[Jap. J. Parasit., Vol. 21, No. 2, 70-81, 1972]

Energy Metabolism of *Toxoplasma gondii*: Metabolism of Pyruvate

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(Received for publication; Feb 12, 1972)

Introduction

Although energy metabolism of *Toxoplasma gondii* is a problem of importance to elucidate the physiology of the organism, no biochemical researches have appeared excepting the article by Fulton and Spooner (1960). The detailed aspects of its energy metabolism, therefore, still remain mostly unknown. The present author intended to clarify the essential causes of obligate intracellular parasitism which is one of the most important physiological characteristics of the organism. Concerning the materials which are pure enough to be employed for biochemical investigations, the present author has already reported the method to obtain pure suspension of trophozoites of *Toxoplasma* (RH strain) from the peritoneal exudate of mice infected experimentally (Takeuchi 1971).

The present work on energy metabolism of the organism deals with the metabolism of pyruvate in *Toxoplasma*.

Materials and methods

(1) Reagents

All of the reagents used in the present experiment were special or analytical grade. Cytochrome C, adenosine mono-phosphate (AMP), adenosine di-phosphate (ADP), adenosine tri-phosphate (ATP), Ca-pantothenate, coenzyme A, nicotinamide adenine dinucleotide (NAD), and diazotate-6-benzamide-4-methoxy-m-toluidine chloride were purchased from Sigma Chem. Co. Ltd. Thiamine pyrophosphate (TPP), and phosphoenol pyruvate were the products of Tokyo Kasei Co. Ltd. Malate dehydrogenase (EC 1.1.1.37) and citrate synthase (EC 4.1.3.7) were obtained from Boehringer Mannheim Biochemicals as crystalline suspension in ammonium sulfate.

(2) Enzyme solution

Sonicated extract of *Toxoplasma* trophozoites (RH strain) which were purified by means of the combined use of tris-sucrose-EDTA solution and anti-mouse peritoneal cell serum (AMPCS) as described in the previous report (Takeuchi 1971) was employed throughout the experiment as the enzyme solution.

(3) Assay methods

(A) Formation of pyruvate

(a) Formation of pyruvate from glucose

In this experiment, the effect of adenine nucleotide in the presence of some sorts of inorganic ions, and the effect of magnesium chloride and potassium chloride in the presence of ATP, ADP, AMP, inorganic phosphate (Pi), and NAD on formation of pyruvate from glucose were investigated. In both experiments, the assay mixtures other than the enzyme solution were prepared to make a final volume 1.5 ml with 0.1 M tris-HCl buffer pH 7.4. The control assay mixture was composed of glucose 5 mM only.

In every case, the assay mixtures other than the enzyme solution were pre-incubated at 37°C for 10 minutes, and the reaction was started by addition of 0.2 ml of the enzyme solution. After incubation at 37°C for 60 minutes, 0.5 ml of 10 % trichloroacetic acid was added to terminate the reaction. Then, the test tubes were centrifuged and 1.0 ml

of the supernatant thus obtained was used for further investigations. Pyruvate determination was carried out according to the slight modification of the method described by Kackmar and Boyer (1953).

(b) Pyruvate kinase

In this assay, the effect of magnesium ion and potassium ion which have been known to be the indispensable cofactors for pyruvate kinase was investigated. Acetate salt was used as the source of magnesium ion instead of magnesium chloride. The control assay mixture was composed of 1.5 ml of 0.1 M tris-HCl buffer pH 7.4 containing phosphoenol pyruvate in 1.5 mM, and 0.2 ml of the enzyme solution. The assay mixtures were incubated at 37°C for 60 minutes. Then, the termination of the reaction and the determination of pyruvate produced during the incubation period were made in the same way as in the assay of pyruvate formation from glucose. Activity of pyruvate kinase was represented by the concentration of pyruvate produced by one million organisms for 60 minutes.

(B) Utilization of pyruvate

(a) Utilization of pyruvate under various conditions

In the first experiment, the effect of coenzyme A on consumption of pyruvate was investigated using two kinds of assay systems. In one case, the complete assay mixture was composed of 1.3 ml of 0.1 M tris-HCl buffer pH 7.4 containing sodium pyruvate in 2.8 mM, NAD in 0.6 mM, coenzyme A in 0.03 mM, TPP in 1.2 mM, L-cystein in 2.8 mM, potassium chloride in 5.6 mM, magnesium chloride in 2.8 mM, and 0.4 ml of the enzyme solution. In the other case of this series of the experiment, the complete assay system was consisted of 1.3 ml of 0.1 M tris-HCl buffer pH 7.4 containing sodium pyruvate in 6.0 mM, NAD in 0.6 mM, ADP in 0.6 mM, Pi in 3.3 mM, L-cystein in 3.0 mM, TPP in 1.2 mM, coenzyme A in 0.03 mM, and 0.4 ml of the enzyme solution.

