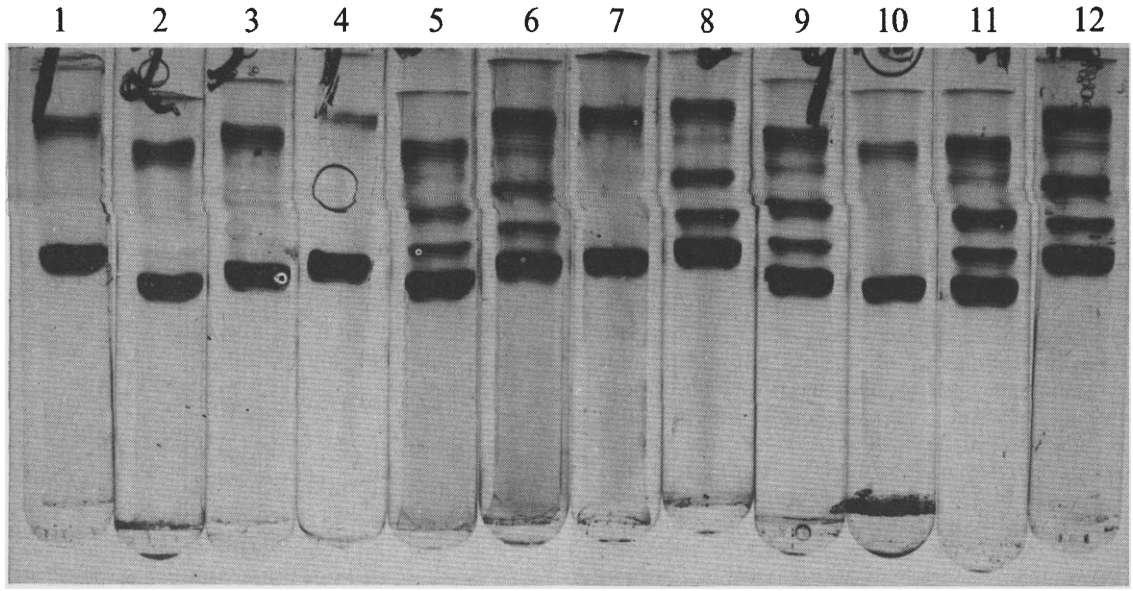

Fig. 3 Effect of temperature and *p*H on the hybridization reaction. Standard buffer throughout. Tubes 1, 2 and 3 (*p*H 6, 8 and 9), 2 h at 0° C. Tubes 4, 5 and 6 (*p*H 6, 8 and 9), 30 min at 37° C. Tubes 7, 8 and 9 (*p*H 6, 8 and 9), 60 min at 37° C. Tubes 10, 11 and 12 (*p*H 6, 8 and 9), 2 h at 37° C.

We feel that the most significant feature of these findings is our hypothesis: *in vivo* regulation of LDH isozyme levels could, in part, be by apogenetic parameters. That is, levels of isozymes may not be absolutely controlled by functioning of gene elements or repressors, but may also be sensitive to environmental stresses.

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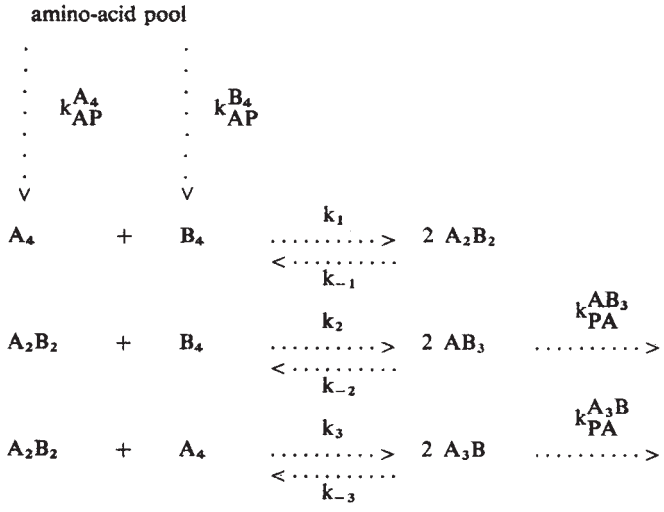
New Theory of the Control of Protein Concentrations in Animal Cells

THE molecular basis for the regulation of intracellular concentrations of protein has been studied intensively since the discovery of the transfer of genetic information from DNA to RNA to protein[1,2]. We wish to present experimental evidence in support of a model which considers regulation by post-translational events. This model takes into account the contributions of synthesis, degradation, and protein interactions to the regulation of intracellular protein concentrations. The model explains how protein concentrations could change within a cell while rates of transcription and translation remained constant. Although the experimental evidence in support of the model was collected from studies of the lactate dehydrogenase (LDH) system in the rat, the model may be generally applicable to other subunit proteins and other organisms.

