# IMPROVED PURIFICATION OF RAT BRAIN CHOLINE ACETYLTRANSFERASE BY USING AN IMMUNOABSORBENT

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## 1. Introduction

Rat brain choline acetyltransferase (acetyl-CoA. choline-o-acetyl-transferase, EC 2.3.1 6) has been purified to a specific activity (S.A.) of 0.6 μmoles/min/mg of protein by Potter et al. [1], which represents a purification of seven hundred times We tried to improve Potter's purification by various biochemical techniques, and reached a S.A. of 0.8 μmoles/min/mg of protein. Such a preparation still gave four protein bands in gel electrophoresis, at pH 4.5.

In order to obtain antibodies to choline acetyltransferase (CAT), we injected several preparations into seventeen animals, rodents as well as gallinaceae, but failed to induce an immunological response against the enzyme Yet, in all cases, we obtained antibodies to some contaminant proteins. Such antisera were used, following Avrameas' technique [2], in order to improve the CAT purification. We polymerised one of the antisera with glutaraldehyde into a gel and prepared with it a chromatography column It retained the immunogenic proteins, while CAT passed through the column without being absorbed, giving a final 2,700-fold purified enzyme, with a S.A. of 1.8 μmoles/min/mg of protein.

## 2. Materials and methods

### 2.1. *CAT assay*

Assay was based on Fonnum's extraction technique: [$^{14}$C]acetyl choline derived from [$^{14}$C]acetyl-CoA was extracted as a tetraphenylborate salt in ethylbutylketone [3]. In our modified assay, we used the following final concentrations in a volume of 20 μl 0 04 mM [$^{14}$C]acetyl-CoA (Radiochemical Centre, Amersham, S.A. 60 Ci/M), 10 mM choline HCl, 200 mM NaCl, 1 mM 1–10 phenanthroline-HCl, 0.1 mM eserine, 50 mM Na phosphate buffer pH 7.2, bovine serum albumin 1 mg/ml and enzyme. After a 5 min incubation at 37°, in a 400 μl polypropylene tube, the reaction was stopped by 100 μl ethylbutylketone containing 10 mg of tetraphenylborate per ml After mixing and centrifuging at 2,000 *g* for 30 sec, 50 μl of the organic phase were transferred to a scintillation vial containing 10 ml of toluene : Triton X-100 (2 5.0.88) with 5 g PPO and 0.24 g POPOP per liter of toluene Incubation with beef erythrocyte acetylcholinesterase (Sigma), instead of eserine, gave the blank value. This technique is suitable for amounts of enzyme between 2 and 60 pmoles of acetylcholine formed per minute. Before an assay, the enzyme was diluted to suitable concentration in a Na phosphate buffer of 50 mM at pH 7 2, containing 1 mM 1–10 phenanthroline-HCl, 200 mM NaCl and albumin 2 mg/ml to protect the enzyme against denaturation

Protein was assayed by the method of Lowry et al [4].

### 2.2. *Biochemical purification of CAT*

As CAT is very unstable at low protein concentrations, care was taken to avoid protein concentration below 1 mg per ml. All operations were performed at 4°. In order to improve enzyme recovery and purity, we have modified Potter's technique [1] as follows: i) 400 rat brains (630 g wet weight, free of cerebellum tissue, were collected and frozen (–40°). After thawing, they were homogenized in a Waring Blendor (2 × 30 sec, full speed) in 4 ℓ of 1 mM EDTA $Na_2$,

