DETERMINATION OF NUCLEOTIDES IN BIOLOGICAL MATERIALS (II)[1]

Determination of ATP in Rat Blood[2]

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The separation of ATP (adenosine triphosphate), ADP (adenosine diphosphate), and AMP (adenosine monophosphate) from biological materials, especially from rat blood, by thin layer chromatography with DEAE-cellulose ion-exchanger, and their determination in micro amounts (0.1～5 mcg/ml) as established. As aspects for application to biological materials, all considerable factors were dissolved. A standard calibration curve of pure ATP and additional experiments to homogenate solution were obtained using the thin layer chromatography.

Pure ATP value obeyed the BEER'S law up to the concentration of 25 mcg/ml.

ATP group in the blood was determined as follows: Two grams of rat blood containing 3.8 % sodium citrate with 4 ml of 6 % perchloric acid solution were homogenized at 0°C for 2 minutes.

The protein was precipitated by the acid and separated by centrifuging.

The resultant supernatant was adjusted to pH 5.5 with 5 mole potassium carbonate. Whole volume of this solution was brought to 6 ml.

Five portions of this solution were spotted in DEAE-cellulose by a micropipet, and developed as usual with 0.04 normal HCl. After dried with air, ATP groups were extracted with 1 ml of 0.1 normal HCl from DEAE-cellulose by shaking for 1 minute.

The extract was centrifuged for 5 minutes to separate the layer. This elution procedure was repeated enough in three portions. The extinction of the extract solution at 260 mμ was read against blank, which was treated with non-absorptive portions in the same way as mentioned above. From these results, ATP values in rat blood were found to be 150～200 mcg/ml whole blood. Nine times of these values were proportional with ATP amounts in rat liver. The method was much more rapid and direct than the enzymatic method containing hexokinase groups.

The micro-determination method of nucleotides in biological materials has been widely required in the field of biological and clinical chemistry. The determination method of nucleotides has been investigated and improved, but in spite of the fact that good results are obtained in the fundamental study, some difficult questions occur in the applied procedure to the biological materials. The experiments in the separation of a small amount of nucleotide and nucleoside are done using RANDERATH's thin-layer-chromatography[3] or HORVATH's Pellicular ion-exchangers column[4]. However, the above mentioned methods leave room for improvement.

The authors[5] studied the separation of ATP in drugs, and then also made an experiment on the simultaneous determination of ATP, ADP and AMP in liver[1]. The method of the ashen inorganic phosphine of RAPPORT[6], the fluoresence method of STREHLER[7], ($^{14}C_{10}$) ATP method and enzymatic analysis[9] are used as the determination methods of ATP in blood. Since their methods have had a difficulty to obtain a simple and speedy procedure and a correct value, authors established the method to separate ATP in the thin layer by the ultraviolet rays and to determine ATP itself in the liver homogenate. And they applied this method to rat blood. The thin layer chromatography to concentrate a small amount of ATP can get the results speedily and can be applied to the determination method, because the amount of ATP in blood is less than that in liver.

Experimental Materials

1) Experimental materials and reagents

* ATP-2 Na (Kowa Chemical Co., Ltd.)
* Hydrochloric acid (special class), perchloric

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acid (60%) (special class) and potassium carbonate (first class) (Wako Junyaku)

* Wistar strain rats (male) (180~200 g)
* DEAE Cellulose ion exchanger (TLC) (Serva Co., Ltd.)

2) Preparatory procedure of thin layer plate

Distilled water (120 ml) was added to DEAE cellulose (20 g) and this solution was stirred in a fast electric mixer for 30 minutes. And this substance, the bubbles which had been removed by the vacuum pump, was set in the thin layer plate of 0.5 mm by the applicator and was kept at the room temperature for one night and activated at 40°C for one hour, and then kept in the dessicator.

(First, DEAE cellulose was washed with diluted acidity and diluted alkaline solutions. But, by this method, it was found that the ability of absorption decreased because of the isolation of diethylamino groups from the combined cellulose. So the cellulose ion-exchangers by Serva Co., Ltd. were used.) As the products by the same company have a difference in the ability of absorption, the calibration curve should be prepared in each lot of the cellulose.