Of the many enzymes known to exist in subunit form, LDH is the most familiar[3–5]. In most mammalian tissues the LDH isozymes are tetramers composed of all combinations of two distinct subunits designated A and B. In this notation isozymes labelled A_4, A_3B, A_2B_2, AB_3, and B_4 correspond to LDH-5, LDH-4, LDH-3, LDH-2, and LDH-1, respectively. LDH-1 or B_4 is the most negatively charged of these isozymes. Vertebrate tissues have characteristic distributions of LDH isozyme. For example, in adult mammalian heart muscle, tetramers containing B subunits usually predominate, whereas A subunits are present in the greatest concentrations in mammalian skeletal muscle and liver.

The LDH isozyme patterns of rat heart during development were of particular interest because they suggested an interdependence of the tissue concentrations of the five isozymes: Fig. 1 shows that in the rat heart A_4 and A_3B decreases sharply just after birth whereas concentrations of A_2B_2 decrease and then increase to adult levels. Concentrations of AB_3 and B_4 begin at low and slowly rise to adult levels. These changes could be accounted for by a model which involved the exchange of subunits between enzymatically active tetramers. After considering several possibilities we assumed the following scheme as the simplest way of inter-relating isozymes by reactions between tetramers:



We propose that homopolymers A_4 and B_4 are synthesized by the well known transcription–translation route and that the overall synthetic processes, represented by the rate constants $k_{AP}^{A_4}$ and $k_{AP}^{B_4}$, are zero order with respect to these proteins. In this scheme A_4 is also produced by second order reactions with rate constants k_{-1} and k_{-3} and B_4 by reactions with rate constants k_{-1} and k_{-2}. Heteropolymers A_2B_2, AB_3, and A_3B are produced by second order reactions as indicated. In our model, interaction of these tetramers expresses the rate determining steps as well as the overall stoichiometry of the process. Rapid intermediate reactions of monomeric and/or dimeric forms are not excluded. Dotted lines indicate the possibility of such intermediate reactions. According to this model only the asymmetrical heteropolymers AB_3 and A_3B are degraded in the sense that the subunits of which they are composed are removed from the system. The details of these degradation reactions are not specified, but they are assumed to be first order processes. All the proposed reactions might be enzyme catalysed or might proceed without specific catalysis. One of the properties of this model, and a principal reason for suggesting it, is the fact that it can be tested experimentally, for all proposed intermediates can be measured.

First, we can use development data. (Rate equations derived from the model are given at the end of this communication.) The ten rate constants can be obtained from the development data by choosing any two points on the time curve for each isozyme, setting the derivative equal to the slope and inserting the measured values of the isozymes at that point into equations (1) to (5). This yields ten linear equations with ten unknowns which can be solved simultaneously. There is the additional requirement that all rate constants are positive. We used the adult steady state values where the slope is zero and a point 16 h after birth to calculate the rate constants shown in Table 1. These rate constants were then used in equations (1) to (5) to calculate the changing values of the five isozymes in the rat heart during development. This was accomplished with the aid of a full Fortran program in an IBM 360/30 computer. The calculated values and

