DETERMINATION OF NUCLEOTIDES IN BIOLOGICAL MATERIALS (III)[1)2)]

Effect of Antibiotic Agents in ATP in Rat Liver[3)]*

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The effect of antibiotic agents of several kinds of tetracyclines involving other chemicals in the ATP in rat liver was studied, and ATP amounts in the liver of the same time (blank) were determined, and the following results were obtained.

1) *In vitro*, ATP values in liver were decreased by treatment with antibiotic agents tested in the tissue homogenate. The ATP value of liver involving the antibiotic agents was lower by 500～700 mcg/g than that of normal liver homogenate, but of liver treated with achromycin, the ATP value in 10 minutes, was increased.

2) *In vivo*, ATP values in rat liver with antibiotic agents, especially one hour after incubation, indicated remarkably low amounts. The downfall of metabolic function of liver was observed from above results.

3) In the determination of ATP concentration, parallel relationship was not observed between in enzymatic method and in chemical method because enzymatic method had many troubles in its procedure.

4) By analyzing ATP value in the liver homogenate, it was found that the value of ATP indicated the degree of metabolic function of liver. Thin layer chromatography was more advantageous than the other methods.

5) Comparatively close, but completely parallel relationship was observed between activating factor of antibiotic agents and ATP value of liver homogenate.

6) ATP groups could be determined by separation with thin layer chromatography on DEAE-cellulose, developing with 0.04 HCl.

It was very important and difficult to determine ATP itself in biological body from the analogous substance. Especially, if an ATP value in normal liver can be compared with its variety, *i.e.* ATP value in a condition of a certain disease, and if the difference of the values can suggest the rate of the disorder of liver, these results give us a significant factor to the field of clinical and biological chemistry.

Authors already published a paper on a change of ATP value in each organ and on the rise or fall of ATP value in biological body[4)].

By the oxidative phosphorilation of mitochondria, the role of ATP for the system of the drug metabolism in biological body became significantly important ; however, administered drug was loaded with a large amount of charge for antidotal effect on liver function as a foreign substance. Accordingly, the rate of loaded charge for liver cells was reflected to the metabolism of energy, converted into the ATP value ; and if it estimates the liver function by the change of ATP value, it will be very useful in the field of clinical analysis.

As for liver toxicity with antibiotic, especially tetracyclines, it was reported that MASHIMO *et al.*[5)] were studying the quantitative change of phospholipid, cholesterol and triglyceride in the liver after and before the administration of antibiotics.

For the determination of ATP value in the field of biological chemistry, enzymatic method is mainly used ; it requires a comparatively difficult technic.

The authors performed these experiments using their own experimental procedure by which ATP, in case of liver disturbed, could be separated and determined chemically and speedily ; change of ATP amount in rat liver influenced by antibiotics of tetracycline derivatives could be assayed ; and the disturbance degree of liver could be presumed by ATP value in liver.

Experiments

1) Experimental materials and reagents

Tetracycline Series-Randomycin (R.M.) of Taito Pfizer Co., Ltd., Achromycin (A.M.) of Nippon

** : Location : 1, Yayoicho, Chiba, Japan.

Lederle Co., Ltd. and Hostacyclin-P.R.M. (HC) of Haechst Japan Co., Ltd. were used. Kanamycin (KM, flavin-mono nucleotide FMN) and EDTA were supplied by Takeda Chemical Industries Ltd., Difco Co., Ltd. and Dotite Co., Ltd. respectively as agents for comparison. These materials were dissolved in distilated water, and then resultant solutions were used as occasion demands in the experiment. The male albino rats (Wistar strain) weighing 150～200 g were used. Water and foods were given freely.

2) Liver homogenate

Liver was extracted from male rat and washed with iced physiological salt solution to remove blood. Fractions were prepared by cutting the liver with scissors and were homogenized by Potter Elvehjem glass homogenizer with Teflon pestol.

Procedure

1) Effect of each drug on rat liver *in vitro*

According to the sample preparation methods, each one milliliter of the drug solution (RM 3 mg/ml, AM 10 mg/ml, HC 3 mg/ml and KM 10 mg/ml) was added to 2 g of the prepared liver homogenate, and the mixture solution was incubated for 10～30 minutes at 30°C.

