acidic pH, while the curve at the alkaline pH remains unaltered. This is exactly what happens in the haemolysates of Peruvian Indians[2] and in diabetic patients[3]. This fact strongly supports the idea that the factor(s) present in the haemolysates of the Peruvian Indians and in diabetic patients is the same or similar to that effective in producing the acclimatisation in cold-blooded animals.

The enhancement in the Bohr effect should increase the release of the oxygen to the tissues; at least in poikiloterms animals this factor should be of primary adaptive value.

*Riassunto.* In emolizzati di Tritone (*Triton cristatus*) e di pesce rosso (*Carassius auratus*) si è dimostrata la presenza di un fattore differente dai fosfati organici che influenza l'effetto Bohr modificando l'affinità dell'Hb per l'ossigeno a pH acido. Il fattore è specie aspecifico e influenza anche l'effetto Bohr della Hb umana.

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## Studies on the Quaternary Structure of the First Enzyme for Histidine Biosynthesis

The results of several studies suggest that the feedback sensitive first enzyme of a biosynthetic operon is involved in repression[1–5]. In *Salmonella typhimurium* the involvement of the first enzyme for histidine biosynthesis (G enzyme)[6], in regulation of the operon[2,4] is well documented. Genetic studies in the *his* system of *E. coli* K12 by Garrick-Silversmith and Hartman[7] and Goldschmidt et al.[8] show the great similarity between the 2 operons of these 2 organisms.

Studies with *E. coli* have been carried out in the present work with the aim of dealing with a slightly different system to which the findings of Ames, Hartman and Goldberger[9] in *Salmonella* could be applicable. We have purified the G enzyme from *E. coli* K12 and report here the results of Sephadex G-200 gel filtration experiments of association-dissociation with substrates and ligands, correlating the quaternary structure of the enzyme with its regulatory role in the *his* operon.

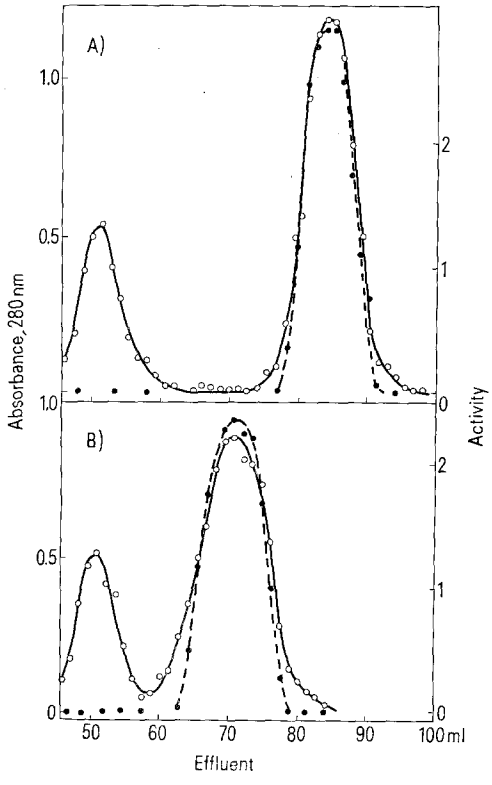


Fig. 1. Elution patterns in the absence of histidine (1A) and in the presence of 0.4 m*M* histidine (1B). (---○---, $A_{280}$; ---●---, specific activity in arbitrary units). The standard proteins were: Cytochrome C, mol. wt. 13500; Chymotrypsinogen A, 25000; Ovalbumin, 45000; Bovine serum albumin, 67000; Aldolase, 147000; Catalase, 240000; and Ferritin, 540000.

*Materials and methods.* The source of the enzyme was a regulatory mutant, OA111, 30-fold derepressed, obtained by ethyl methanesulfonate mutagenesis[10] in strain JC5459 ($F^-$ trp$^-$ thi$^-$ lac 74 str$^r$, from Clark's collection[11]), and selected by its resistance to triazolalanine[12]. The mutant was grown with aeration in a New Brunswick gyrotory shaker at 37 °C, and the pelleted cells were kept frozen.

The purification procedure, following the method of Whitfield[13], is based on the solubility changes in $(NH_4)_2SO_4$ of the enzyme in presence or absence of histidine[14]. Using acid precipitations (S. M. Parsons, personal communication), about 90% of the enzyme precipitated with 1 m*M* histidine at pH 5.1. The enzyme was purified

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more than 100-fold and the enzymatic activity determined according to MARTIN et al.[15].

Calibration proteins were obtained from Boehringer Manheim; DL-1, 2, 4-triazolalanine, from Cyclo; Trizma base, ethyl methanesulfonate, phosphoribosyl pyrophosphate (PRPP), AMP and ATP, were Sigma products. In all the experiments, the sample was previously resuspended in the elution buffer.