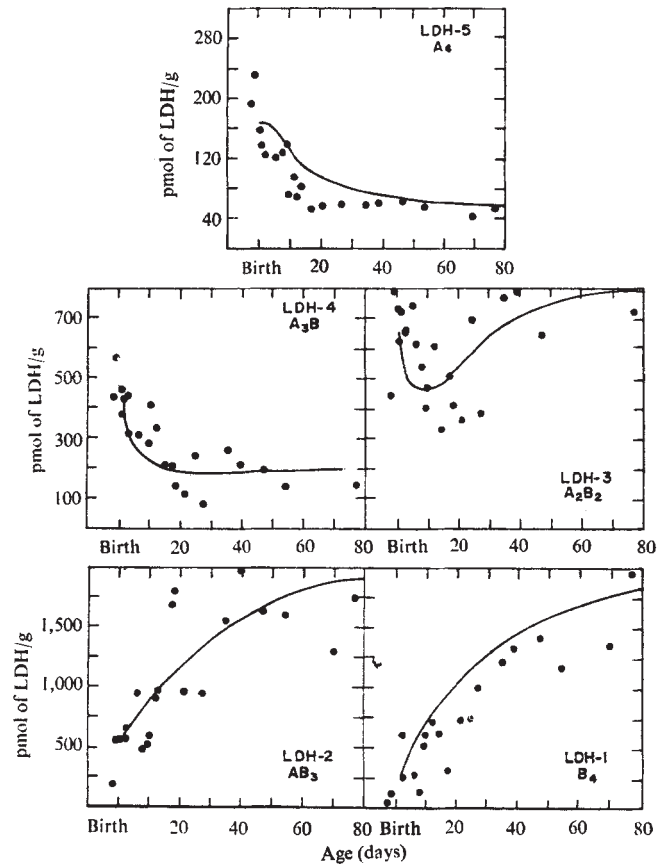


Fig. 1 LDH isozyme concentrations during development of rat heart. At the indicated times before and after birth rats were killed by decapitation and the hearts were weighed and homogenized in ice; 10 ml. of 0.02 M Tris-Cl⁻ buffer, *p*H 7.4, was used for each gram of heart. After centrifuging at 10,000*g* for 10 min in a refrigerated centrifuge the supernatants were placed on 'DEAE-Sephadex' (A-50 coarse) columns (4×15 cm). Isozymes were separated by stepwise elution with NaCl concentrations in the same buffer correlated as follows: A_4, 0.0 M; A_3B, 0.10 M; A_2B_2, 0.14 M; AB_3, 0.18 M; B_4, 0.22 M. Details of these separations are to be published elsewhere[6]. The closed circles represent the experimental points. Early experimental values (up to 20 days after birth) represent the pooled tissues from several rats. Later values are for single animals. During the early times better isozyme separations were obtained using 0.08 M NaCl for A_3B and 0.12 M NaCl for A_2B_2. The lines represent theoretical values for the isozymes calculated using the rate constants shown in Table 1 and equations (1) to (5).

experimental data (Fig. 1) are sufficiently close to support the model.

The point–slope procedure we used to obtain rate constants forces the theoretical curves to fit the data only at two points. The real test of the theory is how well the curves fit the observations for points intermediate between the initial and the steady state concentrations of isozymes. None of the ten rate constants can be manipulated arbitrarily without changing the shapes of all five theoretical curves and thus the close agreement of the predicted and observed curves is striking.

Second, we can use amino-acid incorporation data. Incorporation of ^{14}C-labelled amino-acids into the individual LDH isozymes was accomplished by continuous administration of isotope in the adult rat diet and measured by precipitation with LDH specific antibodies[7]. Rate equations describing the ^{14}C amino-acid incorporation into the five isozymes according to the model are given at the end of this communication.

We calculated theoretical curves for isozyme labelling by substituting measured values of the steady state concentration of isozymes into equations (6) to (10) together with the rate constants which we had calculated from the development data. The initial labelling was taken as zero, and subsequent labelling