3) Instruments and apparatuses

* Potter-Elvehjem glass homogenizer (Teflon Pestle)
* Centrifuger (Kubota Co., Ltd. K-80)
* pH meter (Hitachi Horiba, M-5)
* Spectrophotometer (Shimazu, QB-50, QV-50)
* Ultra violet radiator (Manasuru light, Nikko Sekiei, LS-DI)

4) Reagents for the enzymatic method

* Triethanolamine buffer solution (0.05 M, pH 7.5 ~7.6)
* NADP (7×10^{-3} M)
* Magnesium chloride (0.1 M)
* Glucose (0.5 M)
* G-6-P dehydrogenase (200 μg protein/ml)
* Hexokinase (10~15 mg protein/ml)
* Perchloride acid (6 %)
* Potassium carbonate (5 M)

Procedure and Result

1) Calibration curve

Standard solution of ATP-2 Na is prepared as the following ; Standarized solutions of ATP-2 Na containing 2, 6, 8, 10, 13, 20, 22, and 25 μg per 0.01 ml, respectively are prepared.

A 0.01 ml portion of the solution is spotted on DEAE-cellulose by a micropipette, and developed as usual with 0.04 M HCl for about 13 cm. After drying in air, ATP, which is found as the absorption parts of darkness, is detected by ultraviolet rays of 2537 Å at 0.1 of Rf value and then scraped up by microspatula. It is eluted 3 times with 3 ml of 0.1 N HCl for 2, 1 and 1 minute respectively. After the centrifuging, a collected supernatant is made up to 3 ml with 0.1 N HCl.

The absorption is then measured at 260 mμ against a blank run on the portion without ultraviolet absorption of 2537 Å. The blank is measured at 260 mμ against a control cuvette containing 4.0 ml of 0.1 N HCl, its absorption being 0.021~0.022 at 260 mμ. As Fig. 1 shown the calibration curve is obeyed the BEER's law to 25 μg/ml of ATP-2 Na.

2) Preparation of rat blood sample

Animals are sacrificed by hitting rat in the head and blood is taken from veins without statis in a test tube containing 0.5 ml of 3.8% sodium citrate.

Two grams of the sample are immediately homogenized and deproteinized in POTTER-ELVEHJEM glass homogenizer with TEFLON pestle in 4 ml of ice-cold 6 % perchloric acid for 10 minutes and centrifuged for 10 minutes at 4000 r.p.m., yielding supernatant. The resultant supernatant is adjusted to pH 5.5 with 5 M K_2CO_3 solution, while efficient stirring. The sediment of $KClO_4$ is removed by centrifuging, and the solution is made up to 6 ml with distillated water. The ATP level in blood of rat was shown in Table 1.

Fig. 1. Calibration curve of ATP and blood+ATP
mcg/g=absorbance×51.5×200
○—○ ATP ▲—▲ blood+ATP

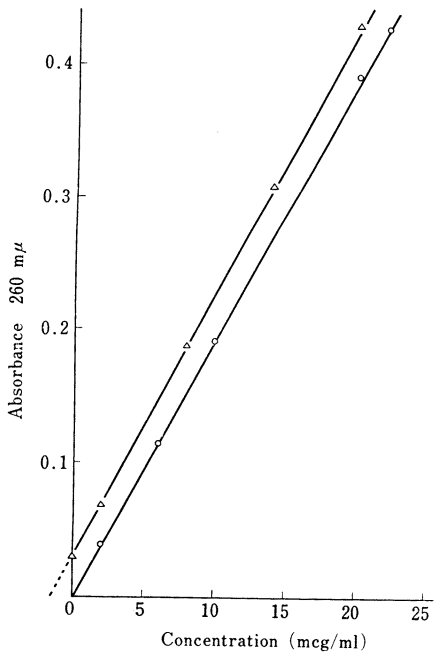


Table 1 ATP in rat blood (mcg/g)

Sample No.	ATP	ADP	AMP	ATP*
1	105	270	275	270
2	115	170	455	370
3	125	232	432	495
4	130	170	235	220
5	140	175	125	315
6	160	232	264	400
7	170	220	250	310
8	184	240	288	470
9	195	320	435	360
10	215	110	180	—
11	216	240	512	440
12	230	130	155	—
13	270	105	230	—
14	285	230	305	385
15	290	115	70	—
16	325	85	115	330
17	330	335	230	110
18	395	45	105	—
19	370	120	240	—
20	415	190	440	—
21	450	205	340	—

* Enzymatic method — : not measured

Table 2 Effect of addition of 1000 mcg/ml ATP-2 Na on ATP in blood (mcg/g)

Sample No.	ATP		ADP		AMP		ATP*	
	C	A	C	A	C	A	C	A
1	300	850	—	225	—	85	310	1020
2	330	180	335	130	230	380	110	210
3	80	485	185	200	175	155	300	955
4	140	525	175	135	125	155	315	845
5	90	300	0	15	30	110	—	—
6	90	215	0	0	30	80	—	—
7	220	625	220	210	220	250	—	—
8	220	245	220	60	220	160	—	—

* : Enzymatic method
C : Control
A : Added
— : not measured

3) Addition of ATP into the blood

To make the sample preparation, each one ml of ATP-2 Na solution, which corresponds to a concentration of ATP in the standard calibration curve, is added to 4 ml of the deproteinized supernatant (pH 5.5) of rat blood (2 g). And then the total volume is diluted to 5 ml with distillated water.

