The samples were taken between 08.00 and 09.00 h and used up for analysis without delay. Starch was determined on ovendry (80 °C) samples according to the method of Sensabaugh and Rush[9].

Phosphorylase activity was estimated following the procedure described by Madhav Singh et al.[10]. A 10% (w/v) leaf homogenate in neutralised cysteine solution (0.015 M) formed the enzyme source. Other things remaining the same, the assay system comprised 0.4 ml citrate buffer (0.1 M, pH 6.2), 0.3 ml 1% soluble starch, 0.15 ml NaF (0.3 M), 0.5 ml homogenate and 0.15 ml G-1-P (0.05 M, pH 6.2). Pi liberated was estimated by chlorostannous acid reagent. The enzyme activity was expressed as μg Pi liberated/100 mg tissue/30 min under the conditions of the experiment.

With respect to phosphoglucomutase, the enzyme extract (10% leaf homogenate) was prepared following the procedure described by Cardini[11], and the activity was estimated according to the method of Ramasarma et al.[12]. The reaction mixture (1.5 ml enzyme preparation + 0.5 ml veronal buffer (pH 7.5) containing 8.25 μmoles G-1-P and 0.07 μmoles G-1,6-di P and 1.7 μmoles $MgSO_4$) was incubated for 30 min at 36 °C, after which the reaction was stopped by adding 1 ml 5N H_2SO_4 and the incubation tubes were kept in boiling water bath for 10 min to hydrolyse residual G-1-P. The tubes were then centrifuged and the supernatant was separated and made up to 25 ml. Pi was estimated in an aliquot of this. In the control, the H_2SO_4 was added to the reaction mixture before the start of incubation. The enzyme activity was expressed as μg Pi converted/150 mg tissue/30 min under the conditions of the experiment.

Results and discussion. The results obtained are presented in the table. It can be seen that in the diseased leaves, compared to the healthy, while phosphorylase activity increased, phosphoglucomutase activity decreased, more so at mature stage. Diseased leaves showed high starch content particularly at mature stage.

In the healthy leaves, phosphorylase activity remaining stationary, the increase in phosphoglucomutase activity and decrease in starch content from young to mature stage, seemed to have a correlation. In the diseased leaves, increased phosphorylase activity and decreased phosphoglucomutase activity could cause G-1-P to accumulate. Though, in the plant, G-1-P undergoes conversion to sucrose for translocation, this conversion seemed not to be freely occurring in the spiked sandal due to restricted phloem translocation. On the other hand, much of the G-1-P in these leaves appeared to be undergoing reconversion to starch for the reason that, of the substrate (adenosine diphosphoglucose/uridine diphosphoglucose) and the acceptor (oligosaccharide/maltose) needed for starch synthesis[4,13,14], the former can arise from the accumulating G-1-P, and the high diastatic activity in the starch-loaded diseased leaves could make the latter readily available. Thus, a considerable decline in the activity of phosphoglucomutase appeared to be a contributing factor for the starch accumulation in the spiked sandal leaves.

9 A. J. Sensabaugh and K. L. Rush, J. A. O. A. C. *55*, 209 (1972).
10 Madhav Singh, M. U. Beg, D. V. Singh, K. K. Tewari and P. S. Krishnan, Indian J. Biochem. *4*, 146 (1967).
11 C. E. Cardini, Enzymologia, *15*, 44 (1952/53).
12 T. Ramasarma, J. Sri Ram and K. V. Giri, Archs Biochem. Biophys. *53*, 167 (1954).
13 L. F. Leloir, M. A. De Fekete and C. E. Cardini, J. biol. Chem. *236*, 636 (1961).
14 T. Murata and T. Akazawa, Archs Biochem. Biophys. *114*, 79 (1966).

Immunological studies on the key enzyme of arginine biosynthesis in Pseudomonas aeruginosa

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Summary. A method to manufacture specific antisera with a minute amount of pure enzyme is presented. The influence of antibodies on activity and inhibition of an allosterically regulated enzyme was studied.

N-acetylglutamate 5-phosphotransferase (E2, EC 2.7.2.8) catalyzes the second step in the biosynthesis of the amino acid arginine (arg.). In Pseudomonas aeruginosa this enzyme possesses the key position of the whole anabolic sequence. It is inhibited allosterically by the endproduct arg.