*Results and discussion.* Column experiments at 2°C: A Sephadex G-200 column (15 × 900 mm) was equilibrated with 0.1 *M Tris* – HCl buffer, pH 8.0, containing 0.1 *M* NaCl, 0.5 m*M* EDTA and 2.8 m*M* 2-mercaptoethanol, with a flow rate of 7 ml/h (Void volume, 50.5 ml). The elution pattern obtained is shown in Figure 1A. Although in the last purification step on Sephadex G-200 the fractions pooled were retarded in the gel (mol. wt. of about 140,000), however in all the experiments reported here approximately 25% of the protein is excluded; as it will be seen later, this fraction corresponds to inactive aggregate enzyme with a mol. wt. bigger than 300,000 (exclusion limit of the gel). The active G enzyme is eluted at a position that corresponds to a mol. wt. of 68,000, without lower species. In *Salmonella typhimurium*[13,16] the enzyme ('stabilized' by histidine) is composed of 6 subunits of 36,000 daltons, and on *E. coli* KLUNGSOYR and KRYVI[17] report similar results. Therefore, the peak considered would correspond to a dimer.

The same experiment in the presence of 0.4 m*M* histidine is represented in Figure 1B. The activity coincides with a size of 138,000 (tetramer).

Column experiments at room temperature (ca. 20°C): When carrying out the experiments at room temperature, the enzyme loses a great part of its activity, but not the ability to associate (the association processes would be enhanced[14]). A column (9 × 300 mm) was equilibrated with basal buffer (0.1 *M Tris*-HCl, pH 8.0, containing 2.8 m*M* 2-mercaptoethanol), and the flow rate was 6.5 ml/h.

Figure 2A presents the elution pattern in the absence of histidine: the fraction superaggregated appears in the void volume (8.5 ml); the second peak shows a mol.wt. of 135,000; however, the main fraction of the protein behaves as a dimer (67,000 daltons), i.e. in the absence of any ligands the dimer-tetramer equilibrium is displaced to the former. In Figure 2B the same experiment with 0.4 m*M* histidine is shown: the main peak has a size of 205,000–210,000 (hexamer), and a minor tetrameric species also appears. There is neither dimer, nor monomer. As we shall see later in the effect of phosphoribosyl pyrophosphate (PRPP), the more active form is the disaggregated enzyme; the histidine effects a drain of dissociated species, with subsequent decrease of activity.

The substrate PRPP (Figure 2C) reverses totally the histidine effect, thus giving a pattern similar to Figure 2A. It should be noticed that the excluded highly associated enzyme is not sensitive to dissociation. The PRPP is in the concentration used in the in vitro assay; we are therefore measuring the activity of the dimer, i.e. the dimer is the active species 'par excellence'. More experiments are required to establish the degree of dissociation with different concentrations of PRPP.

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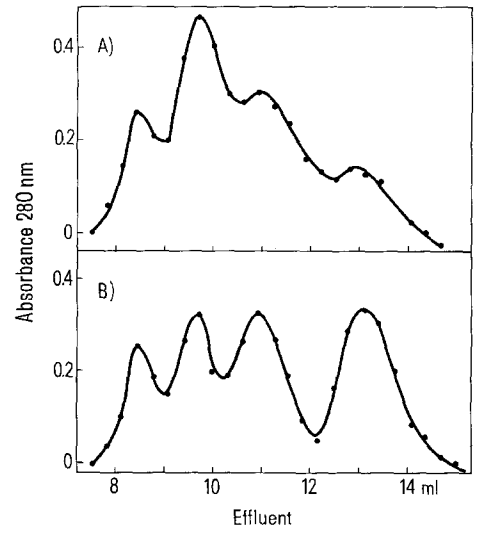


Fig. 2. A) Elution with basal buffer. B) Idem basal plus 0.4 m*M* histidine. C) Idem basal plus 0.4 m*M* histidine and 0.6 m*M* PRPP.

