Preparation of $^{125}$I-Catalytic Subunit of Aspartate Transcarbamylase and Its Use in Studies of the Regulatory Subunit*

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SUMMARY

A quantitative specific assay for the regulatory subunit of aspartate transcarbamylase is described. The assay, in which the native enzyme will not interfere even when present in large excess, is sensitive to 5 ng of regulatory subunit at a concentration as low as 0.1 µg per mg in crude extracts. The assay utilizes a $^{125}$I-labeled catalytic subunit which combines spontaneously with regulatory subunit to form a complex which co-migrates with the native enzyme on acrylamide gel electrophoresis in a position clearly separable from the $^{125}$I-catalytic subunit. The amount of regulatory subunit can then be calculated from the known weight ratio in the enzyme complex. Mutants of Salmonella typhimurium defective in aspartate transcarbamylase activity are shown to still synthesize regulatory subunit, while a mutant constitutive for the enzymes in the pathway for pyrimidine biosynthesis is shown to produce an excess of free regulatory subunit over native enzyme. A new procedure for iodination of proteins under mild conditions is described. It utilizes a preoxidation of iodide to iodine under conditions of stoichiometric or very low excess of oxidizing agent followed by reaction with the protein. It is used on a microscale to prepare $^{125}$I-catalytic subunit having properties virtually indistinguishable from the native protein.

Aspartate transcarbamylase, an allosteric enzyme, exemplifies the principle of regulation by feedback inhibition at branch points of metabolic pathways (1). Because of this property it has been the subject of a number of enzymatic and physical chemical studies (see Reference 2 for a recent review). The enzyme is composed of two types of polypeptide chains, six copies of the catalytic chain arranged as trimers on 2 catalytic subunits and six copies of the regulatory chain arranged as dimers on 3 regulatory subunits (3-6). The enzymatic activity, catalysis of the reaction between aspartate and carbamyl phosphate, is localized in the catalytic subunit (3) while the regulatory subunit, which binds CTP, the end product of the biosynthetic pathway, has no known catalytic function but is strongly implicated in the allosteric properties of the native enzyme (6).

In spite of the central role this enzyme plays in the regulation of pyrimidine biosynthesis, little effort has gone into studying the regulation of its synthesis in the bacterial cell. Part of the difficulty is that an independent assay for the regulatory subunit has not been available. In this paper we describe a quantitative specific assay for the regulatory subunit which may be applied to bacterial crude extracts. The assay will detect amounts of protein at least as sensitive as the enzymatic assay for the catalytic subunit. Some applications of this assay to studies of the synthesis of regulatory subunit in mutants of Salmonella typhimurium are presented.

The assay uses a $^{125}$I-labeled catalytic subunit which reacts with the regulatory subunit to give a $^{125}$I-labeled aspartate transcarbamylase. An assay of this type requires that the $^{125}$I-catalytic subunit be as competent as the native protein to combine with regulatory subunit. If it were not, the catalytic subunit present in the extract would compete in some unknown way for the regulatory subunit. We were concerned since methods of iodination have been shown to cause appreciable damage to proteins due to a high excess of oxidizing agent used to generate I$_2$ from I$^-$ (7). Milder methods of oxidation which have been developed (7-9) to circumvent this problem may be fairly restrictive. They may, for example, require the presence of an additional protein component which could obscure small amounts of $^{125}$I-labeled proteins, or they may be restricted to certain buffer and solvent conditions, or the techniques may be complicated and not easily adapted to small scale preparation. Because of the various limitations in these methods we sought a method of iodination which was easy to perform on a micro scale under a variety of solvent conditions, and which would not expose proteins to high concentrations of oxidizing agents.
EXPERIMENTAL PROCEDURE

Materials

Aspartate transcarbamylase from *Escherichia coli* was purified according to the method of Gerhart and Holoubek (10). The catalytic and regulatory subunits were prepared by reacting the native enzyme with 1-(3-(chloromercuri)-2-methoxypropyl)-urea (neohydrin) and separating the subunits by salt elution from a DEAE-cellulose column as described by Kirschner (11). The neohydrin obtained from K & K Laboratories is purified by reprecipitation by HCl from alkaline solution. Regulatory subunit from X. typhimurium LT-2 were performed on strains derived from a strain HD-58, kindly provided by G. O’Donovan. This strain is constitutive for the enzymes in pyrimidine biosynthesis (12) and we call this pyr-700.