In the second experiment, the effect of addition of Ca-pantothenate, ATP, and L-cystein, i.e. substances indispensable for coenzyme A biosynthesis, on utilization of pyruvate was investigated. The composition of the assay mixture as the complete system consisted of 1.3 ml of 0.1 M tris-HCl buffer pH 7.4 containing sodium pyruvate in 5.3mM, Ca-pantothenate in 5.3 mM, ATP in 1.0mM, ADP in 0.5 mM, NAD in 0.5 mM, L-cystein in 2.6 mM, TPP in 1.0 mM, Pi in 3.5 mM, magnesium chloride in 10 mM, and 0.4 ml of the enzyme solution.

In the third experiment, the effect of various concentrations of NAD on pyruvate consumption was examined. The control assay mixture was composed of sodium pyruvate 5.6 mM only. The concentration of NAD used in the experiment varied from 1.2 mM to 6.0 mM.

The concentration of pyruvate remained in the assay mixture was determined by the method described above.

(b) Pyruvate carboxylase

Activity of pyruvate carboxylase was expressed by the concentration of oxaloacetate produced during the incubation period by one million organisms. The control assay mixture was composed of 1.4 ml of 0.1 M tris-HCl buffer pH 7.4 containing sodium pyruvate 12.5 mM only and 0.2 ml of the enzyme solution.

The assay mixtures other than the enzyme solution were pre-incubated at 37°C for 15 minutes, and then the enzyme solution was added to the assay mixtures to start the reaction. After incubation at 37°C for 30 minutes, the reaction was terminated by de-proteinization with 0.3 ml of 15% perchloric acid. Then, after the assay mixtures were neutralized with 2.0 N KOH and allowed to stand in cold room overnight, centrifugation was made to obtain 1.0 ml of the supernatant to be used for the determination of oxaloacetate. The concentration of oxaloacetate produced in the supernatant was determined by a colorimetric method specific for oxaloacetate (Katsunuma *et al.* 1965).

The supernatant recovered was mixed with

3.0 ml of 99 % ethanol, and added with 0.1 ml of diazotate-6-benzamide-4-methoxy-m-toluidine chloride solution (40 mg/ml) prepared before use. After allowed to stand for 30 minutes in room temparature, 1.0 ml of 2.0 N HCl was added. Several hours later, absorbance at 520 mμ was determined with Hitachi-Perkin Elmer 139 type spectrophotometer.

(c) Pyruvate dehydrogenase

The complete assay mixture was composed of the enzyme solution in amount of 0.1 or 0.2 ml, and 3.1 ml of 0.1 M tris-HCl buffer pH 7.4 containing sodium pyruvate in 11 mM, NAD in 0.6 mM, coenzyme A in 0.03 mM, TPP in 0.6 mM and L-cystein in 0.6 mM. After pre-incubation at 37°C for 15 minutes, the assay mixture other than the enzyme solution was transferred to a cuvette of 1.0 cm light path, and the reaction was started by addition of the enzyme solution.

Increase in absorbance at 340 mμ occured by conversion of NAD to reduced from of NAD was recorded with Hitachi-Perkin Elmer 139 type spectrophotometer and Hitachi QPD 54 type recorder with a special amplifier, with which change in reading of absorbance less than 0.01 could be analyzed accurately.

(d) Lactate dehydrogenase

Activity of lactate dehydrogenase was investigated in three different aspects.

In the first experiment, activity of lactate : NAD oxidoreductase was assayed. In this case, the control assay mixture was consisted of 3.0 ml of 2 mM tris-HCl buffer pH 7.6 containing sodium lactate in 6.0 mM, and 0.2 ml of the enzyme solution.

In the second experiment where activity of lactate : cytochrome C oxidoreductase was investigated, the composition of the control assay mixture was the same as in the former experiment.

In the third experiment, activity of lactate dehydrogenase linked with NADH-cytochrome C reductase was determined with an assay mixture composed of the enzyme solution in amount of 0.1 or 0.2 ml, and 3.6 ml of 0.1 M tris-HCl buffer pH 7.4 containing sodium lactate in 2.6 mM, NAD in 0.5 mM, sodium azide in 2.6 mM, ADP in 0.2 mM, Pi in 2.0 mM, and ferricytochrome C (0.1 %) in amount of 0.2 ml.

Increase in absorbance at 550 mμ or 340 mμ was recorded in the same way as in pyruvate dehydrogenase assay.

(e) Formation of acethyl coenzyme A

The assay mixture arranged completely for the investigation of acethyl coenzyme A formation was composed of Ca-pantothenate, sodium pyruvate (or glucose), NAD, ATP, L-cystein, TPP, and potassium chloride (or magnesium chloride).

