a functional connection between vitamin K, Gla residues and the enzymatic activity of acrosin:
a) As previously reported[10], chronic treatment of cocks with marcoumar leads to a significant reduction (around 50%) of acrosin activity in a spermatozoal extract. To corroborate an effect of vitamin K on acrosin activity, it was further shown[10] that this enzymatic activity, when it had been reduced by the administration of marcoumar, could be fully restored by oral application of vitamin K_1[10].
b) After a single oral application of highly labelled ^{3}H-synkavit (tetrasodium-2-methyl-(5,6,7,8-^{3}H)-naphtho-1,4-chinol-diphosphate) the existence of ^{3}H-vitamin $K_{2(20)}$ was observed in spermatozoa[10]. Since ejaculated sperms are not able to convert synkavit to vitamin $K_{2(20)}$ in an in vitro system, in which most other tissues synthesize it[10], all spermatozoal vitamin $K_{2(20)}$ seems to be packed into these cells during developmental stages.
c) The similarities in temporal appearance of labelled spermatozoa after an oral dose of ^{3}H-synkavit and ^{3}H-acetate suggest that vitamin $K_{2(20)}$ is present in membraneous structures. Additionally, after fractionation of sperm cells or partial solubilisation with Triton X-100 of these cells, marked parallels between subcellular- and subfraction-partition of vitamin $K_{2(20)}$ and phospholipids were observed[10], which further supports the localization of vitamin K in membranes. Such a finding indicates that vitamin $K_{2(20)}$ is located in that intracellular region where the activation (carboxylation) of initially inactive proacrosin is thought to take place.
d) According to the time-course of the appearance of labelled sperm cells after a single dose of ^{3}H-leucine (a marker of protein synthesis)[10] it can be stated that maximal synthesis of these macromolecules takes place in round spermatides. At this developmental stage of the sperm cell vitamin K is found in spermatozoa and it can be speculated that a carboxylation of certain Glu residues to Gla would be possible, provided that the carboxylating enzyme can be shown to be present in spermatozoa (to our knowledge this has not yet been shown).

Whether the Gla residues found in an extract of cock sperms are actually derived from proacrosin molecules, and whether they are located in the N-terminal portion of the molecule, as in zymogens of other vitamin K dependent proteases, is not conclusively shown by the present experiments, although many arguments do support such an assumption. It can, however, be stated that the Gla residues characterized here are parts of macromolecules derived from spermatozoa, as they are found in an amino acid analysis after the cells have been freed from seminal fluids and the extract has been dialyzed. Unfortunately, preliminary attempts to purify the zymogen were not successful enough to encourage us to do experiments on such fractions. Until determinations on the purified zymogen, or even better on defined fragments of this molecule are possible it can only be presumed that Gla residues are found in another precursor of a vitamin K-dependent serine protease.

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Inhibition of molybdenum blue formation by ATP

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Summary. Molybdenum blue formation was not affected by the presence of ATP up to a concentration of 1.2 mM/l. At higher concentrations the color development was inhibited relative to ATP concentration, finally reaching complete inhibition. Auto-hydrolysis of ATP was found at a rate of 1.4%/h. An exact determination of inorganic phosphate in the presence of easily hydrolyzed phosphate esters requires the measurement of extinction at fixed time intervals and extrapolation back to time zero.

Most conventional techniques for the determination of membrane-associated ATPase activity involve a timed incubation of enzyme with excess ATP followed by subsequent measurement of released inorganic phosphate by the molybdenum blue method. This procedure depends on the reaction of ammonium molybdate with inorganic phosphate in an acid milieu and subsequent reduction of the complex to molybdenum blue, the optical density of which is directly related to the concentration of orthophosphate. As reducing agents hydroquinone[2], aminonaphthol sulfonic acid[3], stannous chloride[4,5], 2,4-diaminophenol dihydrochloride[6], ferrous sulfate[7], ascorbic acid[8] and other reducing agents have been used. In some instances certain compounds have been observed to curtail the reaction of ammonium molybdate with inorganic phosphate or the subsequent reduction to the blue complex[5,9-12]. An effect of ATP on the color development has not yet been reported. On the other hand an apparent inhibition of Na-K-ATPase by high ATP concentrations has been observed[13] when inorganic phosphate was determined by the molybdenum

blue method. These data prompted us to investigate the effect of ATP on the color development of the molybdenum blue complex.

Materials and methods. Inorganic phosphate was determined by a modification of the Fiske and Subbarow method[3] using ascorbic acid as reducing agent. For the calibration curve 0.1 ml sodium dodecyl sulfate (13.2%), 1.0 ml trichloracetic acid (12.8%), 0.6 ml molybdate solution (357.0 ml 5 N H_2SO_4, 107.0 ml 4% $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$, 36.0 ml 0.27% $K(SbO)C_4H_4O_6 \cdot 5\ H_2O$, filled up to 1000 ml) and 0.6 ml ascorbic acid (0.2 g/100 ml) were added to 1.0-ml samples of phosphate solutions with increasing concentrations of inorganic phosphate. All solutions used were ice cold except for sodium dodecyl sulfate. The final volume was 3.3 ml. The samples were mixed and the molybdenum blue complex was developed within 20 min at room temperature. Extinctions were read at 578 nm in 1.0-cm cuvettes.

