kilos weight was catheterized and the bladder was thoroughly washed out. One cannula was placed in the right femoral vein, another in the left femoral artery. A sample of blood was drawn from the artery, and immediately afterwards the injection of 12 grams of *dl*-alanine dissolved in 400 cc. of water into the vein of the other leg was commenced. At intervals samples of blood were drawn. At the end of the experiment the bladder was again washed out and the excreted amino-acid nitrogen determined. The results are here summarized.

	TIME	TIME AFTER END OF INJECTION	FREE AMINO-ACID N PER 100 CC.	ESTIMATED ALANINE IN CIRCULATION	UNPRECIPITATED AMINO NITROGEN PER 100 CC. FREED BY HYDROLYSIS
	p. m.	minutes	mgm.	grams	mgm.
Drew blood sample					
I. Normal.....	3:15		4.2	0.00	2.1
Began injection ...	3:17				
Finished injection.	3:30				
Drew sample II. ...	3:35	5	37.2	1.47	1.8
Drew sample III. ...	3:42	12	20.8	0.64	1.5
Drew sample IV....	4:05	35	12.3	0.36	1.4
Drew sample V....	4:30	60	12.3	0.36	1.7

Total nitrogen in urine.....	0.445 gram.
Ammonia nitrogen in urine.....	0.015 gram.
Free amino-acid nitrogen in urine.....	0.236 gram.
equivalent to 1.501 grams alanine.	
Total amino-acid nitrogen in urine.....	0.233 gram.
The excreted alanine was optically inactive.	

The injected alanine disappeared from the blood with such rapidity that five minutes after the injection only 1.47 grams out of the 12 grams injected remained in circulation and in thirty-five minutes only 0.36 gram. A relatively small proportion, 1.5 grams in all, was excreted, about 10 of the 12 grams *being almost immediately taken from the circulation by the tissues*. There was no evidence of synthetic processes in the blood, for the amino nitrogen freed by hydrolysis, which might be expected to increase if intermediate products of protein synthesis were present, remained practically constant.

The total amount of alanine in circulation was calculated on the assumption that the volume of the blood in liters is one-twentieth of the weight of the body in kilograms. While this is, of course, only a rough approximation, it suffices for the purpose of the experiment.

The details of the analyses follow:

Experiment I. Determination of free amino-acid nitrogen. Temperature 25°; pressure 762 mm.

SAMPLE	VOL. OF SAMPLE	VOL. OF BLOOD EQUIVALENT TO FILTRATE USED	N GAS EVOLVED IN 4 MIN.	N GAS IN FOLLOWING 4 MIN.	N GAS (CORR.) FROM AMINO-ACIDS	AMINO-ACID N PER 100 CC. BLOOD
	cc.	cc.	cc.	cc.	cc.	mgm.
I	{ 87	28.4	2.3	0.2	2.1	4.1
		28.4	2.4	0.2	2.2	4.3
II	100	31.6	22.1	0.9	21.2	37.2
III	{ 76	24.0	9.3	0.4	8.9	20.7
		24.0	9.4	0.4	9.0	20.9
IV	{ 91	29.2	6.8	0.4	6.4	12.1
		29.2	7.0	0.4	6.6	12.5
V	{ 75	24.8	5.9	0.4	5.5	12.3
		24.8	5.8	0.3	5.5	12.3

For the above determinations each filtrate was brought to 25 cc., duplicate determinations being made on 10 cc. portions of this.

Of the unused parts, 4 cc. portions were used for hydrolysis. They were mixed with equal volumes of concentrated hydrochloric acid and

heated at 100° for twenty-four hours. The acid was evaporated off as completely as possible, and the ammonia removed by vacuum distillation with calcium hydrate. The latter was then dissolved with acetic acid and the sample used for amino determination.

Experiment I. Determinations of amino nitrogen after hydrolysis. Temperature 27°; pressure 764 mm.

SAMPLE	VOL. OF BLOOD EQUIVALENT TO SAMPLE USED	N GAS IN 3 MIN.	N GAS IN FOLLOWING 3 MIN.	N GAS FROM AMINO- ACIDS	AMINO N PER 100 CC. BLOOD	FREE AMINO N	AMINO N FREED BY HYDROL- YSIS.
	cc.	cc.	cc.	cc.	mgm.	mgm.	mgm.
I	11.4	1.50	0.10	1.40	6.3	4.2	2.1
II	12.6	9.10	0.20	8.90	39.0	37.2	1.8
III	9.6	4.50	0.20	4.30	22.3	20.8	1.5
IV	11.7	3.05	0.15	2.90	13.7	12.3	1.4
V	9.9	2.65	0.15	2.50	14.0	12.3	1.7

The urine voided during the hour after the beginning of the injection was made up to 100 cc. Portions of 2 cc. used for Kjeldahl determination required 6.40 and 6.30 cc. of $\frac{N}{10}$ acid, the average indicating 0.445 gram of total nitrogen.