Studies on regulatory subunit from *S. typhimurium* LT-2 were obtained from Mathcon, Coleman and Bell. It was titered by standard methods and found to be 79.4% pure. All catalytic T concentrations, however, are expressed assuming 100% purity; therefore to obtain the absolute concentration they should be multiplied by 79.4%. All other chemicals were reagent grade.

Methods

Iodination of Catalytic Subunit—In the procedure finally adopted, catalytic subunit samples at a concentration of about 9 mg per ml were dialyzed versus three changes of 0.04 M potassium phosphate buffer, pH 7.0, containing 2 mM NaN₃ EDTA to remove thoroughly any reducing agents. To an aliquot of the protein dipotassium succinate and diethylthiramyl phosphate are added to a final concentration of 10 mM and 8 mM, respectively. The final protein concentration should be 3 to 4 mg per ml. Stock solutions of 1 mM KI, 1.5 mM chloramine T, 2 mM H₂SO₄, 1 M potassium phosphate buffer, pH 7.0, and 1 M mercaptoethanol are prepared. On a sheet of paraffin spread over a wooden block are placed as consecutive drops 15 μl of 1 mM KI, 15 μl of 1.5 mM chloramine T, 15 μl of 1 M K₂HPO₄, pH 7.0, 180 μl of the protein solution, and 10 μl of 1 M mercaptoethanol. To the KI are added 15 μl of carrier-free ¹²⁵I⁻ (initially 40 μCi per ml), and to the chloramine T 1 μl of 2 M H₂SO₄. A glass rod made by flaming the end of a Pasteur pipette is used to mix the drops, which roll easily on the paraffin surface. The iodide is mixed with the chloramine T-sulfuric acid for 30 s, where I₃⁻ is generated. It is then mixed quickly with the concentrated buffer to neutralize the sulfuric acid and then with the protein sample where it is allowed to react for 2 min. It is then mixed with the mercaptoethanol to reduce any excess chloramine T or I₃⁻. The sample is next dialyzed in a microdialyzer against 0.04 M potassium phosphate buffer, pH 7.0, containing 2 mM mercaptoethanol, 1 mM KI, and 0.2 mM NaN₃ EDTA, and finally against two changes of the same buffer not containing KI. The level of iodination from a typical preparation was 1.5 10⁶ g and the specific activity was 6.7 × 10⁶ cpm per mg, for an over-all efficiency of 10% iodine incorporated. In other experiments the concentrations of iodide and chloramine T were varied.

Preparations of Bacterial Extracts—Bacterial cultures were grown with vigorous aeration at 37° in the minimal glucose medium of Vogel and Bonner (15), supplemented with 0.2 mM uracil. Cultures in late log phase of growth (approximately 10⁸ cells per ml), were cooled to 0°, washed once in 0.85% NaCl solution, and resuspended in 0.04 M Tris hydrochloride, pH 8.1, to give 2 × 10⁸ cells per ml. Aliquots of cells were disrupted by sonicing twice for 30 s with a Bronwell Biosonic III sonicator. Cell debris was removed by centrifugation at 18,000 × g for 30 min.

Assays for Regulatory Subunit—To 0.1 ml of solution containing regulatory subunit in 0.05 M Tris hydrochloride buffer, pH 8.1, containing 0.01 M mercaptoethanol and 0.2 mM zinc acetate are added approximately 3 μg of ³¹I-catalytic subunit having a specific activity of about 5 × 10⁶ cpm per μg. For bacterial crude extracts the total protein should be 3 mg per ml. The mixture is incubated for 30 min at 30° and a 50-μl aliquot is removed to be fractionated by acrylamide gel electrophoresis.

When assaying for total regulatory subunit in solution the sample must be pretreated with neohydrin to release free regulatory subunit from complexes with catalytic subunit. The protocol for this assay is as follows. To 0.1 ml of solution containing free regulatory subunit or aspartate transcarbamylase, or both, in 0.05 M Tris hydrochloride, pH 8.1, 0.05 μmole of freshly reprecipitated neohydrin is added at room temperature. After 2 min 0.16 μmole of carbamyl phosphate and 0.5 μmole of succinate are added followed by simultaneous addition of 1 μmole of mercaptoethanol and 6 μg of ³¹I-catalytic subunit. Higher concentrations of ³¹I-catalytic subunit may be used if high concentrations of aspartate transcarbamylase are thought to be present. After incubation at 30° for 30 min a 50-μl aliquot is removed for electrophoresis.