Acethyl coenzyme A produced was determined by means of the combined use of malate dehydrogenase and citrate synthase described by Tubbs and Garland (1964). These two kinds of enzymes were diluted two-fold with cold tris-HCl buffer 0.1 M pH 7.8, and dialyzed overnight against 1 liter of the same buffer at 4°C before use to exclude the influence of ammonium sulfate.

Results

(1) Formation of pyruvate from glucose

In the control assay system solely composed of 5 mM glucose, formation of pyruvate from glucose was recognized in amount of 0.37 μg/ml in 60 minutes by one million organisms (Table 1). The most stimulatory effect on formation of pyruvate was observed in the case of addition of ATP, ADP, AMP, NAD, Pi, and magnesium chloride as the cofactors to the control assay system. In this case, pyruvate was formed in amount of as much as 1.05 μg/ml, about three times more than that in the control assay system. The use of potassium chloride in 5 mM or 20 mM instead of magnesium chloride showed slightly lower degree of formation of pyruvate. Among three sorts of adenine nucleotides examined in the experiment, ADP enhanced formation of pyruvate from glucose most prominently in the presence of Pi.

In table 2, activity of pyruvate kinase under several conditions is indicated. Prod-

Table 1 Formation of pyruvate from glucose
Control assay mixture: Glucose 5.0 mM

Assay mixture	Pyruvate formed: μg/ml/60 min./one million organisms
Control assay mixture	0.37
+ ATP 0.5 mM	0.52
+ $MgCl_2$ 10 mM	0.62
+ AMP 0.5 mM, $MgCl_2$ 10 mM	0.88
+ AMP 0.5 mM, KCl 5 mM	0.58
+ AMP 0.5 mM, KCl 20 mM	0.45
+ ADP 0.5 mM, Pi 3 mM	0.91
+ ADP 0.5 mM, Pi 3 mM, $MgCl_2$ 10 mM	0.95
+ ATP 0.5 mM, KCl 5 mM	0.39
+ ATP 0.5 mM, KCl 20 mM	0.45
+ NAD 0.5 mM, KCl 5 mM	0.50
+ NAD 0.5 mM	0.78
+ ATP 0.5 mM, ADP 0.5 mM, AMP 0.5 mM, $MgCl_2$ 10 mM, Pi 3 mM	1.05
+ ATP 0.5 mM, ADP 0.5 mM, AMP 0.5 mM, NAD 0.5 mM, $MgCl_2$ 10 mM, Pi 3 mM	1.12
+ ATP 0.5 mM, ADP 0.5 mM, AMP 0.5 mM, NAD 0.5 mM, KCl 5 mM, Pi 3 mM	0.89
+ ATP 0.5 mM, ADP 0.5 mM, AMP 0.5 mM, NAD 0.5 mM, KCl 20 mM, Pi 3 mM	0.86

Table 2 Activity of pyruvate kinase
Control assay mixture: Phosphoenol pyruvate 1.5 mM

Assay mixture	Pyruvate formed: μg/ml/60 min./one million organisms
Control assay mixture	0.17
+ ADP 0.6 mM, Pi 2 mM	0.21
+ ADP 1.2 mM, Pi 4 mM	0.31
+ ADP 1.2 mM, Pi 4 mM, KCl 6 mM	0.28
+ ADP 1.2 mM, Pi 4 mM, KCl 29 mM	0.39
+ ADP 1.2 mM, Pi 4 mM, KCl 58 mM	0.36
+ ADP 1.2 mM, Pi 4 mM, KCl 87 mM	0.43
+ ADP 1.2 mM, Pi 4 mM, Mg-acetate 12 mM	2.35
+ ADP 1.2 mM, Pi 4 mM, Mg-acetate 24 mM	2.28
+ ADP 1.2 mM, Pi 4 mM, Mg-acetate 36 mM	2.17
+ ADP 1.2 mM, Pi 4 mM, KCl 29 mM, Mg-acetate 12 mM	2.93
+ ADP 1.2 mM, Pi 4 mM, KCl 58 mM, Mg-acetate 12 mM	2.87
+ ADP 1.2 mM, Pi 4 mM, KCl 87 mM, Mg-acetate 12 mM	2.80
+ ADP 1.2 mM, Pi 4 mM, KCl 116 mM, Mg-acetate 12 mM	2.80
+ ADP 1.2 mM	0.17
+ Pi 4 mM	0.14
+ ADP 1.2 mM, Pi 4 mM, KCl 29 mM, Mg-acetate 12 mM with no substrate	0.28

uction of pyruvate was enhanced most markedly when ADP, Pi, magnesium acetate, and potassium chloride were added as activators to the control assay mixture consisted of phosphoenol pyruvate only. Exclusion of the substrate from the assay mixture described above produced pyruvate in 0.28 μg/ml for 60 minutes by one million organisms, two times more than in the control assay mixture. In case that either magnesium acetate or potassium chloride was added to the control assay mixture together with ADP

and Pi, the former showed rather high degree of stimulatory effect on pyruvate kinase reaction. On the contrary, a little enhancement of the reaction was observed in the case of addition of the latter. However, the simultaneous use of magnesium acetate and potassium chloride enhanced production of pyruvate most prominently in these experiments.

