CHEMICAL DETERMINATION OF NUCLEOTIDES IN BIOLOGICAL MATERIALS (V)

The Distribution of ATP in Centrifuged Fractions of Liver Homogenate

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Introduction

Author has studied the determination of ATP in biological bodies, especially in rat liver, established a new determination[1)2)] method, and applied it in the field of biological and clinical chemistry.[3)4)]

Generally, the procedure to separate ATP from other similar substances and to determine ATP itself in the biological materials was difficult though very important and significant, since there is a small amount of ATP in biological body.

In the biological homogenates, the determination method of nucleotides in the subcellular fractions of brain tissue has been investigated and improved[5)], however, the investigation on the method to determine directly ATP itself has not been detailed to date. Author established the method to obtain the distribution value of ATP itself in subcellular fractions of rat liver tissue by centrifuge and to presume the role of ATP in liver function. In this paper the application of this method and obtained results will be reported.

Materials

1. Reagent : DEAE-cellulose ion exchanger T.L.C. (Serva Co., Ltd.)

2. Liver homogenate : Livers were extirpated from Wistar-strain male rats weighing 120~150 g and were washed with cooled physiological salt solution to remove all blood. Liver was cut with scissors into fractions. The fractions were smashed in Potter-Elvehjem glass homogenizer with Teflon pestle and suspended in physiological salt solution at the ratio of 200 mg/ml.

Method and Result

1) Standard calibration curve

Standard aqueous solutions of ATP-2Na pure substance were prepared in concentrations of 3, 5, 9, 10, 15, 30 and 60 mcg per 0.01 ml by micropippete, respectively.

Each solution (0.01 ml) is spotted on DEAE-cellulose T.L.C. plate. After developed to 13 cm long with 0.04 N HCl, they were dried with air and their absorbance was measured under U. V. at 2537 Å.

A part of the T.L.C. plate having 0.1 of Rf value was scraped up ; it was extracted in 1 ml of 0.1 N hydrochloric acid at intervals of 2, 1 and 1 minute respectively ; after centrifuge, its supernatant was collected up to 3 ml in total ; the supernatant was put into 10 mm cell and its absorbance at 260 mμ was measured by photometric method.

2) Preparation of liver homogenate

Animals were sacrificed by hitting in the head.

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

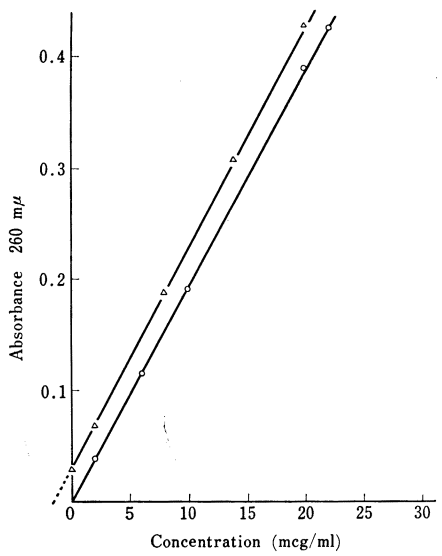


Fig. 2 Separation of fractions of rat liver homgenate

P_1 : nuclei, cell debris

P_2 : mitochondria

S_2 : microsomes

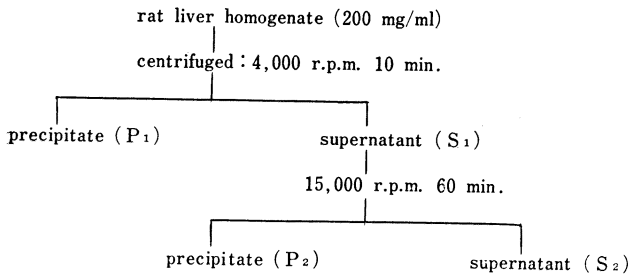
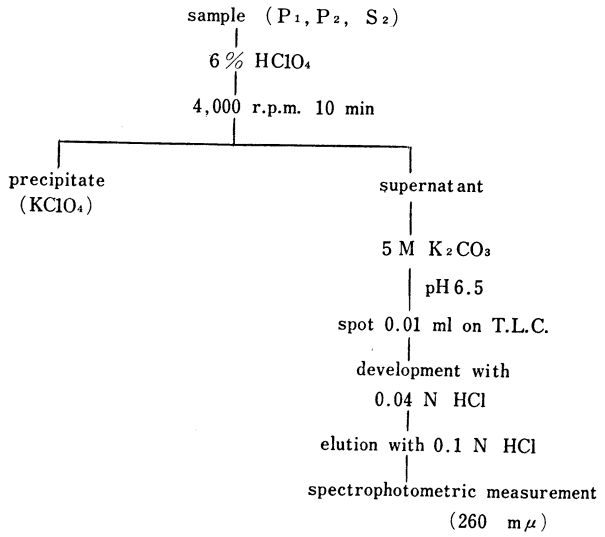