Fifty cubic centimeters were used for determination of ammonia and free amino nitrogen, as described by Levene and Van Slyke.⁷ The ammonia neutralized 5.2 cc. of $\frac{N}{10}$ HCl, indicating 0.0146 gram of ammonia N in the entire urine. The ammonia-free urine was brought back to 50 cc. volume and 10 cc. portions used for determination of free amino-acid nitrogen.

I	cc.	II	cc.
N in first 3 minutes	45.0	N in first 4 minutes	45.2
N in second 3 minutes	1.9	N in second 4 minutes	2.2
Amino-acid N	43.1	Amino-acid N	43.0

These determinations were made at 27°, 758 mm. The average, 43.05 cc., indicates 0.236 gram of free amino-acid nitrogen in the total urine. This includes all the amino nitrogen, comparison with the following determination showing that no measurable amounts were conjugated.

For determination of the total amino nitrogen 40 cc. of urine were used, the sample being difuted to 50 cc. after removal of urea and ammonia. Ten cc. portions were used for amino determinations. They gave 33.5 and 33.4 cc. of nitrogen at 25°, 762 mm. indicating 0.233 gram of total amino nitrogen in the urine.

In order to determine whether the organism had destroyed or retained the natural component of the amino-acid (*d*-alanine)

⁷ This *Journal*, xii, p. 275.

and excreted the other (*l*-alanine), as found by Wohlgemuth⁸ to be the case when certain *dl*-amino-acids were given *per os*, 25 cc. of ammonia-free urine, containing 0.375 gram of alanine, were concentrated, and acidified with 0.5 cc. of concentrated hydrochloric acid. The weight of the solution was 4.273 grams, the concentration of alanine hydrochloride 12.3 per cent. This, if all *l*-alanine, should have given a rotation of -1.2° in a 1 dm. tube. The observed rotation was only -0.04° . The *dl*-alanine was, therefore, excreted practically unchanged and the alanine retained was equal parts dextro and levo.

Experiment II. Absorption of alanine from the small intestine.

The animal used was a dog of 10 kilos weight which had fasted for twenty-four hours. Cannulas were placed in the left femoral artery and the mesenteric vein. Fifty cc. samples of blood were drawn from each and diluted to 500 cc. with alcohol. A loop of the small intestine was then ligated at both ends and 15 grams of alanine, dissolved in 100 cc. of water, injected into it. After forty minutes, samples of blood were again drawn. The loop was then washed out, its contents diluted to 500 cc., and 10 cc. used to determine the unabsorbed alanine nitrogen. The determination yielded 26 cc. of nitrogen at 26° , 760 mm. From this:

Total alanine N injected.....	grams 2.461
Alanine N unabsorbed.....	0.718
Alanine N absorbed.....	1.743

The blood analyses gave the following results. The temperature was 30° , barometer 758 mm.

SOURCE	VOL. OF BLOOD EQUIVALENT TO FILTRATE USED	N GAS EVOLVED IN 2.5 MIN.	N GAS IN FOLLOWING 2.5 MIN.	N GAS FROM AMINO- ACIDS	AMINO-ACID N PER 100 CC. BLOOD
	cc.	cc.	cc.	cc.	mgm.
Femoral artery be- fore injection*...	40	3.7	0.7	3.0	4.0
Mesenteric vein be- fore injection....	40	3.7	0.8	2.9	3.9
Mesenteric vein after injection....	43	5.6	0.6	5.0	6.3

* The determination on the blood from the femoral artery after the injection was lost.

⁸ *Ber. d. deutsch. chem. Gesellsch.*, xxxviii, p. 2064, 1904.