It was of interest to investigate the influence of specific antibodies on the enzymic activity and the allosteric properties of the enzyme in vitro. Immunochemical techniques are considered valuable tools in all sectors of biochemical research. Therefore, it was hoped to add to the methodology in metabolic regulation. The arg biosynthesis was chosen as a model pathway because much work has been devoted to the knowledge of the respective enzymes at this institute (e.g.[3-7]).

Pseudomonas aeruginosa PAO-1, a wildtype strain, was cultivated by the method of Haas and Leisinger[3] with slight modifications. The assay procedure for E2 activity was described[3] as: The reaction catalyzed by the enzyme yields N-acetylglutamyl 5-phosphate and ADP. Hydroxylamine present in the assay mixture traps the product forming N-acetylglutamyl hydroxamate which in turn is complexed with Fe^{3+} to produce a brownish colour measurable at 540 nm.

The purification procedure[3] was followed, except for the early heat denaturation step. E2 in the crude extract was observed to be more heat-stable in the presence of N-

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2 Present address: R. U., Institute of Toxicology, Swiss Federal Institute of Technology, CH-8603 Schwerzenbach, Switzerland.
3 D. Haas and Th. Leisinger, Eur. J. Biochem. *52*, 365 (1975).
4 D. Haas and Th. Leisinger, Eur. J. Biochem. *52*, 377 (1975).
5 Th. Leisinger, Ch. O'Sullivan and D. Haas, J. gen. Microbiol. *84*, 253 (1974).
6 D. Haas and Th. Leisinger, Biochem. biophys. Res. Comm. *60*, 42 (1974).
7 Th. Leisinger and D. Haas, J. biol. Chem. *250*, 1690 (1975).

acetyl-glutamate (20 mM) alone than with 20 mM substrate and 5 mM arg. Therefore, a more efficient heat treatment at 72 °C (instead of 65 °C) could be conceived which enabled us to scale up the purification to 1200 g of bacteria as starting material. The enzyme was purified 1700fold (over the crude supernatant of the cell homogenate) to a final specific activity of 76.5 IU/mg of protein. 2 antisera were raised: a) anti-E2, a specific antibody against pure E2 was obtained from mice via the i.p. route (4 injections with 10 days intervals, each with 150 µl E2 solution containing 90 µg of pure enzyme + 150 µl of complete Freund adjuvant). b) anti-ppt, a rabbit was immunized intradermally with the method proposed by Axelsen et al.[8]. The immunogen was an immunoprecipitate resulting from the reaction of the above mouse anti-E2 with the enzyme. Precipitation was achieved by mixing 30 µl E2 solution containing 54 µg of enzyme with 70 µl of phosphate buffered saline (PBS) and 50 µl of anti-E2 serum (diluted 1:5 with PBS). The precipitate formed immediately and was washed 3 times with PBS. The amount of E2 administered was between 30 and 50 µg/kg b. wt of the rabbit per injection. The antisera were dialyzed against PBS to remove small molecular components (e.g. arg) before they were subjected to further experiments.

In Ouchterlony double diffusion assay[9], anti-E2 displayed 1 sharp precipitation line as run against the E2 preparation purified as described above (figure 1).

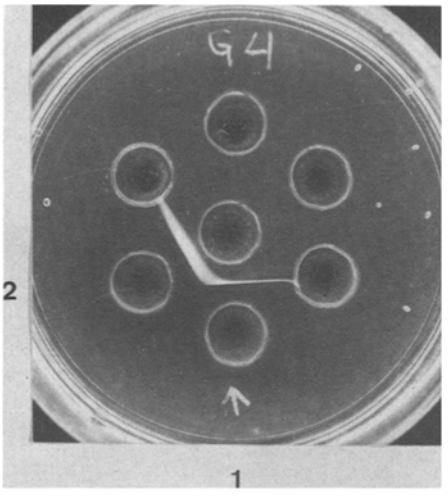

Fig. 1. Ouchterlony assay of pure E2 against mouse anti-E2. 1, 2, antisera from 2 different mice; center well, pure E2 (1.7 mg/ml).