After the incubation, the mixture was immediately homogenized and was deproteinized in Potter-Elvehjem glass homogenizer with Teflon pestol in 10 ml of ice-cold 6% perchloric acid, and was centrifuged for 10 minutes at 4,000 r.p.m. to supernatant.

The resultant supernatant was adjusted to pH 6.5 with 5 M K_2CO_3 solution with efficient stirring. The sediment of $KClO_4$ was removed by centrifuge and the solution was diluted up to 10 ml with distillated water.

A 0.01 ml portion of the solution was spotted in the thin layer and the chromatoplate was developed with 0.04 N HCl for about 15 minutes and 13 cm long. After drying in the air, ATP, which was found as the absorption part of darkness, was detected by ultraviolet rays of 2537 Å at 0.1 of Rf value, and then scraped up by microspatula.

It was eluted 3 times with 3 ml of 0.01 N HCl for 2, 1 and 1 minute, reapectively. After centrifuge, a collected supernatant was diluted up to 3 ml with 0.1 N HCl.

The absorption was then measured at 260 μm against a blank run in the portion without UV absorption of 2537 Å. In a control homogenate was given 1 ml of physiological salt solution and treated

Table 1 Separation of fractions

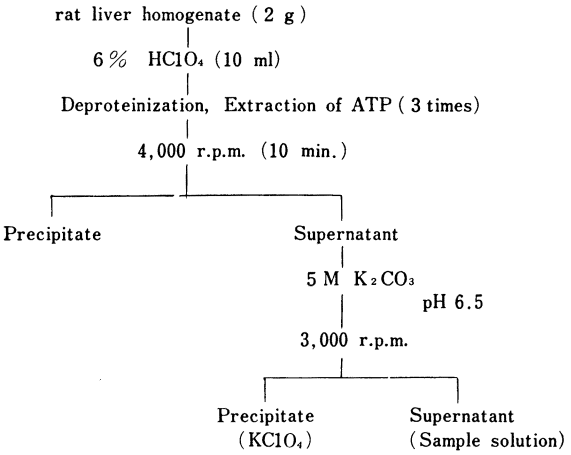
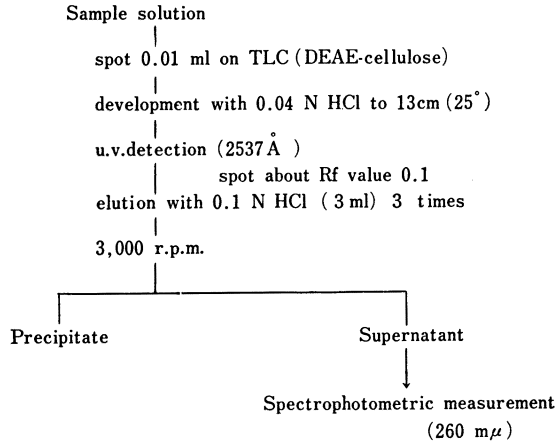


Table 2 Determination of ATP in sample solutions



in the above mentioned condition. The summarized procedure was shown in Tables 1 and 2.

2) Effect of AM on rat liver *in vivo*

Wistar strain male rats weighing 150～200 g divided into 3 groups (5 rats/group) were used. One ml of AM (10 mg/ml) was administered intraperitoneally. The treated rats were allowed for 30～60 minutes and killed. Liver was extracted and washed with iced physiological salt solution to remove blood from it. Therefore, treatments were carried out by the same method as *in vitro*, thin layer method.

Nucleotides were determined by the calibration curve of pure ATP.

Result

1) Effect of RM on ATP value of rat liver *in vitro*

According to the above-mentioned procedure, ATP value in liver was determined. The obtained

Fig. 1 Effect of rondomycin added to liver on liver nucleotides (*in vitro*)
The figure shows 3 cases of typical examples. Rondomycin 2 mg/10 ml
□ normal ▤ enzymatic ▥ added
■ added (enzymatic)
ADP and AMP values determined by chemical method