The amount of amino-acid nitrogen in the mesenteric blood increased by 60 per cent as the result of the injection of alanine into the intestine. This, however, demonstrates only the possibility that amino-acids can pass the intestine. The sudden flooding of the intestine with a solution of one or more amino-acids is so entirely different from the gradual entrance of partially digested proteins from the stomach which occurs in normal digestion, that the results can not be utilized to explain the normal process of protein assimilation. The same restriction applies to the results of Folín and Denis,⁹ who flooded the intestines of cats with solutions containing unusual amounts of amino-acids and observed a subsequent rise in the fraction of blood nitrogen left after subtracting the urea and protein.

THE RISE OF THE AMINO-ACID CONTENT OF THE BLOOD DURING DIGESTION.

Experiment III.

We have, therefore, performed the following experiment. Samples of 50 cc. of blood were drawn from the right femoral arteries of two dogs, of about 15 kilos weight each, which had fasted for

Experiment III. Effect of digestion on the amino-acid content of the blood.

SOURCE OF BLOOD	VOL. OF BLOOD EQUIVALENT TO FILTRATE USED	N GAS IN 3.5 MIN.	N GAS IN FOLLOWING 3.5 MIN.	N GAS FROM AMINO- ACIDS	AMINO-ACID N PER 100 CC. BLOOD
<i>Dog A</i>	cc.	cc.	cc.	cc.	mgm.
Right femoral artery before feeding....	51.0	4.6	0.4	4.2	3.7
Mesenteric vein after feeding.	38.3	8.3	1.5	6.8	9.5
Left femoral artery after feeding.....	37.7	7.2	1.2	6.0	8.6
<i>Dog B</i>					
Right femoral artery before feeding....	35.5	4.0	0.7	3.3	5.2
Mesenteric vein after feeding.....	38.5	9.4	2.3	7.4	10.2
Left femoral artery after feeding.	37.3	9.1	2.2	6.9	9.9

⁹ This *Journal*, xi, p. 87, 1912.

twenty-four hours. The dogs were in normal condition the next day, and each then devoured a kilo of fresh beef. Five hours after the meal the animals were etherized and samples of blood drawn from both the left femoral artery and the mesenteric vein.

The amino-acid content is about doubled during digestion. It will be noticed that the correction for urea also increases. This is to be expected from the results of Folin and Denis, who noted a marked rise in the urea content of the blood during digestion of protein.

SUMMARY OF RESULTS.

The gasometric method for direct determination of amino-acid nitrogen is easily applicable to blood from which the proteins have been precipitated by alcohol. Duplicate results usually agree within 0.2 mgm. of amino-acid nitrogen per 100 cc. of blood.

The blood of the dog normally contains amino-acid nitrogen. The amount, in animals which have been fasting for twenty to twenty-four hours, is 3 to 5 mgms. per 100 cc. of blood.

Twelve grams of alanine, injected during 13 minutes into the vein of a dog, were so rapidly removed from the blood stream that five minutes after the injection only 1.5 grams were left in the blood, and after 35 minutes but 0.4 gram. Only 1.5 grams were excreted in the urine, the greater part of the injected amino-acid being evidently taken up by some of the tissues.

Absorption of 10 grams of alanine from the small intestine increased the amino-acid nitrogen of the mesenteric blood from 3.9 to 6.3 mgm. per 100 cc.

During normal digestion of meat the amino-acid content of the blood undergoes a marked increase compared with its value before feeding. It was doubled in the case of one dog and somewhat more than doubled in that of another. The increase affected the blood from the femoral artery almost as much as that directly from the mesenteric vein.

CONCLUSIONS.

With the finding of amino-acid nitrogen in the normal blood the hypothesis, that the amino-acids formed in digestion are synthesized into blood protein while passing the intestinal wall,

becomes superfluous. The increase in amino-acid nitrogen of the blood, noted during digestion of protein, is, furthermore, positive evidence that amino-acids as such do normally pass the intestinal wall and enter directly into the blood current. The fact that the amino-acid content decreases but little during passage of the blood from the mesenteric vein out to the femoral artery indicates that the amino-acids are not held back or destroyed by the liver before reaching the other tissues. On the contrary, it seems that the amino-acids absorbed from the intestine circulate through the entire organism and are offered directly to the body cells in general. The fact that the amount of amino-acids normally present in the circulation is small, is accounted for by the rapidity with which the tissues take up amino-acids from the blood as soon as they become unusually abundant therein. This is illustrated by the disappearance of intravenously injected alanine from the blood stream.

The experiments here reported are preliminary to a more complete investigation of the problem of protein assimilation and of the effect of different physiological and pathological conditions upon the amino-acids of the blood.