Fig. 3 Determination and separation of ATP in samples



Being extirpated from the sacrificed rats, the livers were washed up with ice-cold saline to remove blood. Fractions prepared by cutting the liver with scissors were smashed by Potter-Elvehjem glass homogenizer with Teflon pestle. The homogenate was suspended in physiological salt solution at the ratio of 200 mg/ml.

The suspension was centrifuged for 10 minutes at 4,000 r.p.m. to separate the layers into the precipitate P_2 and supernatant S_2. These fractions samples were served for the determination of ATP which is summarized in Fig. 2.

After weight determination of each fraction, three times of the volume of 6% perchloric acid are added to remove the organic components ; after ATP was extracted, the solution was centrifuged at 4,000 r.p.m. for 10 minutes ; supernatant was adjusted to pH 6.5 with 5 M potassium carbonate and was served as sample solution after removal of white precipitate of potassium perchloric acid.

Sample solution was spotted five times at a certain point of DEAE-cellulose T.L.C. by a micropippet and developed as usual with 0.04 NHCl to 13 cm long.

The value was measured on standard calibration curve which was summarized in Fig. 3.

3) Separation and identification of nucleotides in each fraction of rat liver.

Qualitative properties and separated condition of P_1, P_2 and S_2 of liver homogenate in the thin layer plate of each sample are shown in Fig. 4.

ATP, ADP, AMP and bases become an absorptive ray of dark violet under U. V. rays and HMN given an emission of yellow in P_1 and P_2 0.85 and 0.25 Rf value, respectively.

It was observed that the separation of each spots is complete. As for S_2, only AMP and its bases were detected as its chromatogram.

4) ATP value in each centrifuged fraction of liver homogenate.

Liver homogenate was supplied from 3～4 rats/group and its sample solution was used in one experiment.

The measured data which was obtained by the above mentioned procedure, as shown in Table 1.

ATP value in liver homogenate fractions of mitochondria was highest indicating 230 mcg/g. ADP and AMP values in mitochondria were higher than those of the nucleous fractions of P_1, while only AMP was observed on microsome layer of S_1.

5) Change of ATP value in rat liver according to lapse of time.

After incubated for 10～30 minutes at 30℃, the rat liver homogenate was immediately deprotenized and ATP value *in vitro* was determined in each time lapse. Typical examples are shown in Figs. 5 and 6.

As results, it seemed that the change of ATP value in liver was caused by the incubation of liver homogenate. ATP values became either maximal

Fig. 4 Separation and identification of ATP, ADP and AMP in each fraction of rat liver.
DEAE-cellulose (T.L.C.), Thickness : 0.5 mm, P_1 : nuclei, cell debris, P_2 : mitochondria, S_2 : microsome, Solvent : 0.04 N HCl

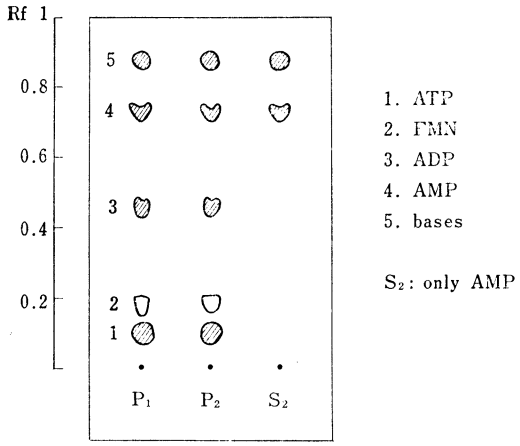


Table 1 ATP value in each centrifuged fraction of liver homogenate

	P_1	P_2	S_2
ATP	202	230	—
ADP	300	430	—
AMP	620	670	300

— : not detected mcg/g liver

or minimal 10 minutes after incubation in almost equal ratio. It was found that the metabolic function of rat liver was operated for 30 minutes after extraction of the liver. These results could be well compared with those reported previously.[3)4)]

Discussion

The author established the methodology for separating ATP on the thin layer by the ultraviolet rays and determining ATP itself in the biological materials, and applied this method to ATP determination in subcellular fractions of liver homogenate.