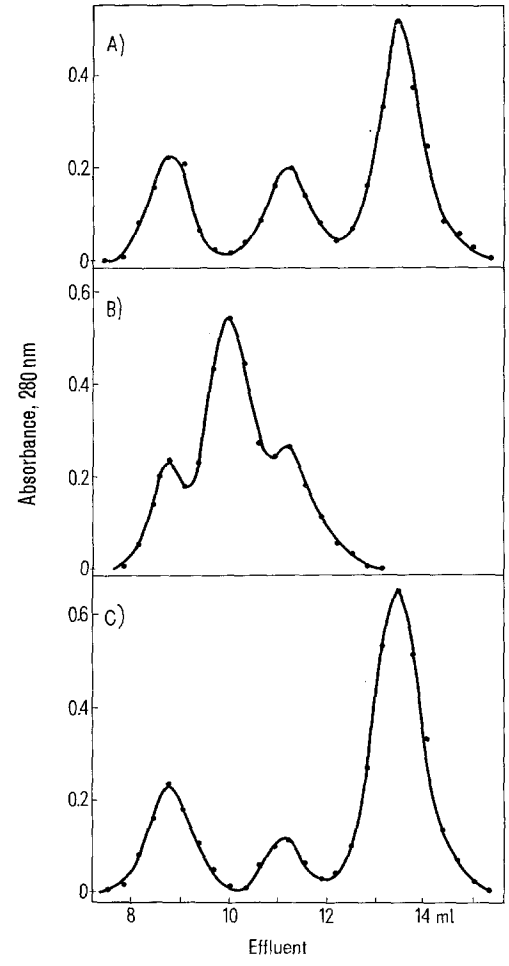


Fig. 3. A) Experiment with basal buffer plus 6.4 m*M* ATP (the same concentration used in the in vitro assay). B) Elution with basal buffer plus 20 m*M* 2-thiazolalanine (50 times less effective in feedback inhibition than histidine[19]).

The ATP (Figure 3A) also associates the enzyme, although not so highly and surely not so specifically as histidine. This effect, contrary to that of the PRPP, is hard to explain if ATP binds only at the catalytic center; therefore another site(s) (allosteric) for ATP would be required, as KLUNGSOYR and KRYVI[17] have postulated. This finding is also in agreement with the KRYVI and KLUNGSOYR kinetic data[18] referring to ATP inhibition at high concentrations.

In Figure 3B it is clearly displayed that 2-thiazolalanine (also a feedback inhibitor), associates the protein not so efficiently as histidine does; this fact agrees with our thiazolalanine studies in vivo, in which the wild type is more resistant to this inhibitor than other *E. coli* K12 strains and *S. typhimurium* LT 2.

In the elution of a denatured sample (lyophilized and stored for one month at −15 °C) with only 5% of the initial activity (experiment not shown), nearly all the protein is excluded, and there are small amounts of hexamer and tetramer that could be responsible for the residual activity elicited by dissociation by the PRPP. It is conceivable that reversible tetramer and hexamer formation are intermediate steps towards irreversible high order polymerization.

Kinetic data of derepression of the histidine operon (unpublished experiments) point towards a positive control for the first enzyme. We are working on a biosynthetic model of regulation, of the JACOB-MONOD type, in which the association-dissociation processes reported in this paper play a central role[20].

*Resumen.* Se han realizado estudios de asociación-disociación por filtración en gel con el primer enzima de la biosíntesis de histidina en *Escherichia coli*, en presencia de sustratos y ligandos. Se observa una interconversión reversible entre las formas dímero, tetrámero y exámero, y una agregación irreversible de orden superior.

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## RNase Activities in Blood Serum of Several Vertebrates

The presence of Ribonuclease in the serum of several animals has been reported[1–5]. Problems of like the origin and biological function of these serum enzymes were approached by some authors, but they remain controversial. Origin of serum RNase has been accepted to be pancreatic[6], but no direct evidence was ever reported. On the contrary, the hypothesis that the pancreas is the exclusive source of serum RNase was not sustained by pancreatectomy experiments[7].

Whatever the uncertainties in this field, this protein proves to be very useful for philogenetic studies, as recently emphasised by BARNARD[8,9] who demonstrated the existence of three well-defined classes among the vertebrates as concerns RNase content of the pancreas. As similarities between pancreatic and seric RNase have been proved to exist in different animal species[5], a comparative study of serum RNase activities in several vertebrates has been undertaken in the present investigation.

Representative species of the 3 groups classified by BARNARD were chosen for our purpose. Among those with very high pancreatic RNase activities – 200 to 1,200 μg/g of pancreatic tissue (group A) – we have studied ox, hamster, goat, rat, sheep and guinea-pig. Belonging to the group with a very low content of pancreatic nucleasic activity – 0 to 20 μg per g (group C) – we studied cat, pigeon, rabbit, dog and human sera. Other species included in this study, horse, pig and chicken, belong to the intermediary group, the group B, in BARNARD's classification.

*Materials and methods.* Blood samples from ox, sheep, goat, pig and horse were obtained from the slaughter-

Total serum protein in vertebrates

| | Total protein[a] (mg/ml serum) |
|---|---|
| Ox (*Bos taurus*) | 74.4 |
| Hamster (*Mesocricetus auratus*) | 63.7 |
| Goat (*Capra hircus*) | 80.5 |
| Rat (*Rattus norvegicus*) | 71.4 |
| Sheep (*Ovis aries*) | 61.4 |
| Guinea-pig (*Cavia porcellus*) | 50.0 |
| Horse (*Equus caballus*) | 72.8 |
| Pig (*Sus scrofa*) | 81.1 |
| Chicken (*Gallus domesticus*) | 43.1 |
| Cat (*Felis catus*) | 72.2 |
| Pigeon (*Columba livia*) | 27.6 |
| Rabbit (*Oryctolagus cuniculus*) | 60.7 |
| Dog (*Canis familiaris*) | 60.7 |
| Mouse (*Mus musculus*) | 69.9 |
| Human | 86.0 |

[a] Mean values of at least 3 different samples.

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