In another experiment a calibration with increasing concentrations of ATP was performed; inorganic phosphate concentration was kept constant. In the stock solution the concentration of orthophosphate was $3.5 \cdot 10^{-5}$ mole/l and that of ATP varied between 0 and $5.0 \cdot 10^{-3}$ mole/l. The color was developed as described above. Again, extinctions were measured following 20 min of incubation at room temperature and then once more after 15 min, 30 min, 45 min and 95 min.

Results. The calibration curve for inorganic phosphate was linear to an extinction of 0.27. At higher optical densities it deviated from linearity.

The effect of ATP on the optical density of molybdenum blue is shown in figure 1. In the absence of ATP the extinction of the sample containing $3.5 \cdot 10^{-8}$ mole inorganic phosphate was 0.136. Following the preincubation period of 20 min for color development, the optical densities increased at first almost linearly with increasing concentration of ATP indicating that the ATP contained inorganic phosphate. When ATP concentration approached 1.2 mmole/l the optical densities decreased and were almost zero at high ATP concentrations.

The extinction of the ATP-free sample remained constant throughout the evaluation period of 95 min. In presence of ATP, however, extinctions of individual samples increased with increasing time intervals due to spontaneous hydrolysis of ATP. Again, for each incubation period a linear function was obtained up to an ATP concentration of 1.2 mmole/l provided the extinctions were within the linear region of the calibration curve. At higher ATP concentrations the optical densities decreased and reached a minimum of zero at high ATP levels.

A linear function was obtained ($y = 0.925x + 57.46$; $r = 0.9998$) when the slopes of the regression lines of d E/d[ATP] were plotted against the incubation period (fig. 2). The intersection of 57.46 with the ordinate represents the extinction for inorganic phosphate in an ATP solution at a concentration of 1.0 mole/l. The slope 0.925 gives the change of extinction per min at an ATP concentration of 1.0 mole/l due to spontaneous ATP breakdown within the sample during formation of the molybdenum blue complex at room temperature in the presence of trichloracetic acid and molybdenum. By means of the calibration curve for inorganic phosphate it can be calculated that within 1 h 1.4% of ATP is being cleaved spontaneously.

Discussion. At 578 nm the calibration curve for inorganic phosphate determined as molybdenum blue was linear to an extinction of 0.27 when optical densities were measured in 1.0-cm cuvettes, representing a phosphate concentration of almost $7.0 \cdot 10^{-5}$ mole/l in the stock solution. At higher optical densities the calibration curve deviated from the linearity indicating the validity of Lambert-Beers law to this critical value. Therefore, solutions with phosphate concentrations higher than $7.0 \cdot 10^{-5}$ mole/l must be diluted prior to the development of the molybdenum blue complex. Since the optical density of molybdenum blue remains stable for at least 95 min extinctions do not have to be read immediately following the color development provided the samples do not contain any organic phosphate esters.

ATP in a concentration up to 1.2 mmole/l (i.e. 0.36 mmole/l in the final solution for phosphate determination) did not interfere with the phosphate determination with molybdenum. At higher concentrations, however, ATP curtailed the

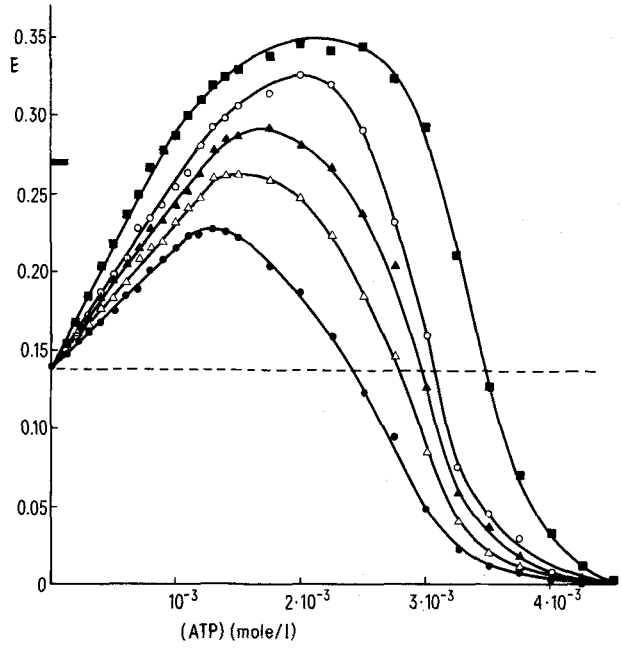

Figure 1. Effect of ATP on the optical density of molybdenum blue (E) in presence of $3.5 \cdot 10^{-8}$ mole inorganic phosphate, measured after 20 min of incubation (●) and subsequently after 15 min (△), 30 min (▲), 45 min (○) and 95 min (■). The broken line represents the optical density of the solution containing $3.5 \cdot 10^{-8}$ mole inorganic phosphate. The black bar in the ordinate indicates the upper limit of linearity of the calibration curve.