(2) Utilization of pyruvate

In the first experiment, utilization of pyruvate by way of acethyl coenzyme A formation was investigated. No enhancement of consumption of pyruvate was observed in every assay system even in the completely arranged assay mixture composed of sodium pyruvate, NAD, coenzyme A, TPP, L-cystein, potassium chloride, and magnesium chloride (Table 3-a, b).

The second experiment regards the effect of addition of the members needed for coenzyme A biosynthesis on utilization of pyruvate. No difference of the extent of the enhancement on consumption of pyruvate was observed in any assay mixture (Table 4).

In the third experiment with respect to utilization of pyruvate, the effect of various concentrations of NAD was investigated. The concentration of NAD added to the assay mixtures was varied in 1.2 mM to 6.0 mM. However, even addition of NAD in

Table 3-a Utilization of pyruvate
Complete assay mixture: Sodium pyruvate 2.8 mM, NAD 0.5 mM, Coenzyme A 0.03 mM, TPP 1.2 mM, L-cystein 2.8 mM, KCl 5.6 mM, $MgCl_2$ 2.8 mM

Assay mixture	Pyruvate utilized: μg/ml/30 min./one million organisms
Complete assay mixture	3.6
− $MgCl_2$	3.6
− KCl	3.6
− $MgCl_2$, KCl	3.8
− Coenzyme A	3.7
− Coenzyme A, $MgCl_2$	3.7
− Coenzyme A, KCl	3.7
− Coenzyme A, $MgCl_2$, KCl	3.7
− TPP, L-cystein	3.7
− TPP, L-cystein, $MgCl_2$	3.8
− TPP, L-cystein, KCl	3.8
− TPP, L-cystein, $MgCl_2$, KCl	3.8
− Coenzyme A, TPP, L-cystein, $MgCl_2$, KCl	3.7
− Coenzyme A, NAD, TPP, L-cystein, $MgCl_2$, KCl	3.8
− NAD, TPP, L-cystein, $MgCl_2$, KCl	3.8

Table 3-b Utilization of pyruvate
Complete assay system: Sodium pyruvate 6.0 mM, NAD 0.6 mM, ADP 0.6 mM, Pi 3.3 mM, L-cystein 3.0 mM, TPP 1.2 mM, Coenzyme A 0.03 mM

Assay mixture	Pyruvate utilized: μg/ml/60 min./one million organisms
Complete assay system	4.6
− Coenzyme A, TPP, L-cystein	4.5
− ADP, Pi	4.6
− Pi	5.1
− NAD	4.9
Sodium pyruvate only	4.7

Table 4 Utilization of pyruvate
Complete assay system : Sodium pyruvate 5.3 mM, Ca-pantothenate 5.3 mM, ATP 1.0 mM, ADP 0.5 mM, L-cystein 2.6 mM, TPP 1.0 mM, Pi 3.5 mM, $MgCl_2$ 10 mM

Assay mixture	Pyruvate utillized : μg/ml/60 min./one million organisms
Complete assay system	6.8
− ATP, L-cystein, TPP	7.3
− $MgCl_2$	7.2
− Ca-pantothenate	6.9
− Ca-pantothenate, ATP, L-cystein	7.3

Table 5 Utilization of pyruvate
Control assay mixture : Sodium pyruvate 5.6 mM

Assay mixture	Pyruvate utilized : μg/ml/60 min./one million organisms
Control assay mixture	3.3
+ NAD 1.2 mM	3.6
+ NAD 3.6 mM	4.0
+ NAD 6.0 mM	4,0

Table 6 Time course of pyruvate carboxylase activity
Assay mixture : Sodium pyruvate 12 mM, $NaHCO_3$ 25 mM, ATP 0.6 mM

Incubation period	Oxaloacetate produced : μg/ml/one million organisms
10 min.	9.6
20 min.	8.8
30 min.	9.1
40 min.	4.0
50 min.	4.6
60 min.	4.6

6.0 mM did not show any stimulatory effect (Table 5).

In table 6, time course of pyruvate carboxylase activity is indicated. The reaction reached maximum level in 10 minutes, and continued until 30 minutes. Thereafter, it appeared that utilization of oxaloacetate formed by pyruvate carboxylase reaction came to be visible. However, it can not be known in which metabolic pathway oxaloacetate is utilized.