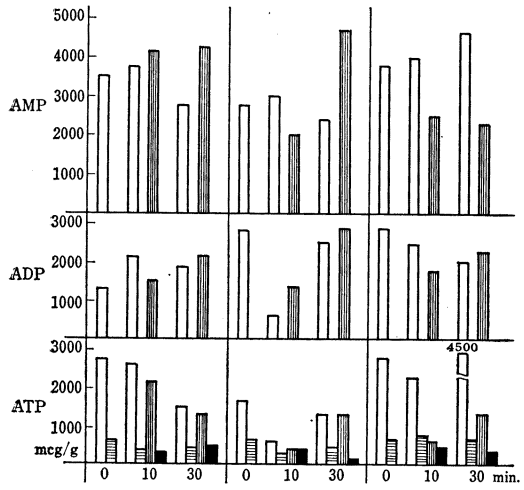


Fig. 2 Effect of hostacylin-PRM added to liver on liver nucleotides (*in vitro*)
The figure shows 3 cases of typical examples.
□ normal ▥ added
Hostacyclin 3 mg/ml

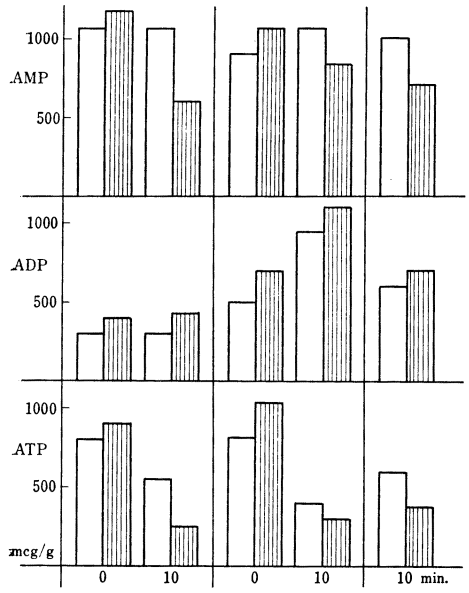
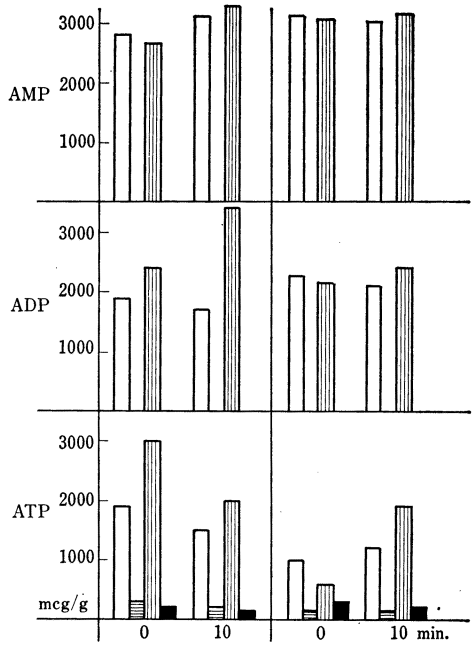


Fig. 3 Effect of achromycin added to liver on liver (*in vitro*)
The figure shows 2 cases of typical examples.
□ normal ▥ added ▤ enzymatic
■ added (enzymatic)
Achromycin 3 mg/ml



resultant was shown in Fig. 1.

After the incubation, ATP value in liver decreased as compared with a control of no addition. The tendency to decrease of ATP value was also observed by enzymatic method. On the other hand, ATP value in liver of control varied following the incubation period ; that is, for 30 minutes the extracted liver seemed to act as liver function *in vitro*.

2) Effect of HC on ATP value of rat liver *in vitro*

According to the procedure, ATP value in liver was shown in Fig. 2.

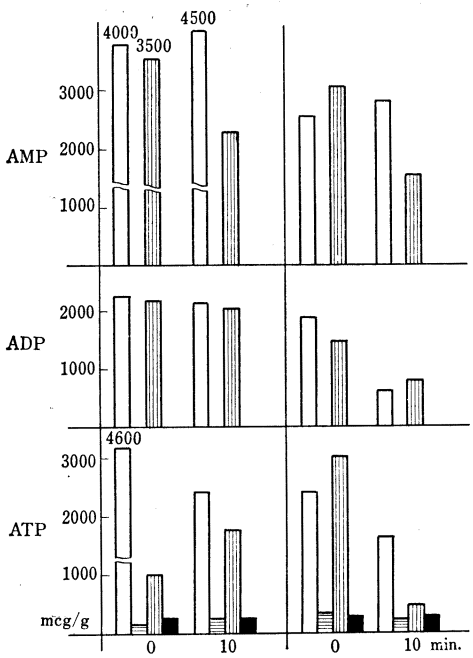
Ten minutes after incubation, ATP and AMP values decreased as compared with those of the control. But ADP value increased following a disappearance of ATP.