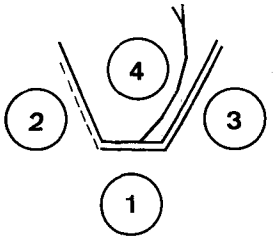

Fig. 2. Ouchterlony assay of preincubated E2 against anti-E2. 1, pure E2 (1.7 mg/ml); 2, pure E2 + 200 mM N-acetylglutamate; 3, pure E2 + 143 mM argininium chloride; 4, mouse anti-E2 diluted 1:5 with PBS.

The influence of the substrate and the allosteric effector arg on the antigen antibody reaction was investigated. It could be shown that the enzyme incubated with 140 mM arg developed a second precipitation band near the antibody well corresponding to an antigenic entity with a greater diffusion coefficient than the native enzyme and, therefore, with a smaller molecular size. Although it was reported that allosteric mechanisms are based only rarely on dissociation processes of oligomeric enzymes[10], in this experiment the inhibitor in non-physiological excess had a dissociating effect on the tetrameric enzyme[4]. 200 mM N-acetyl glutamate, on the other hand, did not affect the precipitation pattern (figure 2). This is a confirmation of the results of Haas and Leisinger[3] who, by means of gel filtration, found molecular weights of 230,000 d in presence of the substrate and of 65,000 d in presence of arg. In several instances, the formation of 2 distinct precipitation lines in Ouchterlony diffusion was observed, irrespective of the degree of purification of the enzyme and without any previous treatment of the antigenic solution. This phenomenon was probably due to dissociation association processes of the tetrameric enzyme, for immunoelectrophoresis[11] revealed a pattern of parallel (concentric) precipitin bands showing that the 2 antigens possessed identical electrical behaviour.

The second antiserum (anti-ppt) showed bispecificity, as expected, directed towards mouse immunoglobulins on the one hand and against E2 on the other. The immunization technique applied was developed recently. Its special advantage is that only small amounts of pure immunogen are required. The activity of the antiserum harvested after 3 injections was strong against mouse immunoglobulin and weak towards E2. It appears that for the case of immunization with immunoprecipitates, the amount of enzyme injected should be increased up to 70–100 µg/kg b. wt. The fact that the titre of anti-E2 activity in this serum was low can be understood from our knowledge of the structure of immunoprecipitates. Part of the antigenic determinants of the enzyme molecules are occupied by antibodies and, therefore, withdrawn from the contact with immunocompetent cells. In addition the immunogenic interactions are sterically hindered by the immunoglobulin molecules bound to the surface of the enzyme. In such determinant-deprived systems, low-zone tolerance may be found in higher concentration ranges of the applied immunogen than would be the case with free molecules.

During the stepwise addition of the 2 antisera to E2 solutions, both enzyme activity and allosteric inhibition decreased in an asymptotical manner. In solid precipitates half of the enzymatic activity was conserved (50 ± 6%); 2% of the original activity remained in the supernatant. Inhibition by arginine of the enzyme in the precipitate was reduced by about 10%.

Since the effects of the 2 antisera on the catalytic properties of the enzyme were the same, it was concluded that their specificity was identical or at least very similar. Thus it is possible to obtain monospecific antisera against enzymes or other proteins, using only very small amounts of purified immunogen. In the experiments presented

8 N. H. Axelsen, J. Krøll and B. Weeke, in: Quantitative Immunoelectrophoresis, IV. Production of antisera, p. 161. Universiteitsforlaget Oslo 1973.
9 O. Ouchterlony, Prog. Allergy *5*, 1 (1958).
10 J. Monod, J. Wyman and J. P. Changeux, J. molec. Biol. *12*, 88 (1965).
11 J. J. Scheidegger, Int. Archs Allergy *7*, 103 (1955).

here, the first antiserum (anti-E2) was monospecific. However, a method could be conceived which requires only partially purified enzyme: A first oligospecific serum can be reacted with a prepurified antigen solution. The precipitation band of the desired antigen is isolated (e.g. from immunoelectrophoresis slides) and used for immunization. Further investigations on another enzyme of arginine biosynthesis will be reported in a subsequent publication[12].