Pyruvate carboxylase activity was expressed by the concentration of oxaloacetate produced by one million organisms for 30 minutes. The highest degree of enhancement of the reaction was recognized by addition of $NaHCO_3$, ATP, ADP, AMP, and magnesium chloride to the control assay mixture composed of phosphoenol pyruvate only. In this case, oxaloacetate was produced about five times more than in the control assay mixture (Table 7). The experiments were not performed to elucidate the necessity of acethyl coenzyme A which is known to be an indispensable cofactor for the maximum activity of the enzyme in metazoan animals.

In table 8, the activity of pyruvate dehydrogenase is shown. The activity of the enzyme was represented by the concentration of reduced form NAD produced by one million organisms for one minute. In any case including the completely arranged assay

Table 7 Activity of pyruvate carboxylase
Control assay mixture: Sodium pyruvate 12.5 mM

Assay mixture	Oxaloacetate produced: μg/ml/30 min./one million organisms
Control assay mixture	2.2
+ $NaHCO_3$ 25 mM, ATP 0.6 mM	9.4
+ $NaHCO_3$ 25 mM, ADP 0.6 mM	6.4
+ $NaHCO_3$ 25 mM, AMP 0.6 mM	6.7
+ $NaHCO_3$ 25 mM, ATP 0.6 mM, KCl 6 mM	8.6
+ $NaHCO_3$ 25 mM, ATP 0.6 mM, KCl 12 mM	8.8
+ $NaHCO_3$ 25 mM, ATP 0.6 mM, $MgCl_2$ 12 mM	8.0
+ $NaHCO_3$ 25 mM, ADP 0.6 mM, KCl 6 mM	2.3
+ $NaHCO_3$ 25 mM, ADP 0.6 mM, $MgCl_2$ 12 mM	7.8
+ $NaHCO_3$ 25 mM, AMP 0.6 mM, KCl 6 mM	6.7
+ $NaHCO_3$ 25 mM, AMP 0.6 mM, $MgCl_2$ 12 mM	8.5
+ $NaHCO_3$ 25 mM, ATP 0.6 mM, ADP 0.6 mM, AMP 0.6 mM, $MgCl_2$ 12 mM	11.7
+ $NaHCO_3$ 25 mM	6.1

Table 8 Activity of pyruvate dehydrogenase
Complete assay mixture: Sodium pyruvate 11 mM, NAD 0.6 mM, Coenzyme A 0.03 mM, TPP 0.6 mM, L-cystein 0.6 mM, and tris-HCl buffer 0.1 M pH 7.4 to make a final volume 3.1 ml

Assay mixture	Reduced form of NAD produced: μμM/min./one million organisms
Complete assay mixture	0.560
− Coenzyme A	0.168
− L-cystein	0.140
− Coenzyme A, L-cystein, TPP	0.410
− Sodium pyruvate	Nil

system in which enhancement of reduced form NAD formation was observed slightly, almost low activity of the enzyme was recorded as a whole.

In table 9-a, b, c, lactate dehydrogenase activity i.e. lactate: NAD oxidoreductase activity, lactate: ferricytochrome C oxidoreductase activity, and the activity of lactate dehydrogenase linked with NADH-cytochrome C reductase, is indicated respectively. As known in these tables, the presence of lactate: NAD oxidoreductase is surely recognized. However, it appeares that the organism does not possess lactate: ferricytochrome C oxidoreductase. On the contrary, cytochrome C is certainly reduced in the assay of lactate dehydrogenase linked with NADH-cytochrome C reductase. Therefore, it seems reasonable judging from the presence of lactate: NAD oxidoreductase in mitochondria of the organism (Akao 1971), lactate: NAD oxidoreductase might have some relation with NADH-cytochrome C reductase *in vivo*.

With respect to lactate: NAD oxidoreductase, magnesium acetate and ADP enhanced the activity of the enzyme. In addition, the simultaneous addition of magnesium acetate and ADP also enhanced production of reduced form of NAD. However, ADP inhibited the activity of the enzyme in the presence of Pi.

In table 10, the results on formation of acethyl coenzyme A are indicated. In any assay system, no increment of absorbance at 340 mμ was recognized. Addition of glucose

Table 9-a Activity of lactate : NAD oxidoreductase
Control assay mixture : Sodium lactate 6.0 mM

Assay mixture	Reduced form of NAD produced : mμM/min./one million organisms
Control assay mixture	Nil
+ NAD 0.3 mM	0.173
+ NAD 0.3 mM, KCl 0.16 M	0.195
+ NAD 0.3 mM, Mg-acetate 6.5 mM	0.477
+ NAD 0.3 mM, ADP 0.3 mM	0.282
+ NAD 0.3 mM, Pi 2.0 mM	0.099
+ NAD 0.3 mM, ADP 0.3 mM, Pi 2.0 mM	Nil
+ NAD 0.3 mM, ADP 0.3 mM, KCl 0.16 M	0.166
+ NAD 0.3 mM, ADP 0.3 mM, Mg-acetate 6.5 mM	0.589
+ NAD 0.3 mM, ADP 0.3 mM, Pi 2.0 mM, KCl 0.16 M, Mg-acetate 6.5 mM	Nil