Polyacrylamide Gel Electrophoresis—The procedure for native polyacrylamide gel electrophoresis was that given by Jovin et al. (16). We used a 5-cm column of 5% polyacrylamide in 12-mm diameter glass tubes. The stacking gel was 1 cm long. The upper buffer was 0.05 M Tris-0.05 M glycine, pH 8.9, and a lower buffer 0.10 M Tris, pH 8.1. Electrophoresis was performed at 1 ma per tube for 5 hours. After electrophoresis the gels were stored in 5% methanol-7.5% acetic acid and destained electrophoretically. For counting the gels were frozen in Dry Ice and sliced into 1.5-mm sections with a gang of spaced razor blades. The gel slices were solubilized in 90% NCS (Nuclear Chicago) at 65° for 2 hours and counted in toluene 1,4-bis[(5-phenyloxazolyl)]benzene scintillation fluid in a Nuclear Chicago Mark I scintillation counter. Alternatively the gel slices were counted directly in a Nuclear Chicago Well γ counter.

RESULTS AND DISCUSSION

Iodination of Catalytic Subunit—In the Hunter and Greenwood procedure for iodinating peptides and proteins (17) I₂ is generated from I by the continuous action of chloramine T. This allows for high efficiency of incorporation through the continued recycling of I⁻ produced as a by-product of the iodination reaction or from the adventitious reduction of I₂ by reducing agents in the sample or buffer. Since the continuous presence of high concentrations of oxidizing agents has been shown to cause appreciable protein denaturation (7) we have used a procedure to generate I₂ first by reaction with low levels of chloramine T under conditions where the reaction is most favorable, low pH. This uses up all or most of the oxidizing agent. After neutraliza-
tion of the acid with concentrated buffer, the iodine is mixed with protein sample. This procedure has the disadvantage that maximum efficiency of iodine incorporation into the sample is 50% and the presence of any reducing agents in the sample will lower this still further. However, the extent of iodine incorporation is sufficient for most purposes. The sample is not exposed to high concentrations of oxidizing agents and the actual reaction of the iodine with the protein can be carried out in a variety of solvent conditions. The procedure by which the reaction is carried out on a microscale is described under "Methods."

Iodination of the catalytic subunit to the extent of 0.5 I per subunit by the method of Hunter and Greenwood (17) destroyed 97% of the enzymic activity. The same level of iodination under the same conditions using the preformed iodine method led to only a 15% loss of activity. In all later experiments succinate and carbamyl phosphate were used to protect enzymatic activity further.

Fig. 1 shows the effect of chloramine T concentration on the extent of iodination and on the enzymic activity of the catalytic subunit. As the chloramine T concentration is raised the enzymic activity drops. At 0.5 mM chloramine T (5-fold molar excess over I⁻) the activity is only 55%. Under conditions of chloramine T (35 mM) suggested by Hunter and Greenwood (17) for iodination of human growth hormone there is considerable protein precipitation. Samples iodinated to high levels, however, still retain their capacity to be reconstituted into an enzyme complex as judged by cellulose acetate electrophoresis using the procedure of Meighen et al. (5).

The extent of incorporation increases rapidly in the region of 0 to 0.1 mM and more slowly above 0.1 mM chloramine T. Since 1 mole of chloramine T oxidizes 2 moles of I⁻, stoichiometric reaction should take place at 0.05 mM chloramine T. Under conditions of this experiment, however, optimal incorporation without loss of enzymatic activity will take place at about 1.5:1 molar ratio of chloramine T to iodide at 0.1 mM KI. The sample produced at 0.1 mM chloramine T has 2 I per subunit and 85% enzymatic activity. There was 25% incorporation of the added iodine into protein which is 50% of theoretical. If sulfuric acid is eliminated in the initial step generating iodine, little iodination will take place at low levels of chloramine T.

The extent of incorporation increases with the iodide concentration when there is a fixed ratio of 1.2 moles of chloramine T per mole of iodide as shown in Fig. 2. Up until 3 to 4 I are incorporated per subunit, the enzymatic activity remains above 85%. As higher levels of iodine are incorporated, the activity drops quickly to about 40%. This is consistent with the fact that there are six exposed tyrosines in catalytic subunit which are accessible to chemical modification (11). Modification of the first three occurs with little loss of enzymatic activity while reaction of the next three occurs with 80% loss of activity. The derivative used in the following experiments has an average 1.5 I/10⁶ g. The derivative has 95% of the native enzymatic activity and an initial specific activity of 6.7 × 10⁵ cpm per mg. As shown in the next section, this derivative is indistinguishable from the native subunit in its capacity to combine with regulatory subunits. The above procedure has been applied successfully to a number of other proteins.