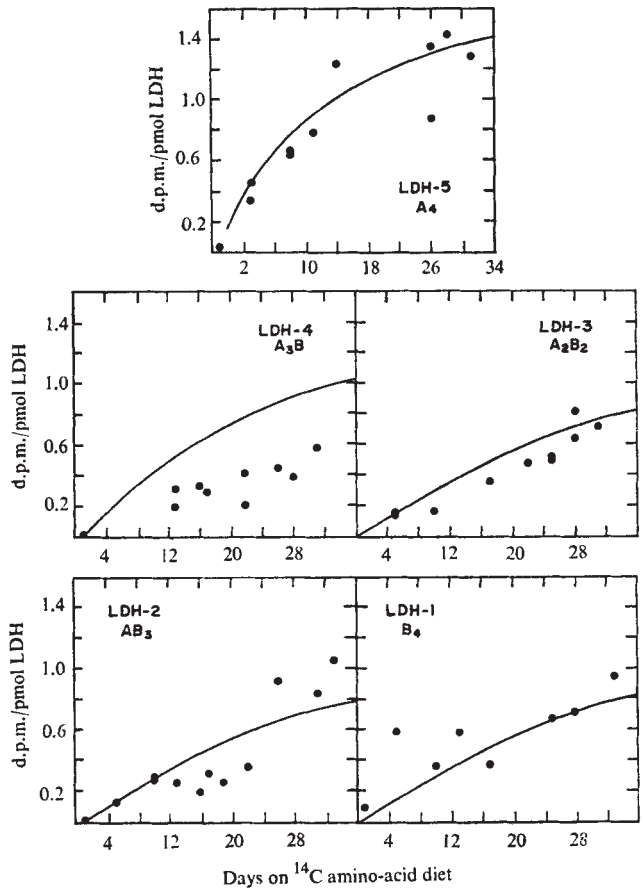


Fig. 2 ^{14}C-Amino-acid incorporation into LDH isozymes of adult rat heart. Male Sprague–Dawley rats (350–450 g) were housed individually in cages and fed a commercially prepared liquid diet containing 19.6% amino-acids by weight ('Nutrico', Nutritional Biochemicals, Inc.). The animals received 35 ml. of the food daily which after 5 days was supplemented with 500,000 d.p.m. of ^{14}C-labelled yeast protein hydrolysate (1,000 μg/mg; Schwarz)/ml. of diet. They had water *ad libitum*. At intervals after being placed on the radioactive liquid diet, 2 rats were decapitated between 0645 h and 0700 h. Heart isozymes were separated by 'DEAE-Sephadex' chromatography, precipitated with specific antibodies and prepared for counting as described before. Closed circles represent the experimental points, and the lines represent theoretical values for the isozymes calculated using the rate constants shown in Table 1 and equations (6) to (10).

was calculated by computer from equations (6) to (10). These curves, together with experimentally determined values, are shown in Fig. 2. The agreement between observation and theory provides further support for the model. This agreement is especially significant because we used a set of rate constants derived from independent data.

Third, the model can be tested by *in vitro* experiments on reactions between isolated isozymes. In mild laboratory conditions we observed the conversion of A_3B to A_4 and A_2B_2 (k_{-3} reaction in the model). The conversion can be observed in a few minutes at 50° C and in a few days at 37° C. This type of reaction is enhanced by a sulphydryl reagent and is sensitive to phosphate ion concentration (personal communication from D. B. Millar and M. R. Summers). In these simulated physiological conditions, AB_3 was produced from A_3B (Fig. 3). This result can be explained by the series of reactions we propose.

Proteins interacting in the manner we propose would be sensitive to intracellular factors such as *p*H, pressure, ionic strength, temperature, substrate and coenzyme concentrations, and any variable which could influence the rate constants controlling interactions between proteins. Each tissue has its own characteristic intracellular environment, and so it would also have its own unique set of rate constants. Thus, the type of post-translational control which we propose would offer the organism a sensitive way of regulating tissue protein concentrations. A change in the temperature of the water has been found to produce a shift in the LDH patterns of fish[8]. This would be understandable within the framework of the new model, for bimolecular rate constants are generally temperature dependent.

The new theory explains how proteins might be "degraded" within a cell in the absence of proteolytic enzymes and thus gives a partial answer to the question of what determines the specificity of protein biodegradation.

Perhaps the most significant implication relates to protein differentiation. A widely accepted explanation of differentiation is that the amount of protein within a cell is directly proportional to the activity of the gene coding for that particular protein. From our theory it is clear that even though a protein may be present in a cell in very small amounts, the rate of synthesis of that protein may be appreciable. This point is illustrated by rat heart LDH-1 which is present in the adult organ in a concentration at least thirty times greater than that of LDH-5, yet the zero order rate constants for synthesis of the two isozymes are the same (Table 1).

Finally, we should like to emphasize that the model for subunit interchange which we have used to interpret our data is only one of several possibilities. Fourteen different bimolecular reactions can be written with pairs of isozymes as products and reactants. Allowing for reversibility of these reactions means that there are twenty-eight different possibilities for subunit interchange. Degradation and synthesis reactions can also be written for each isozyme, adding ten more possible reactions. Thus, specific models can be constructed by choosing appropriate reactions from among the thirty-eight possibilities. It seems that there are many models and that any particular model would have to be chosen arbitrarily. There are, however, restrictions on the choice of reactions, and we