A 0.01 ml portion of the solution is spotted 5 to 10 times repeatedly on the thin layer, After drying in air, chromatoplate is developed with 0.04 N HCl for about 13 cm.

Table 3 Effect of $HClO_4$ quantity for deproteinization of blood (mcg/g)

Sample No.	$HClO_4$(ml)	ATP	ADP	AMP	ATP*
1	2	227	130	115	370
	4	290	0	225	400
2	2	205	58	130	—
	4	370	120	240	—
3	2	108	81	36	350
	4	125	0	65	—
4	2	79	27	29	—
	4	160	125	90	—
5	2	135	61	266	—
	4	290	115	70	—
6	2	117	—	—	—
	4	395	45	105	—
7	2	115	40	50	350
	4	215	110	180	380
8	2	93	40	60	—
	4	230	130	155	—

* : Enzymatic method
— : not measured

Calibration curve of ATP plus blood is followed by the methods of standard calibration curve (Fig. 1). One ml of 1,000 μg/ml ATP-2 Na is added to 2 g of the rat blood and then deproteinized. Five ml (pH 5.5) of the deproteinized solution are treated by the above mentioned method and the following results are obtained (Table 2). As the control, same rat blood is used.

On addition of a large amount of ATP to the rat blood, the enzyme reaction in rat blood proceeded to decrease the added ATP. Therefore, the accumulation of ATP into the rat blood was not quantitative. The portions of ADP and AMP, however, are unchangeable.

4) Examination for the condition of assay

i) Effect of $HClO_4$ quantity for deproteinization in blood

The deproteinization should be carried out completely to remove a decomposing enzyme of ATP (a certain ATPase) in blood. Therefore, effect of $HClO_4$ quantity and concentration in ATP in blood are examined. Two grams of the sample blood are homogenized and deproteinized in 2 ml and 4 ml of ice-cold 6% perchloric acid, respectively. The resultant ATP values are shown in Table 3.

According to Table 3, the extraction rate of ATP in blood, which is deproteinized with 2 ml of 6% $HClO_4$, was very small including also ADP and AMP. The value of each nucleotides in bloood extracted

Fig. 2 Effect of extraction times for deproteinization on ATP in rat blood.

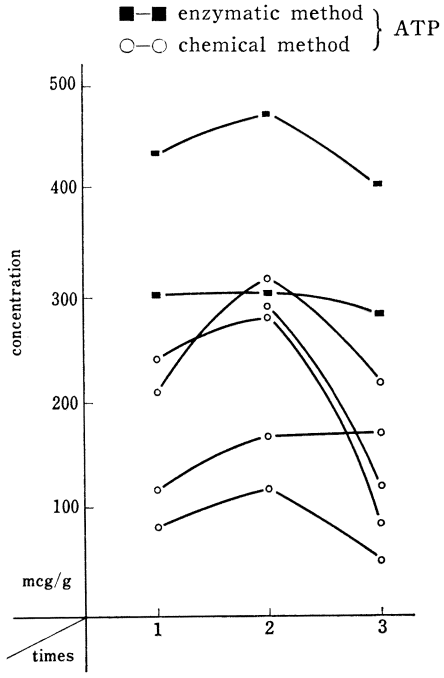


Table 4 Effect of standing time on ATP in blood

Sample No.	time	ATP	ADP	AMP	ATP *
1	0	285	230	305	—
	30	490	470	515	—
2	0	265	275	—	385
	30	140	125	—	340
3	0	460	255	130	230
	30	370	190	270	260
4	0	90	0	30	—
	30	100	0	100	—

* : Enzymatic method
— : not measured

increased to the volume of perchloric acid. However, the detection of a nucleotide was difficult, since the sample solution was diluted by more times volume of perchloric acid. An optimal concentration of perchloric acid was 6% solution. From the resultant data, a photometric measurement of the nucleotides in blood was interfered by a pigment substance, *i.e.* blood dye, which is isolated using 15% and 30 % perchloric acid, respectively.

ii) Effect of the extraction times for deproteinization on ATP in rat blood

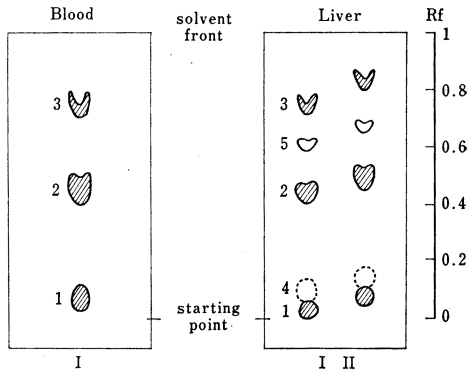
The effect of extraction times for deproteinization in ATP in rat blood, was examined using 2 ml of 6% perchloric acid.