Microassay for Regulatory Subunit—We take advantage of the association reaction between ¹²⁵I-catalytic subunit and regulatory subunit to give a radioactive enzyme complex. As shown in Fig. 3 the electrophoretic properties of ¹²⁵I-catalytic subunit (B) are identical with the native subunit (A). Aspartate transcarbamylase reconstituted from the native (E) or ¹²⁵I-catalytic subunit (F) are indistinguishable from the native enzyme complex (D). It is also clear from Fig. 3 that ¹²⁵I-catalytic subunit

1 Titration of our chloramine T samples showed them to be only 79.4% pure. Therefore stoichiometry should be at 0.06. Some reducing agent in the sample may account for the small lag (to about 0.02 to 0.03 mg) which is found in Fig. 1. Taking this into account stoichiometry would be achieved at just under 0.1 mm chloramine T for 0.1 mm I⁻.

2 Some monoiodo- and some diiodotyrosine is produced. In the samples used for most of the assay experiments, 54% of the ¹²⁵I was in the form of monoiodotyrosine and 46% in the form of diiodotyrosine and none as iodohistidine as judged by hydrolysis and chromatography according to the method of Covelli and Wolff (18).

3 M. W. Kirschner and J. C. Gerhart, unpublished results.
The most sensitive assay shown in Fig. 4 corresponds to 0.015 μg of regulatory subunit and the precision is ±5%. This assay could detect less than 0.1 μg of regulatory subunit per mg of protein. It is limited by the capacity of the gel (approximately

**Table I**

**Competition of native and 125I-catalytic subunit with limiting amounts of regulatory subunit**

| Native catalytic subunit | 125I-catalytic subunit | 125I-catalytic subunit reconstituted |
|--------------------------|------------------------|-------------------------------------|
| μg                       | μg                     | %                                   |
| 0                        | 2.0                    | 49.2                                |
| 0.8                      | 1.2                    | 50.1                                |
| 1.0                      | 1.0                    | 51.5                                |
| 1.2                      | 0.8                    | 51.9                                |
| 1.4                      | 0.6                    | 51.0                                |
| 1.6                      | 0.4                    | 53.6                                |
| 1.8                      | 0.2                    | 54.9                                |

is easily separated from the enzyme complex by polyacrylamide electrophoresis.

**Competition of 125I-Catalytic Subunit with Native Subunit**—Under conditions of excess regulatory subunit more than 95% of the 125I-catalytic subunit is reconstituted into an enzyme complex as shown by either counting the radioactivity or scanning stained gels of the type shown in Fig. 3F. A more stringent test of the “nateness” of 125I-catalytic subunit would be to see whether it competes with the native subunit under conditions of limiting amounts of regulatory subunit.

Table I shows the results of a series of such competition experiments. Sufficient regulatory subunit is added to reconstitute about 50% of the catalytic subunit. The total amount of catalytic and regulatory subunit is kept constant but the fraction as 125I-catalytic subunit is varied from 0.1 to 1.0. It can be seen that the percentage of 125I-catalytic subunit incorporated (last column) is independent of the ratio of labeled and unlabeled protein; a result which shows that 125I-labeled catalytic subunit is just as competent as the unlabeled protein in the reconstruction reaction. If the native subunit were incorporated into the complex before any of the iodinated protein, the fraction of 125I-catalytic subunit reconstituted would be strongly dependent on the ratio of iodinated to native subunit. If the amount incorporated when no native catalytic subunit was present was 55% (first row), then when the native subunit was there at a 10-fold excess (last row) the ratio would be expected to drop to 5.5% if the native subunit were preferentially incorporated. The slight increase in the fraction incorporated shown in Table I as the amount of native subunit increases is opposite in direction from what would be expected from preferential incorporation of the native subunit.