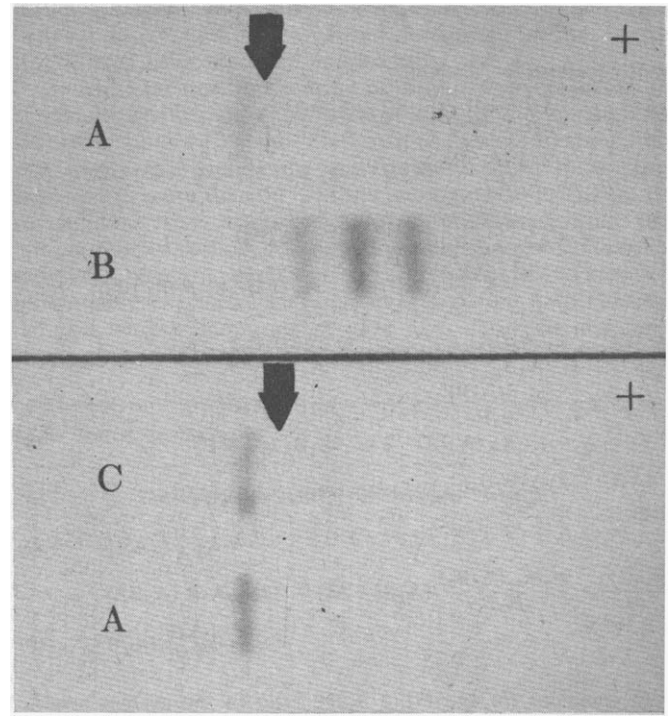


Fig. 3 *In vitro* interconversion of LDH isozymes. Electrophoresis was performed on cellulose acetate strips in 0.02 M glycine buffer, *p*H 10.0, for 12 min at 100 V, using a Millipore electrophoresis apparatus. The strips were stained by the nitroblue tetrazolium technique at *p*H 10.0. A, LDH-4 obtained from rat skeletal muscle. The concentration of enzyme is 3.3 U/ml. B, Rat heart homogenate at a concentration of 25 LDH U/ml. and 7.4 mg of protein/ml. C, Sample A after heating at 37° C for 40 min in 0.02 M phosphate buffer, *p*H 7.4, and 10^{-3} M Cleland reagent.

Table 1 Rate Constants for LDH Isozyme Reactions in Rat Heart

$k_{AP}^{A_4}$	=	153.0 pmol/g/day
$k_{AP}^{B_4}$	=	157.0 pmol/g/day
k_1	=	7.45×10^{-4} g/pmol/day
k_2	=	1.94×10^{-3} g/pmol/day
k_3	=	2.50×10^{-3} g/pmol/day
k_{-1}	=	7.41×10^{-6} g/pmol/day
k_{-2}	=	7.53×10^{-4} g/pmol/day
k_{-3}	=	8.89×10^{-4} g/pmol/day
$k_{PA}^{A_3B}$	=	1.00 day^{-1}
$k_{PA}^{AB_3}$	=	0.0931 day^{-1}

are developing a general theory which will include techniques for using these restrictions to select those models which are consistent with the data.

This work was supported by a grant from the American Cancer Society. We thank Joe Dixon for writing the computer programs.

Rate equations for development

$$\frac{dA_4}{dt} = k_{AP}^{A_4} + k_{-1}(A_2B_2)^2 + k_{-3}(A_3B)^2 - k_1 A_4 B_4 - k_3 A_4 (A_2B_2) \quad (1)$$

$$\frac{dB_4}{dt} = k_{AP}^{B_4} + k_{-1}(A_2B_2)^2 + k_{-2}(AB_3)^2 - k_1 A_4 b_4 - k_2 B_4 (A_2B_2) \quad (2)$$

$$\frac{dA_2B_2}{dt} = 2k_1 A_4 (B_4) + k_{-2}(AB_3)^2 + k_{-3}(A_3B)^2 - 2k_{-1}(A_2B_2)^2 - k_2 B_4 (A_2B_2) - k_3 A_2B_2 (A_4) \quad (3)$$

$$\frac{dAB_3}{dt} = 2k_2 B_4 (A_2B_2) - 2k_{-2}(AB_3)^2 - k_{PA}^{AB_3} AB_3 \quad (4)$$

$$\frac{dA_3B}{dt} = 2k_3 A_4 (A_2B_2) - 2k_{-3}(A_3B)^2 - k_{PA}^{A_3B} A_3B \quad (5)$$