This procedure gave satisfactory results by 2 times extraction.

Table 5 Comparison of ATP in blood with ATP in liver (mcg/g)

Sample No.	ATP		ADP		AMP	
	Liver	Blood	Liver	Blood	Liver	Blood
1	1550	300	850	—	850	—
2	1900	330	3600	335	5650	230
3	2700	80	1800	185	3300	175
4	1600	140	2350	175	3300	125
5	2750	105	1300	270	3400	275
6	1650	125	2900	140	2550	340
7	550	245	1250	315	2350	285
8	4800	290	2300	0	4000	225
9	1900	370	2650	120	3400	240
10	2400	108	1750	81	2600	36
11	1650	79	350	27	1600	29
12	2900	115	900	40	2150	50
13	2250	93	1500	40	2900	60
14	1250	325	850	85	1500	115
15	1800	220	1900	100	2800	165
16	1050	285	2250	230	4250	305
17	2950	460	1500	255	2300	130

Fig. 3 Separation of ATP, ADP and AMP in rat's blood and liver DEAE-cellulose (TLC), thickness : 0.5 mm, I : 0.02 N HCl, II : 0.04 N HCl
1 : ATP, 2 : ADP, 3 : AMP, 4 : FMN, 5 : pyridoxal



iii) Examination for the subfraction of blood

In the fraction of serum, the spots of ATP are not detected and only the spots of ADP and AMP are detected on the plate. The value of determined ADP and AMP was very small, less than 10~20 μg/g. ATP is only detected in the blood cell.

iv) Effect of the standing times on ATP in blood

In the collected blood of rat immediatly after killing *i.e.* 0 minute, and the incubated blood for

30 minutes at 30°C, ATP value in the deproteinized blood was compared.

Those results were shown in Table 4.

5) The comparison of ATP in blood with that in liver

When animals are sacrificed by hitting in the head, blood and liver are taken in the simultaneous time. Rat liver is washed up to remove blood with ice-cold saline. Each determined value at the time is shown in Table 5. According to Table 5, ATP values in blood are significantly breadth among the each determined amounts. However, ATP value in blood is corresponded to about first-nine of that of liver in the time.

Thin layer chromatogram of nucleotides in blood and liver are shown in Fig. 3. In the chromatogram of liver, flavin mononucleotide (FMN) and pyridoxal are detected and that of blood is not detected.

Discussion

The qualitative separation and quantitative extraction of ATP were made from biological materials, especially from rat blood, by thin layer chromatography with DEAE-cullulose ion-exchanger, and their fundamental determination as established.

In the present paper, the determinations of ATP in rat blood were examined to elucidate the mechanism of rat liver function. It was shown to estimate the fate of ATP in biological body, *i.e.* the illustration of energic metabolism. In the establishment of determined method of ATP, we could compare between the concentration of ATP in blood and that of liver and estimated the effect of energic metabolism of liver function ; the fate of ATP in rat liver.

Since ATP amount in blood was very small, ATP was not detected by ultra-violet rays from only 0.01 ml portion of the solution. Therefore, the solution was spotted 5 times repeatedly on the thin layer chromatography and was determined.

The spot of ATP in blood on the thin layer chromatography was widely compared with that of pure ATP, but one could observe the absorbed spots of the clear darkness violet in the layer. In already published reports by various researchers as a general determination of the substances in biological materials, metabolites in biological body are extracted from body fluid and determined under the condition, which is stable in chemical and physical properties. Besides, one had been estimated the amount or the form of original material from the final degradated compounds.

One had been only done the experiments *in vitro*, using the pure substance and it was impossible to show that assay of biological homogenate was difficult, as the applied works.

It was encountered with a difficult operations to determine freed ATP, ADP and AMP, which is presented in enzyme system and extracted a unstable substance from biological organs.

Therefore, the applied determination methods for the biological material should be obtained parallel lines with standard calibration curve for a curve which was obtained adding pure substance into the homogenate, furthermore, had to a linearly curve.

The preparation method for sample was easy and very firstly determined for the purpose substance, as the mentioned methods were given the very importance factor for assay of variable material in biological body.

In order to the resultant examination for the condition of ATP determination in blood homogenate was established in this paper.

Those results were very widely contributing the clinical examination or the investigation of biochemical mechanism.

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