Table 9-b Activity of lactate : ferricytochrome C oxidoreductase
Complete assay mixture : Sodium lactate 6.0 mM, 0.2 ml of ferricytochrome C (0.1 %), KCl 0.16 M, and tris-HCl buffer 0.01 M, pH 7.6 to make a final volume 3.0 ml

Assay mixture	Ferroytochrome C formed : mμM/min./one million organisms
Complete assay mixture	0.082
− KCl	0.049
− Sodium lactate	0.059
− Sodium lactate, KCl	0.081

Table 9-c Activity of lactate dehydrogenase linked with NADH-cytochrome C reductase
Complete assay mixture : Sodium lactate 2.6 mM, NAD 0.5 mM, 0.2 ml of ferricytochrome C (0.1 %), sodium azide 2.6 mM, ADP 0.2mM, Pi 2.0 mM, and tris-HCl buffer 0.1 M, pH 7.4 to make a final volume 3.5 ml

Assay mixture	Ferrocytochrome C formed : mμM/min./one million organisms
Complete assay mixture	0.052
− Pi	0.104
− ADP	0.104
− Sodium lactate	Nil

or sodium pyruvate did not increase the absorbance.

Discussion

As described in the introduction, no researches except for the work of Fulton and Spooner (1960) have appeared on the energy metabolism of *Toxoplasma gondii* during past fifteen years. They purified *Toxoplasma* trophozoites by means of filtration with a sintered glass filter, and studied physiological characteristics of the energy metabolism in the organism. They suggested the presence of the Embden-Meyerhof-Parnas pathway, and the activity of hexokinase. Furthermore, they showed the presence of acetate, lactate, and another unidentified acid as the metabolic products, the traces of three sorts of fatty acids, i.e. propionic acid, buthyric acid, and valeric acid, by gas phase chromatography, the ability of utilization of reduced

Table 10 Formation of acethyl coenzyme A
Control assay mixture: Ca-pantothenate 6.0 mM

Assay mixture	Increase in absorbance at 340 mμ/10 min.
Control assay mixture	Nil
+ ATP 1.2 mM, L-cystein 3.0 mM	Nil
+ ATP 1.2 mM, L-cystein 3.0 mM, TPP 1.2 mM	Nil
+ ATP 1.2 mM, L-cystein 3.0 mM, TPP 1.2 mM, Sodium pyruvate 6.0 mM	Nil
+ ATP 1.2 mM, L-cystein 3.0 mM, TPP 1.2 mM, NAD 0.6 mM, Sodium pyruvate 6.0 mM	Nil
+ ATP 1.2 mM, L-cystein 3.0 mM, TPP 1.2 mM, NAD 0.6 mM, Sodium pyruvate 6.0 mM, $MgCl_2$ 12 mM	Nil
+ ATP 1.2 mM, L-cystein 3.0 mM, TPP 1.2 mM, NAD 0.6 mM, Sodium pyruvate 6.0 mM, KCl 60 mM	Nil
+ ATP 1.2 mM, L-cystein 3.0 mM, TPP 1.2 mM, Glucose 6.0 mM	Nil
+ ATP 1.2 mM, L-cystein 3.0 mM, TPP 1.2 mM, NAD 0.6 mM, Glucose 6.0 mM	Nil
+ ATP 1.2 mM, L-cystein 3.0 mM, TPP 1.2 mM, NAD 0.6 mM, Glucose 6.0 mM, $MgCl_2$ 12 mM	Nil
+ ATP 1.2 mM, L-cystein 3.0 mM, TPP 1.2 mM, NAD 0.6 mM, Glucose 6.0 mM, KCl 60 mM	Nil

form of NAD, and other facts suggesting the aerobic nature of the energy metabolism in the organism. Judging from the fact that cytochrome system is present in the organism as was proved by Fulton and Spooner (1960), and the present author (unpublished data), it seems reasonable to suppose that *Toxoplasma* has rather similar energy production pathway as in the higher animals which owe the production of ATP to TCA cycle and cytochrome system mainly. In addition, existence of mitochondria in the organism, definite operation of malate dehydrogenase, succinate dehydrogenase, succinate-cytochrome C reductase, and NADH-cytochrome C reductase in the metabolic pathway of the organism, and the presence of the ability of ATP biosynthesis from the intermediates in TCA cycle (Takeuchi unpublished data) may support this supposition.

In the present study, the metabolism of pyruvate in *Toxoplasma* was investigated in several aspects, i.e. formation of pyruvate from glucose, consumption of pyruvate under various conditions, enzyme activity in relation to the metabolism of pyruvate, and formation of acethyl coenzyme A.