**Linearity and Sensitivity of Assay**—Fig. 4 shows the amount of enzyme complex formed when different amounts of regulatory subunit are added to a given amount of 125I-catalytic subunit. From the known stoichiometry of the aspartate transcarbamylase complex the amount of regulatory subunit corresponding to a given amount of enzyme complex can be calculated. The assay is linear with respect to regulatory subunit until the weight fraction of regulatory subunit exceeds 0.5 that of catalytic subunits. At that point no more enzyme complex is formed as shown by the break in the curve. This weight ratio is in perfect agreement with the subunit composition of aspartate transcarbamylase (3) and attests to the fact that freshly prepared regulatory subunit prepared by newer procedures (11, 19) is fully competent to recombine with catalytic subunit. As shown also in Fig. 4, the assay is independent of the amount of 125I-catalytic subunit added. In that figure the triangles represent assays using 3 μg of 125I-catalytic subunit, while the circles represent assays using 1 μg of 125I-catalytic subunit.

**Fig. 3.** Polyacrylamide gels of electrophoresis of: A, native catalytic subunit; B, 125I-catalytic subunit; C, regulatory subunit; D, native aspartate transcarbamylase; E, aspartate transcarbamylase reconstituted from native catalytic subunit and excess regulatory subunit; F, aspartate transcarbamylase reconstituted from 125I-catalytic subunit and excess regulatory subunit. All gel experiments were performed as described under “Methods” and stained with Coomassie blue.

**Fig. 4.** Linearity of complex formation between 125I-catalytic subunit and regulatory subunit. Purified regulatory subunit in amounts between 0.016 μg to 3 μg was added to 1 μg (●) or to 3 μg (△) of 125I-catalytic subunit in 75 μl of buffer (0.025 M Tris-Cl, 14 mM Na2EDTA, 2.4 mM zinc acetate, pH 8.0). The samples were incubated at 30° for 30 min before adding to the acrylamide gel. The percentage of radioactivity found in the aspartate transcarbamylase region of the gel is plotted against the weight ratio of regulatory subunit to 125I-catalytic subunit.
150 μg of protein) and by background of 125I-catalytic subunit in the region corresponding to the enzyme complex which is very low. It is not limited by the specific activity of the 125I-catalytic subunit which can be raised by using more 125I-iodide. An assay of 0.015 μg of regulatory subunit corresponds to about 104 cpm in the region of the enzyme complex with the 125I-catalytic subunit used. By using longer gels for better resolution, it should be easy to extend the sensitivity of the assay if that were needed.

**Assays of Regulatory Subunit in Bacterial Extracts**—The assay for regulatory subunit was developed in the course of studies on mutants of *S. typhimurium* defective in aspartate transcarbamylase activity, pyrB mutants (20, 21). The enzyme from *S. typhimurium* is very similar to that from *E. coli* and hybrid molecules made up of regulatory subunits from one species and catalytic subunits from the other have been described (22). These latter studies indicate that we can use 125I-catalytic subunit from *E. coli* to assay for regulatory subunit in extracts of *S. typhimurium*.

A great deal of experience has shown that in vitro preparations of regulatory subunit are unstable over a period of days or weeks as assayed by their ability to reconstitute aspartate transcarbamylase. However regulatory subunit in the enzyme complex is very stable. We have tested the stability of free regulatory subunit in the crude extracts. Purified regulatory subunit from *E. coli* aspartate transcarbamylase was added to an extract of the deletion mutant pyrB655 (which will be shown below to have no detectable regulatory subunit) and assayed both immediately and after 90 min incubation. If 125I-catalytic subunit used. By using longer gels for better resolution, it should be easy to extend the sensitivity of the assay if that were needed.

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The results of a typical experiment of assaying regulatory subunit are given in Fig. 5. The extract was made from a stable point mutation in *S. typhimurium* mutant pyrB124 (strain TR2126). As can be seen this strain still synthesizes a significant amount of regulatory subunit as judged by the complex formed with 125I-catalytic subunit. This complex corresponds to 0.22 μg of regulatory subunit protein per 50 μl of extract used in the assay or 0.10% of total protein in the extract. As a means of comparison the parent strain, pyr-700, grown under identical conditions has approximately 0.5% of the cellular protein of as regulatory subunit, as is shown below. Thus the mutant pyrB124 synthesizes a significant amount of regulatory subunit which is somewhat less than the amount synthesized in a strain making native aspartate transcarbamylase. Possible reasons for this are discussed by Syvanen and Roth (13).