3) Effect of AC on ATP value in rat liver *in vitro*

Accoring to the above-mentioned procedure, ATP value in liver was shown in Fig. 3.

The above-mentioned drug is a typical tetracycline derivative. Ten minutes after the addition

Fig. 4 Effect of kanamycin added to liver on liver nucleotides (*in vitro*)
The figure shows 2 cases of typical examples. ADP and AMP values are determined only by the chemical method.
□ normal ▥ added ▤ enzymatic
■ added (enzymatic)
Kanamycin 10 mg/ml



of AC ATP, ADP and AMP value increased as compared with the control. The values determined by enzymatic method did not change according to the lapse of the incubation time, and from those values, more significant difference was not obtained compared with those by chemical method.

4) Effect of KM on ATP value in rat liver *in vitro*

According to the above-mentioned procedure, ATP value in liver was shown in Fig. 4.

ATP value after 10 minutes decreased as compared with that of the control. Also there was a vigorous effect on the liver function of energy supply by ATP group in the biological organ, was influenced by the drugs.

The value by enzymatic method made no significant difference in the lap time.

5) Effect of FMN on ATP value in rat liver *in vitro*

One ml of FMN (500 mcg/ml) was added to 2 g of the prepared liver homogenate and after 10 minutes, ATP value in rat liver was determined and shown in Fig. 5.

Fig. 5 Effect of FMN added to liver on liver ATP (*in vitro*)
The figure shows 4 cases of typical examples. In this experiment, only the ATP value is determined.
□ normal ▤ enzymatic
FMN 500 mg/ml

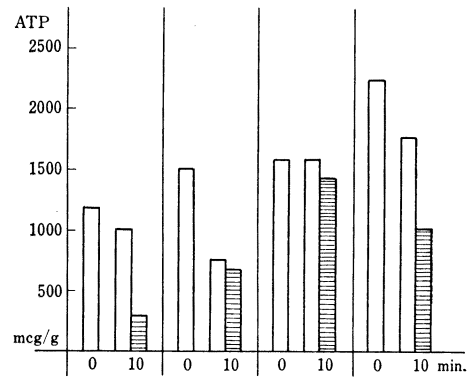


Table 3 Effect of EDTA added to liver on liver ATP

	immediately after cut off				10 min. after cut off			
	normal		added		normal		added	
	Chemical	Enzymatic	Chem.	Enzym.	Chem.	Enzym.	Chem.	Enzym.
ATP	1950	507	2000	588	1900	327	800	367
ADP	2830	—	2600	—	3200	—	2800	—
AMP	3000	—	3100	—	3600	—	4000	—
ATP	2100	314	1650	100	2600	248	1100	229
ADP	3000	—	3000	—	3000	—	3000	—
AMP	2800	—	3100	—	3100	—	3200	—
ATP	2100	280	2000	327	2200	260	1600	104
ADP	2900	—	2850	—	3000	—	2650	—
AMP	2400	—	2000	—	2900	—	3050	—

added ATP : 1 ml of 0.01 mol/ml unit : mcg/g liver

ATP value decreased as those did by the influence of drugs previously mentioned. In energy metabolism system, FMN acted as the co-factor of oxidized enzyme, or yellow enzyme ; however in case in which a large amount of FMN was introduced all at once into the rat liver, FMN must be treated as the foreign substance above the limit of the liver function. Therefore, the supply of the energy from ATP in rat liver was needed according to the lapse of the incubatfon time. According to the above-mentioned phenomenon, it showed possibility that FMN acted as a co-factor influencing a vigorous effect on the liver function under a certain condition.

According to the previous report[1], it was shown

Fig. 6 Effect of intraperitoneal achromycin on liver nucleotides (*in vivo*)
These experiments are determined only by the chemical method.
□ normal
▤ liver treated after 30 min. from the administration of achromycin
▥ liver treated after 60 min. from the administration of achromycin
Achromycin 3 mg/ml