0.2 mM dithiothreitol. The solution was adjusted to pH 5 with 1 N HCl and centrifuged for 30 min at 10,000 *g*. The supernatant was discarded.
ii) Pellets were rehomogenized with a Polytron in 3.5 ℓ 200 mM NaCl, 1 mM EDTA $Na_2$, 0.2 mM dithiothreitol and centrifuged for 30 min at 10,000 *g*. The supernatant was diluted in an equal volume of glass distilled water. The pH was adjusted to 5.9 with 1 M $Na_2HPO_4$. We added to the enzyme solution 130 ml of slurry CM-Sephadex C-50 in 10 mM Na phosphate buffer pH 5.9, 100 mM NaCl, 1 mM EDTA $Na_2$, 0.2 mM dithiothreitol. After stirring for 15 min, the colorless supernatant was discarded and the resin which had adsorbed the enzyme turned red. The CM Sephadex was washed with 1 ℓ of the same buffer.
iii) CM-Sephadex was poured into a column (90 cm height, 1.5 cm diameter). The enzyme was eluted by a linear gradient from buffer to 400 mM NaCl (100 ml each). The enzyme appeared at 200 mM NaCl in a large peak, just before hemoglobin. Fractions with a S.A. over 75 nmoles/min mg of protein were pooled.
iv) The enzyme was precipitated between 38.5% and 50% saturation by a saturated $(NH_4)_2SO_4$ solution pH 6, containing 1 mM 1–10 phenanthroline-HCl and 1M dithiothreitol. After centrifugation for 15 min at 20,000 *g*, the pellet was resuspended in 1 ml of 0.5 M NaCl, 1 mM 1–10 phenanthroline HCl, 1 mM dithiothreitol, Na phosphate buffer 10 mM, pH 6.
v) The enzyme on a Sephadex G-200 column (90 cm height, 1.5 cm diameter) in the same buffer appeared after two void volumes. At this stage, fractions with a S.A. over 0.6 μmoles/min/mg of protein were pooled.

### 2.3. *Immunological techniques*

Nine rabbits, three hamsters, three guinea pigs and two roosters were injected three times, every three weeks, with two types of CAT preparations (step $(NH_4)_2SO_4$ and G-200). We injected amounts of proteins from 0.5 to 5 mg in a complete Freund's adjuvant emulsion, at subcutaneous, intradermal and intramuscular sites. The animals were bled two weeks after the last injection.

The antisera were tested by double immunodiffusion in a 1.2% agar gel [5].

We used Avrameas' technique [2] in order to polymerize the serum: 20 ml of whole antiserum, predialyzed against cold phosphate buffer saline, were adjusted to pH 5. 6 ml of an 2.5% aqueous solution of an 2.5% aqueous solution of glutaraldehyde(Taab) were added dropwise. The resulting gel was homogenized and washed before being used on a chromatography column.

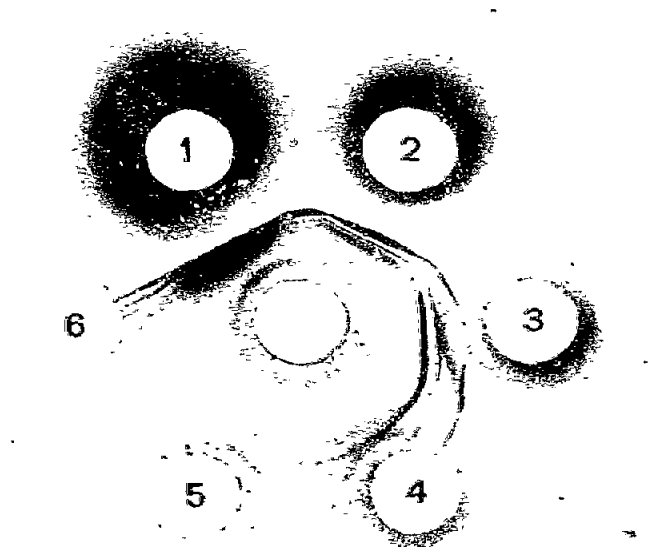


Fig. 1. Immunodiffusion of CAT against antiserum 27. Center well: whole rabbit antiserum. Outside wells: logarithmic dilution of CAT, step $(NH_4)_2SO_4$. Serial 3-fold protein dilution (1/3): concentrations from 36 mg/ml (well 1) to 0.15 mg/ml (well 6).

## 3. Results and discussion

### 3.1. *Characterization of the antisera*

In immunodiffusion tests, all the antisera gave at least one precipitation band with the CAT preparation step $(NH_4)_2SO_4$. Antiserum no. 27 obtained in a rabbit injected three times with 5 mg of CAT step $(NH_4)_2SO_4$ gave the greater number of bands (fig. 1). As rat serum gave several bands with it, we used later our antiserum 27 after absorption with rat serum. Then the antiserum still reacted with the previous CAT preparation in giving two bands (fig. 2). At least two of these remaining bands were in coincidence with an extract of rat adrenals, while extract of other organs such as kidney or liver did not react. Finally we detected no precipitation band between antiserum 27 and extract of C6 rat tumor glial cells [6]. Our antiserum, therefore contains antibodies reacting with serum proteins adrenal proteins and proteins of brain