The assay can be used to show that structural genes for regulatory subunit and catalytic subunit lie near each other in *S. typhimurium*. Fig. 6 shows the results of an assay on pyrB655 (strain TR2156), a deletion mutation which covers all known point mutations in the pyrB gene. As can be seen in the histogram of the gel there is no detectable radioactivity above background in the region of the enzyme complex (<0.01% of total protein as regulatory subunit). It appears that no regulatory subunit chains are synthesized in this strain and the simplest explanation is that this deletion also covers the structural gene for the regulatory subunit protein. Since strains carrying the pyrB124 and pyrB655 mutations have an identical genetic background outside of the pyrB region, we may conclude that there is no nonspecific aggregation of 125I-catalytic subunits with other proteins in the extract and that radioactivity in the enzyme complex region of the gel is a true measure of the amount of regulatory subunit in the extract.

**Assay for Regulatory Subunit Complexed in Aspartate Transcarbamylase** It was important to show that in extracts of the pyrB mutants that reduced levels of regulatory subunit were not due to their combination with catalytically inactive but otherwise competent catalytic subunit protein. Therefore, we pretreated the extracts with a mercurial compound, neohydrin, which has been shown to dissociate catalytic and regulatory subunits (23). After addition of mercaptoethanol these regulatory subunits should combine with added 125I-catalytic subunit and not preferentially with the native catalytic subunits as shown above. In a paper dealing with the properties of these mutants, Syvanen and Roth (13) show that none of the mutants tested liberated additional regulatory subunit on treatment with mercurials.

This assay was first applied to an extract of a pyrB+ strain derepressed for aspartate transcarbamylase, pyr-700. The results of this experiment are shown in Fig. 7. There is a large increase in the amount of complex formed after addition of neohydrin. This represents 0.47% of cell protein as regulatory subunit or if one corrects for the competition of endogenous catalytic subunit the amount is 0.57%.

An unexpected result shown in Fig. 7 is that the sample not treated with neohydrin shows the appearance of the 125I-complex. This would suggest that free regulatory subunit is present in these extracts. The appearance of the complex is not due to subunit exchange between *E. coli* 125I-catalytic subunit and *S. typhimurium* aspartate transcarbamylase present in the extract. A test was made by mixing *E. coli* 125I-catalytic subunit and *E. coli* aspartate transcarbamylase both in standard assay buffer and in extracts of pyrB655. After 1 hour incubation at 30° no complex was detected in either test. It is possible, however, that subunit exchange could occur between *E. coli* 125I-catalytic subunit and *S. typhimurium* aspartate transcarbamylase. To show whether free regulatory subunit was indeed present in the extracts of the pyrB+ strain we attempted to see whether the complex-forming activity was associated with free regulatory subunit or with the enzyme complex. This was done by fractionating the extract by sedimentation through a sucrose gradient. A freshly prepared extract of pyr-700 was layered on the sucrose gradient and after centrifugation the various fractions were assayed for enzymatic activity and for the ability to form a complex with 125I-catalytic subunit. As can be seen in Fig. 8 the enzymatic activity which sediments with a sedimentation coefficient of 12 S is fully separated from the complex-forming activity which sediments at about 3 S, characteristic of regulatory subunit (3). No complex-forming activity is found under the peak for aspartate transcarbamylase (12 S), indicating that no subunit exchange has taken place. In addition to showing that free regulatory subunit is present in pyrB+ extracts, there does not appear to be free catalytic subunit present which would be detected in the sucrose gradient as a second slower peak of enzymatic activity of about 6 S. This result is in basic agreement with the findings of Nelbach et al. (24) and Perbal and Herve (25) who could see no free catalytic subunit in strains of *E. coli* derepressed for aspartate transcarbamylase and grown in standard medium.

At the present time, we have no explanation for why regulatory...
FIG. 5 (left). Distribution of radioactivity in sections of the polyacrylamide gel from the regulatory subunit assay on the crude extract of Salmonella typhimurium carrying the pyrB124 mutation. The amount of 125I seen in the area where aspartate transcarbamylase is found (Slices 6 to 11) corresponds to 0.28 µg of regulatory subunit. Excess 125I-catalytic subunit is found in Slices 16 to 23. Total protein added to the gel is 130 µg. Electrophoresis is from left to right.