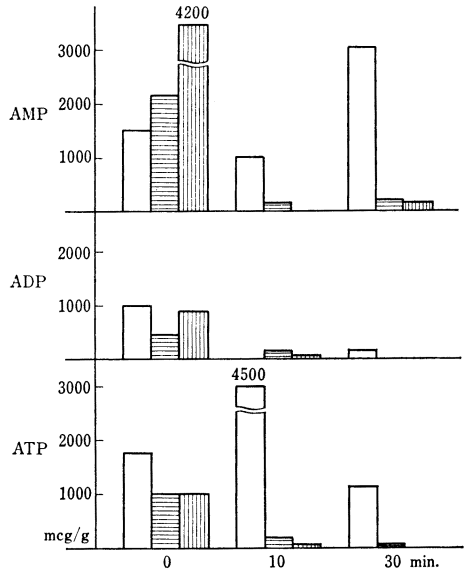
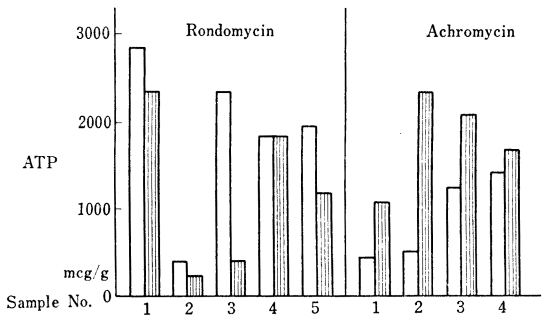


Fig. 7 Contrary effects of rondomycin and achromycin added to liver on liver ATP after 10 min. (*in vitro*)



that added ATP value in liver digested after 10 minutes was almost equal to ATP value in normal liver used as a control.

6) Effect of EDTA in ATP value in rat liver *in vitro*

One ml of EDTA (0.01 mol/ml) was added to 2 g of the prepared liver homogenate. ATP values at each time were determined. The obtained results were shown in Table 3.

From the data, ATP value decreased 10 minutes after an addition of EDTA, while ADP and AMP values remained unchangeable as compared with those of control.

7) Effect of AM via i.p. on ATP value in rat liver *in vitro*

One ml of AM (3 mg/ml) was injected intraperitoneally to male rats weighing 150~200 g and the obtained results by the procedure were shown in Fig. 6.

From the results, ATP value in the extracted rat liver after injection by i.p. decreased rapidly by the lapse of incubation time as compared with that of the control. The value was 150~200 mcg/g and then fell finally to an undetectable value.

8) The comparison between RM and AM *in vitro*

ATP value 10 minutes after administration of each drug was shown in Fig. 7.

ATP value in liver of RM given decreased; on the other hand, that of AC given liver increased. It was not certain that the fact of the above-mentioned data was directly attributed to a structure of the drug; however, it was significant that entirely opposite phenomena were observed in these 2 tetracyclines.

9) Spots of each drug on thin layer plate

Spots of AM, RM and HC were detected by ultraviolet rays of 2537 Å at the front of solvent on the plate, having an emission of a golden color.

FMN and EDTA were detected at 0.25 and 0.45 Rf values, respectively. The former was given an emission of yellow and the latter given an emission of greenish blue.

In spite of the above fact, spot of ATP was detected at 0.1 Rf value where dark violet was absorbed. Therefore, there was no obstacle for determination of ATP.

Discussion

There were a few papers on the effect of antibiotic on a produced energy system; however, we have never read reports about direct determination method for ATP value in liver and relationship between a fate of ATP and antibiotic.

We thought that the establishment of determination of ATP would be useful to an index for inspection of liver function.

In the additional experiments *in vitro*, there was little difference among ATP values influenced by

respective drugs. ATP values decreased generally. ATP value influenced by antibiotic, *i.e.* 500～700 mg/g of liver, was less than that in normal liver. With regard to ATP value of 10 minutes after incubation, when RM and HC were added into liver, ATP value decreased. On the other hand, when AM added, it increased.

In the experiments *in vivo*, ATP of rat liver after 1 hour of administration, as well as ADP and AMP, decreased greatly in contrast with that of no administration. This phenomenon is caused by the disfunction of liver (Fig. 6).

Experimental value in extracted solution of liver homogenate can not be assayed completely by the enzymatic method. It seemed to be indirect being influenced worse by coexisting agents.

In consideration of the above results, the procedure established by the authors is more excellent, speedy and easy in examining of liver function.

As described in the part of Experiments, this method has the following advantages: ATP is directly separated in thin layer: ATP is observed macroscopically; simultaneously ADP and AMP are assayed; a very small amount of ATP can be determined distinguishing from other coexisting compounds.

We consider that this method may be applied widely in clinical chemistry.

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