With regard to formation of pyruvate from glucose, the results indicated that pyruvate was formed most prominently in the case of addition of ATP, ADP, AMP, NAD, Pi, and magnesium chloride to the control assay mixture composed of glucose only. Judging from the result, it seems reasonable that phosphorylated glycolytic pathway is apparently active in the organism. Furthermore, as described below, the presence of pyruvate kinase may support the above thought. These findings agree with the conclusions reported by Fulton and Spooner (1960). Then according to the above facts, it can be concluded that the mechanism of obligate intracellular parasitism of the organism does not derive from the metabolic pathway from glucose to pyruvate at least.

In the investigation of pyruvate formation from glucose, three sorts of adenine nucleotides were used for the examination of the nature of glycolytic pathway in the organism. Generally speaking, the site of activation or inhibition by adenine nucleotide such as ATP, ADP, and AMP to glycolytic pathway is rather complicated. Therefore, it can not be decided in such an assay system as in the present experiment, which enzyme is activated or inhibited by adenine nucleotide, and which enzyme is rate limmiting one. However, it is noteworthy that ADP enha-

nces production of pyruvate most prominently among three sorts of adenine nucleotides in the presence of Pi. This might imply that the reaction in which ADP and Pi are necessary for the maximum activity of phosphoglycerate kinase or pyruvate kinase plays an important role in the glycolytic pathway of *Toxoplasma*.

Activity of pyruvate kinase was rather prominent. In the control assay system, pyruvate was produced in as much as 0.14 μg/ml during the period of incubation with one million organisms. Addition of ADP, Pi, magnesium acetate, and potassium chloride was needed for the full activity of the enzyme. Although both magnesium and potassium ions are regarded as indispensable cofactors of the enzyme, the former seems to be more important for pyruvate kinase reaction of *Toxoplasma*, because the latter showed much less stimulatory effect on production of pyruvate in the presence of ADP, and Pi. However, the nature of pyruvate kinase in *Toxoplasma* that the most prominent activity was recognized in the case of addition of ADP, Pi, and the two sorts of inorganic ions seems to be similar to other microbiral or animal pyruvate kinase. Anyway, taking other facts also in consideration, the definite operation of pyruvate kinase in the glycolytic pathway of the organism indicates the presence of the Embden-Meyerhof-Parnas pathway in the energy production pathway of *Toxoplasma*.

In the case of the assay for pyruvate utilization, many experiments were made in several aspects. Although the results obtained in the present experiment indicate that *Toxoplasma* can consume pyruvate, the activity of pyruvate utilization by way of acethyl coenzyme A formation might be rather low, judging from the facts as follows: (1) Addition of high concentration of NAD even in as much as 6.0 mM as final concentration did not enhance utilization of pyruvate. (2) No enhancement of pyruvate utilization was observed by addition of coenzyme A. (3) No formation of acethyl coenzyme A occured. Furthermore, the low activity of pyruvate dehydrogenase also supports this supposition. However, with regard to pyruvate dehydrogenase assay in the present experiment, the oxidation of reduced form of NAD by lactate dehydrogenase coexisting in the enzyme solution on account of use of crude sonicated extract of *Toxoplasma* may disturbe increment in absorbance at 340 mμ by pyruvate dehydrogenase reaction. Therefore, there is a possibility that the actual activity of pyruvate dehydrogenase *in vivo* may be in higher level than that obtained in the results of the present study. However, even if the oxidation of reduced form of NAD by lactate dehydrogenase should disturbe increment in absorbance, the results obtained in pyruvate consumption assay also suggested the rather low activity of pyruvate dehydrogenase.

Activity of lactate dehydrogenase was investigated in three aspects described previously. The presence of lactate : NAD oxidoreductase was certainly admitted. On the other hand, the definite activity of lactate : ferricytochrome C oxidoreductase was not observed. As known in the result, the effect of cofactors such as ADP or magnesium ion seems to be distinctive. ADP has been regarded as inhibitor to lactate : NAD oxidoreductase. However, in the present experiment, it enhanced the activity of the enzyme. Inhibition of the activity of lactate : NAD oxidoreductase by addition of ADP was observed only when Pi was added at the same time. It is not known exactly what from this nature of lactate : NAD oxidoreductase in *Toxoplasma* arises. This seems to be interesting, however, taking account of the presence of the enzyme in mitochondria (Akao 1971), and the correlation of TCA cycle in which ADP and Pi play some important roles in relation to ATP biosynthesis with gluconeogenetic pathway in which lactate : NAD oxidoreductase reaction is said to be the first step.

Activity of pyruvate carboxylase was determined by the concentration produced by one million organisms during the period of incubation. As known in the results, the

activity of pyruvate carboxylase was more prominent than any other enzymes assayed in the present study. However, it can not be decided whether the prominent activity of the enzyme means the superiority of gluconeogenetic pathway in the organism or not.