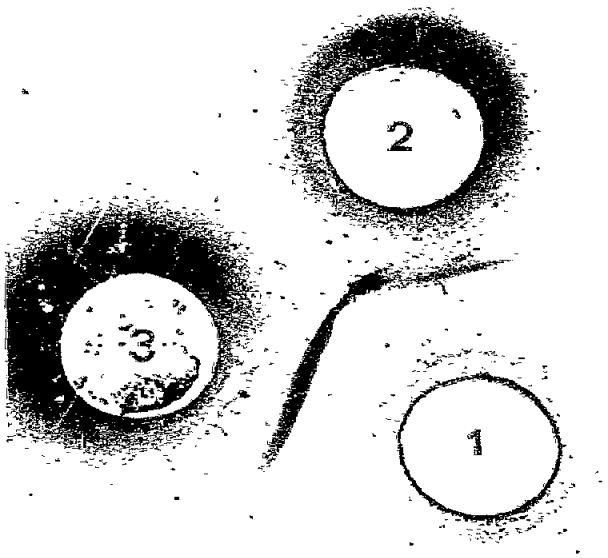


Fig. 2. Immunodiffusion of CAT and adrenals extract against antiserum 27 absorbed with rat serum. Well 1: Antiserum 27 absorbed with polymerized rat serum [2]. Well 2: CAT, step $(NH_4)_2SO_4$. Protein concentration 3.6 mg/ml. Well 3: 105,000 *g* supernatant of a rat adrenal homogenate, made in 50 mM Na phosphate buffer, pH 7.4. Protein conc. 10 mg/ml.

tissue, perhaps specific to neuronal cells, as it does not react with a glial cell extract.

We then performed precipitation tests by adding increasing amounts of antiserum 27 to a constant concentration of enzyme, and by measuring, after centrifugation, the residual enzymatic activity [7]. We could not demonstrate any decrease in the activity, which ruled out the presence of specific antibodies to CAT. We got the same answers with our other rabbit, guinea pig, hamster as well as rooster immunosera. It has been shown that CAT is very species specific [8]. Thus the lack of immunonoiogical response against rat CAT is unlikely to be due to its immunological identity with the CAT of each kind of animal injected, rodents as well as gallinaceae. Furthermore, the CAT molecular weight (50,000) should be large enough to induce antibodies. It would be worth trying another immunisation schedule in the hope of getting specific antibodies to CAT. The need of injecting a CAT preparation free from immunogenic contaminant prompted us to use, as a purification step, antiserum 27 which reacted with the greatest number of contaminant proteins.

### 3.2. *Purification of CAT in using an immunoabsorbent*

Classical absorption technique by antiserum 27 or even by purified γ globulin from it, was not suitable as it adds rabbit serum proteins which cannot be removed without leading to a decrease of the S.A. of the enzyme. Therefore, we polymerized our antiserum into a gel used as an immunoabsorbent.

CAT, step G-200 (0.8 μmoles/min/mg of protein) was put on the immunoabsorbent column (10 cm height, 0.9 cm diameter) equilibrated with 0.5 M NaCl, 1 mM 1–10 phenanthroline, 1 mM dithiothreitol, Na phosphate buffer 10 mM pH 6. The enzyme passed through the column, while contaminant proteins reacting with their specific antibodies remained on the column. We verified that the enzyme is no more reacting in immunodiffusion tests, with antiserum 27 This last step gave a purification of 2.2-fold, the entire procedure (table 1) of 2,700-fold leading to a S.A. of 1.8 μmoles/min/mg of protein.

Table 1
Summary of enzyme purification.

| Step | Total units μmoles Acetylcholine formed per min | Recovery (%) | Protein (mg) | Specific activity (μmoles/min/mg) |
|---|---|---|---|---|
| I Homogenate | 45.5 | 100% | 66,000 | 0.0007 |
| II Supernatant 200 mM NaCl | 30.5 | 75% | 3,960 | 0.0077 |
| III Elution CM-Sephadex | 25.8 | 64% | 257 | 0.105 |
| IV 38.5%–50% $(NH_4)_2SO_4$ precipitation | 19.6 | 48% | 46.4 | 0.422 |
| V G-200 | 13.1 | 32% | 14.5 | 0.810 |
| VI Immunoabsorbent | 11.9 | 29% | 6.35 | 1.870 |

Enhancement of purification by affinity chromatography seems difficult as long as no CAT stable reversible inhibitors are available. We think that our immunological purification can be further developed and possibly lead to a pure enzyme.

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