There was rather a large amount of ATP in fraction of mitochondria and also a large amount of nucleous of first sediments. However, total ATP value in subcellular fraction of liver tissue was smaller than that of whole liver where ATP could be obtained directly by the treatment with perchloric acid.

As previously reported, the fact that oxidative phosphorylation with ATP synthesis is activated

Fig. 5 Effect on ATP value in rat liver by the passing time. This figure shows 1 case of typical example.
●—● ATP ▲—▲ ADP ■—■ AMP
○···○ enzym. method (ATP)

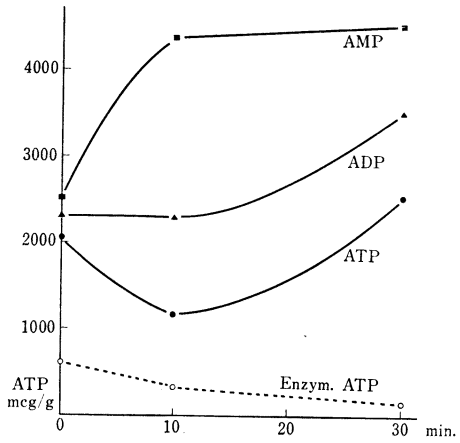
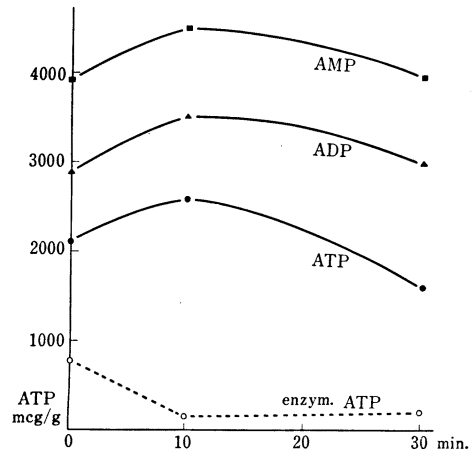


Fig. 6 Effect on ATP value in rat liver by the passing time. This figure shows 1 case of typical example.
●—● ATP ▲—▲ ADP ■—■ AMP
○···○ enzym. method (ATP)



can be presumed and supported by determined ATP value.

As described above, the extracted organs had to be quickly treated with perchloric acid and ATP to be determined.

As for a change of ATP according to the lapse

of time, the change of liver function was shown by author's method. On the other hand, enzymatic method was not able to offer us the delicate change of liver function. The increase and the decrease of the real contents of ATP in T.L.C. employing author's method could be observed macroscopically.

When the prepared liver homogenate was incubated for 30 minutes at 30°C, the two typical patterns of ATP value in liver were observed : One was that ATP value increased until 10 minutes and then began to decrease until 30 minutes. The other was that the value kept on decreasing until 10 minutes, and then increased until 30 minutes (Fig. 5, Fig. 6). The rate of two patterns was almost equal. It seemed that this result was served to estimate the rate of metabolism in rat liver at the same time. It also seemed that the metabolizing mechanism was the pathway of two patterns and that these patterns were crossing each other. These mechanics in biological material, especially in rat liver were measured by the determination method of ATP itself. Author's method seems to be useful and important in the field of clinical and biological chemistry.

Conclusion

Whole rat liver was divided into several fractions and ATP values in the fractions were analyzed. Results of ATP determination of biological materials were summarized as follows :

1) ATP value in liver homogenate fractions was remarkably high in mitochondria.

2) Qualitative fraction spots of nuclei and mitochondria were observed on thin layer chromatoplate.

3) ATP was observed only in the fraction spots of microsome.

4) Total ATP values in these fractions were about 1/5～1/6 of those in liver homogenate directly treated.

5) In analyzing ATP by continuous incubation of rat liver homogenate, it was found that ATP was largely exchanged after 10 minutes and metabolic action of liver was activated for 30 minutes *in vitro*.

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