With regard to biosynthesis of coenzyme A in sporozoa, Trager (1952, 1954, 1966), and Bennet and Trager (1967) reported the absence of the ability of coenzyme A biosynthesis and the deficiency of pantothenate kinase in *Plasmodium lophurae*. In the present study, the results suggest the deficit of acethyl coenzyme A formation in *Toxoplasma*. However, it is not yet clarified whether this is derived from deficiency of some enzyme in coenzyme A biosynthetic pathway of *Toxoplasma* or not, because of the low activity of pyruvate dehydrogenase. If the deficiency of some enzyme in relation to coenzyme A biosynthetic pathway of *Toxoplasma* is proved experimentally, this type of metabolic characteristic in *Toxoplasma* as well as in *Plasmodium lophurae* might be regarded as one of the causes of obligate intracellular parasitism of these organisms.

Even if pyruvate is not utilized by way of acethyl coenzyme A formation judging from the facts described in the present report, when *Toxoplasma* invades into the host cell and proliferates vigorously, this metabolic pathway from pyruvate of citrate must be activated by some regulatory mechanism, because a large quantity of ATP must be supplied for the maximum replication of the organism in host cells, and the metabolic pathway from pyruvate to cytochrome system by way of acethyl coenzyme A formation and TCA cycle seems to be most efficient to produce ATP in aerobic cells such as *Toxoplasma*. Therefore, it seems to be probable that the energy metabolism of *Toxoplasma* is repressed by some mechanism when it is present in extracellular space, and de-repressed by invasion into host cells. This metabolic conversion from repressed state to active state seems to be intersting in the studies on the physiological characteristics of *Toxoplasma*.

Summary

The metabolism of pyruvate in *Toxoplasma gondii* was investigated in several aspects.

(1) Formation of pyruvate from glucose was enhanced most actively by addition of ATP, ADP, AMP, NAD, Pi, and magnesium chloride. The presence of pyruvate kinase activity was apparently recognized. On the basis of these results, it appeares to be rea sonable to conclude that the Embden-Meyerhof-Parnas pathway operates in *Toxoplasma*.

(2) Addition of high concentration of NAD, or coenzyme A, or substances indispensable for coenzyme A biosynthesis did not enhance utilization of pyruvate.

(3) The low activity of pyruvate dehydrogenase was recognized.

(4) The presence of lactate : NAD oxidoreductase was recognized. The enzyme was activated by addition of ADP or magnesium acetate or addition of both of them. However, addition of ADP and Pi inhibited the activity of the enzyme. No presence of lactate : ferricytochrome C oxidoreductase was observed. The presence of NADH-ferricytochrome C oxidoreductase was admitted.

(5) The prominent activity of pyruvate carboxylase was observed.

(6) No formation of acethyl coenzyme A could be observed.

Acknowledgements

The author is indebted to Dr. Keizo Asami, Chief, Department of Parasitology, Keio University School of Medicine, for his invaluable support and critical observation of this manuscript, and Dr. Hiroshi Ohya, Professor, Department of Parasitology, Juntendo University School of Medicine for his discussion about the results obtained in the present study.

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トキソプラスマにおけるエネルギー代謝の研究：ピルビン酸の代謝

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トキソプラスマの偏性細胞内寄生の原因の解明のため，エネルギー代謝の研究の一環としてピルビン酸の形成および利用について研究を行つた。実験には著者が先に報告した方法で分離・精製したトキソプラスマ虫体（RH 株）を音波破砕して使用した。その結果，1）グルコースからのピルビン酸の形成は確かに見られた．この形成は三種のアデニン・ヌクレオチド（ATP, ADP, AMP），NAD，無機リン，およびマグネシウムイオンの存在下で最も顕著に見られた。これは次に述べるピルビン酸キナーゼの存在と共にいわゆる E. M. P. 系路の存在を裏付けるものと考えられる．2）ピルビン酸キナーゼの活性も確かに見られた．最大活性はカリウムイオン，マグネシウムイオン，ADP, および無機リンの存在下において見られた．3）ピルビン酸の利用は高濃度の NAD の添加，コエンザイムAの添加，およびコエンザイムA生合成に必要な物質の添加，いずれの場合も促進が見られなかつた．4）Lactate: NAD oxidoreductase の活性が見られ，これは ADP, 又はマグネシウムイオンの添加によつて活性化された．ADP およびマグネシウムイオンを同時に添加した場合も活性化が見られた．Lactate: ferricytochrome C oxidoreductase の活性は見られなかつた．5）ピルビン酸カルボキシラーゼの高い活性が見られた．6）アセチルコエンザイムAの形成は全く見られなかつた．7）ピルビン酸脱水素酵素の低い活性が見られた．