FIG. 6 (center). Distribution of radioactivity in sections of the acrylamide gel from the regulatory subunit assay on extracts of Salmonella typhimurium carrying the deletion mutation pyrB655. There is no detectable 125I aspartate transcarbamylase peak in this sample which would occur at Slices 9 to 15. The background radioactivity in this region corresponds to less than 0.005 µg of regulatory subunit. Total protein added to gel is 110 µg.

FIG. 7 (right). Distribution of radioactivity in acrylamide gel from the regulatory assay made on Salmonella typhimurium extract from pyr-700, a pyrB+ strain. The assay for free regulatory subunit is given by the broken line and the solid line gives the assay for total regulatory subunit (the sample pretreated with neohydrin, as indicated). The amount of 125I-aspartate transcarbamylase formed in the sample not treated with neohydrin corresponds to 0.10 µg of free regulatory subunit and the amount in the neohydrin-treated sample corresponds to 0.70 µg. Total protein added to gel is 112 µg.

FIG. 8. Sucrose gradient sedimentation velocity fractionation of extract of pyr-700. A freshly prepared extract of pyr-700 (0.1 ml at a protein concentration of 15 mg per ml) was layered on a 0 to 24% sucrose gradient of volume 5 ml containing 0.04 M KH₂PO₄ buffer, pH 7, and 2 mM mercaptoethanol and 0.2 mM NaN₃-EDTA. The sample was spun for 6 hours at 90,000 rpm at 0° to 3° using a Beckman 12-75 B preparative centrifuge and a SW 65 LT rotor. Twenty-seven fractions, 0.2 ml each, were collected. The aspartate transcarbamylase activity denoted by —— was measured in each fraction and regulatory subunit was assayed in 11 of the fractions, denoted by — — —. The assay for the regulatory subunit was done directly in the sucrose solution as described under "Methods." Sedimentation is to the left.
assay to study the kinetics of derepression of excess regulatory subunit to see whether it occurs coordinately with derepression of enzymatic activity.

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REFERENCES

1. Gerhart, J. C., and Pandey, A. D. (1962) J. Biol. Chem. 237, 891
2. Jacobson, G., and Stark, G., in The Enzymes, in press
3. Gerhart, J. C., and Schachman, H. K. (1965) Biochemistry 4, 1054
4. Weber, K. (1968) Nature 218, 1116
5. Meighan, E. A., Pigiet, V., and Schachman, H. K. (1970) Proc. Nat. Acad. Sci. U. S. A. 65, 234
6. Coblentz, J., Pigiet, V., and Schachman, H. K. (1972) Biochemistry 11, 3396
7. Marchalonis, J. J. (1969) Biochem. J. 113, 299
8. McFarlane, A. S. (1966) Biochem. J. 92, 125
9. Rosa, U., Scassellati, G. A., Pennisi, F., Riccioni, N., Giannoni, P., and Giordani, R. (1964) Biochim. Biophys. Acta 66, 619
10. Gerhart, J. C., and Holoubek, H. (1967) J. Biol. Chem. 242, 2886
11. Kirschen, M. (1971) Ph.D. thesis, University of California at Berkeley
12. O'Donovan, G. A., and Gerhart, J. C. (1972) J. Bacteriol. 109, 1065
13. Syvanen, J. M., and Both, J. R., J. Mol. Biol., in press
14. Porter, R. W., Modede, M. O., and Stark, G. R. (1969) J. Biol. Chem. 244, 1846
15. Vogel, H. J., and Bonner, D. M. (1966) J. Biol. Chem. 238, 91
16. Jovin, J., China, A., and Naughton, M. A. (1964) Anal. Biochem. 9, 351
17. Hunter, W. M., and Greenwood, F. C. (1962) Nature, 194, 495
18. Covelli, I., and Wolff, J. (1966) Biochemistry 5, 860
19. Pigiet, V. (1971) Ph.D. thesis, University of California at Berkeley
20. Yan, Y., and Demerec, M. (1965) Genetics 52, 643
21. Syvanen, J. M. (1972) Ph.D. thesis, University of California at Berkeley
22. O'Donovan, G. A., Holoubek, H., and Gerhart, J. C. (1972) Nature New Biol. 238, 264
23. Gerhart, J. C. (1970) in Current Topics in Cellular Regulation, Vol. 2, Academic Press, New York
24. Nielbach, M. E., Pigiet, V., Gerhart, J. C., and Schachman, H. K. (1972) Biochemistry 11, 315
25. Pendal, B., and Herve, C. (1972) J. Mol. Biol. 70, 511
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