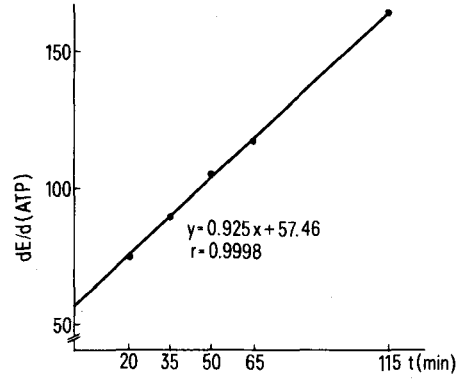

Figure 2. Influence of incubation period for molybdenum blue formation on the slope of the regression lines of optical density as a function of ATP concentration (d E/d[ATP]). The slope of this function gives the change of extinction per min due to spontaneous ATP-breakdown at an ATP concentration of 1.0 mole/l.

color development and inhibited it at least quantitatively at high concentrations. Therefore, samples containing high ATP concentrations (> 1.2 mmole/l) must be diluted prior to the phosphate determination. A similar inhibitory effect on the formation of molybdenum blue has already been reported for citric acid[5,9], tartaric acid[9], mannitol[5,12] and for other compounds[5,12]. Analogous to ATP, these substances inhibited the reaction depending on their concentration.

The impairment of maximal color production by high concentrations of ATP suggests that the inhibition of Na-K-ATPase by excess substrate as reported previously[13] might be due to an impaired color development by excess ATP and not to an inhibition of the enzyme. This assumption is supported by the similar course of enzymatic activity[13] and of optical density as observed in the present study as a function of the ATP concentration. On the other hand, a spontaneous hydrolysis of ATP could be demonstrated and previously it was reported that the cleavage of certain labile phosphates is catalyzed by molybdenum[5,10,14]. In the present study the hydrolysis of ATP was a first order reaction and the amount of inorganic phosphate formed was directly related to the concentration of ATP and to the incubation period during color development. Therefore, in samples containing readily hydrolysable phosphate esters, falsely high concentrations of inorganic phosphate might be obtained. Thus, in the presence of hydrolysable organic phosphate esters the incubation period for color development should be as short as possible. Additionally, by measuring the optical density at intervals and extrapolating back to zero-time or by subtracting blank values from parallel ATP-containing incubation mixtures (without enzyme) the true amount of inorganic phosphate present or enzymatically liberated can be determined. This is possible since spontaneous hydrolysis is a linear function of the incubation period provided it is not performed for a prolonged time resulting in a measurable decrease of the concentration of organic phosphate esters.

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thiD locus of *Escherichia coli*

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Summary. A mutant strain of *Escherichia coli* K-12 lacking phosphomethylpyrimidine kinase activity was produced from the polyauxotrophic female strain, JC1552. The locus of its lesion, for which we propose the designation *thiD*, was mapped at about 46 min on the chromosome.

Four *thi* mutations, *A*, *B*, *C*, and *O*, each affecting a different step in thiamine biosynthesis and its control mechanism, have been identified and mapped within the small region of the chromosome of *Escherichia coli* K-12[1-3]. This leads to the assumption that the genes operating in the biosynthesis of TPP (thiamine pyrophosphate), a coenzyme form of thiamine, might constitute the *thi* operon. Our previous experiments have revealed that in *E. coli* TPP is formed by the direct phosphorylation of thiamine monophosphate, the first thiamine compound synthesized from hydroxymethylpyrimidine pyrophosphate and hydroxyethylthiazole monophosphate[4,5]. A conversion from hydroxymethylpyrimidine monophosphate to the pyrophosphate, catalyzed by phosphomethylpyrimidine kinase, has been thought to be involved as an essential step in the de novo synthetic pathway of TPP. This paper describes a new locus designated as *thiD*, that affects phosphomethylpyrimidine kinase activity. The results showed that the locus is situated at a different chromosomal site from the 4 known *thi* genes already mapped, at about 90 min.

Materials and methods. The strains used in this work are all derivatives of *E. coli* K-12. A mutant strain, JC1552B5, lacking phosphomethylpyrimidine kinase was produced

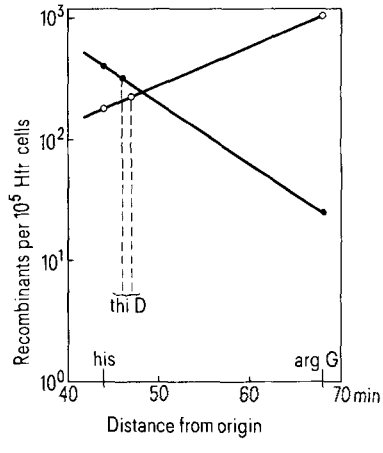

Figure 1. Location of *thiD* locus on the *Escherichia coli* chromosome by the gradient of transmission using Hfr KL209 (○——○) and AB259 (●——●) as donors. JC1552B5 was mated with the male strains for 100 min. The number of Thi^+ recombinants was plotted on the straight line drawn through the points for His^+ and Arg^+ recombinants.