Rate equations for labelling

$$\frac{dA_4^*}{dt} = k_{AP}^{A_4} \frac{Nf(\bar{a}^*)}{A_4} + \frac{k_{-1}(A_2B_2)^2}{A_4}(A_2B_2^*) + \frac{k_{-3}(A_3B)^2}{A_4}(A_3B^*) - k_1 B_4 (A_4^*) - k_3 A_2B_2 (A_4^*) \quad (6)$$

$$\frac{dB_4^*}{dt} = k_{AP}^{B_4} \frac{Nf(\bar{a}^*)}{B_4} + \frac{k_{-1}(A_2B_2)^2}{B_4}(A_2B_2^*) + \frac{k_{-2}(AB_3)^2}{B_4}(AB_3^*) - k_1 A_4 (B_4^*) - k_2 A_2B_2 (B_4^*) \quad (7)$$

$$\frac{dA_2B_2^*}{dt} = \frac{k_1 A_4(B)_4}{A_2B_2}(A_4^* + B_4^*) + \frac{k_{-2}(AB_3)^2}{A_2B_2}(AB_3^*) + \frac{k_{-3}(A_3B)^2}{A_2B_2}(A_3B^*) - k_2 B_4 (A_2B_2^*) - k_3 A_4 (A_2B_2^*) - 2k_{-1} A_2B_2 (A_2B_2^*) \quad (8)$$

$$\frac{dAB_3^*}{dt} = \frac{k_2 A_2B_2 B_4}{AB_3}(A_2B_2^* + B_4^*) - 2k_{-2} AB_3 (AB_3^*) - k_{PA}^{AB_3} (AB_3^*) \quad (9)$$

$$\frac{dA_3B^*}{dt} + \frac{k_3 A_4 (A_2B_2)}{A_3B}(A_2B_2^* + A_4^*) - 2k_{-2} A_3B (A_3B^*) - k_{PA}^{A_3B} (A_3B^*) \quad (10)$$

In equations (6) and (7), f is the ratio of the average specific radioactivity at maximum labelling of amino-acids in A_4 and B_4 to the average specific radioactivity of the free amino-acid pool at maximum labelling, N is the number of amino-acid residues in the LDH tetramer, and $\bar{a}^*$ is the average specific radioactivity of an amino-acid residue in the pool at time t. Only equations (6) and (7) contain N, f, and $\bar{a}^*$ terms since the model assumes that only A_4 and B_4 are made directly from constituent amino-acids. We assumed as a first approximation that the radioactive label in the intermediate forms was evenly distributed among the A and B subunits. This approximation was necessary because we have not measured the actual distribution.

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Induction of Closely Linked Multiple Mutations by Nitrosoguanidine

N-methyl-N-nitro-N-nitrosoguanidine (nitrosoguanidine) is a powerful and widely used mutagen. Using synchronized populations of *Escherichia coli* we have shown that large numbers of mutations occur at specific loci when these loci are replicated[1]. This specificity makes it possible to use nitrosoguanidine to direct mutagenesis, to study the mode of replication of the chromosome and to map the chromosome[1-4].

The addition of 100 μg nitrosoguanidine per ml. of growing culture causes a complete and abrupt cessation of DNA synthesis (Fig. 1). Movement of the replication region is therefore halted for the entire treatment period. We treat cells in buffer, as a further precaution to stop replication and to minimize lethality.

Selective mutagenesis at the replication region implies that if multiple mutations are induced by nitrosoguanidine, they will be closely linked. We have examined the azide-resistant clones induced by nitrosoguanidine for the presence of other mutations, and find a large excess of these at genes closely linked to *azi*. The test organism is *E. coli*, strain TAU-bar (a derivative of 15 T-, requiring thymine, uracil,

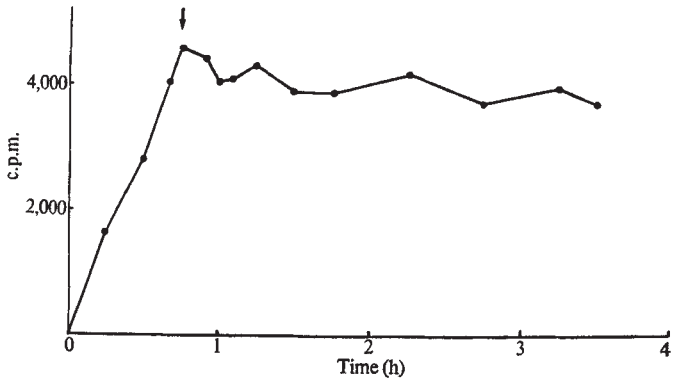

Fig. 1 Effect of nitrosoguanidine on the uptake of ^{3}H-thymine by *E. coli* TAU-bar. To a culture growing exponentially in minimal medium containing 1 μg of thymine/ml., 1.25 μCi of ^{3}H-thymine/ml. was added; 45 min later, when the culture contained 1.1×10^8 cells/ml., 0.1 mg of nitrosoguanidine/ml. was added. Samples were precipitated in trichloroacetic acid, washed and counted for radioactivity. For technical details see ref. 13.