12 R. Voellmy and R. Utzinger, Experientia *33*, 1002 (1977).

Immunochemical studies on acetylornithine 5-aminotransferase from Pseudomonas aeruginosa

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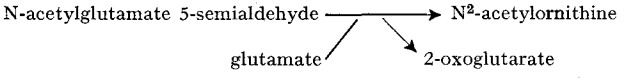
Summary. Mouse antibodies with specificity towards acetylornithine 5-aminotransferase (ACOAT) from Pseudomonas aeruginosa were used to study the structural similarities of serveral isofunctional enzymes from different sources. With the antibody directed against ACOAT, the amounts of enzyme present in cells grown under different conditions were determined. These experiments established that the enzyme is induced by arginine and is subject to repression by carbon sources.

In a previous paper[3] immunochemical studies on N-acetylglutamate 5-phosphotransferase, the second enzyme of arginine biosynthesis were presented. This communication deals with N^2-acetylornithine 5-aminotransferase (ACOAT, EC 2.6.1.11) which catalyses the fourth step in arginine biosynthesis.



A couple of important questions could be answered by immunological experiments.

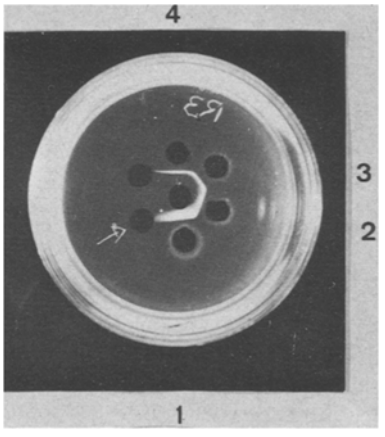


Fig. 1. Ouchterlony assay of pure ACOAT against mouse anti-ACOAT. 1 through 4, dilution series of mouse anti-ACOAT with PBS 1:1, 2, 4, 10; center well, pure ACOAT (0.5 mg/ml).

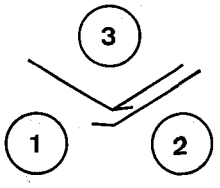


Fig. 2. Ouchterlony assay of 1, pure ACOAT preparation; 2, crude extract from Pseudomonas aeruginosa PAO1 against; 3, mouse anti-ACOAT.

Antibodies were raised in 3 mice using the procedure described previously[3]. ACOAT which was purified[4] by acid fractionation, ammonium sulfate precipitation, gelfiltration on sephadex G-100, and ionexchange chromatography on DE-52 (90% pure) served as immunogen. 1.3 ml of antiserum were obtained. The antibody displayed 1 sharp precipitation band in Ouchterlony double diffusion assay[5] when run against pure ACOAT (figure 1). Development against the immunogenic solution as well as against crude extracts revealed 2 distant bands indicating non-monodispersity of the solutions (figure 2). The second weak antibody population with specificity against a non-ACOAT antigen, however, did not disturb the experiments to be reported.

Specificity and cross-reactivity of the antiserum were examined. The P. aeruginosa wildtype strains PAC1 and PAT2 possessed an antigen which completely cross-reacted with ACOAT from strain PAO1. This result is indicative for the structural identity of the enzymes from different sources. Since the aminotransferases from E. coli and P. aeruginosa display similar structural and kinetic properties ([4,6], unpublished observations from this laboratory), it was of interest to learn about common antigenic specificities of the enzymes. No visible immunoprecipitate was formed with extracts of E. coli K12. This result indicates that the surfaces of the enzymes from the 2 species are not very similar. The question is legitimate whether or not at least the catalytic sites of the isofunctional enzymes have closely related shape. These common antigenic determinants should lead to cross-reactivity towards a specific antiserum. Immunochemical and enzymological studies demonstrate that both the antigen binding sites of immunoglobulins, as well as active sites of enzymes, are concave in shape, thus explaining why antibodies against active sites are not likely to be raised.

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3 R. Utzinger, Experientia *33*, 1000 (1977).
4 R. Voellmy and Th. Leisinger, J. Bacteriol. *122*, 799 (1975).
5 O. Ouchterlony, Prog. Allergy *5*, 1 (1958).
6 J. T. Billheimer and E. E. Jones, Archs Biochem. Biophys. *161*, 647 (1974).