filters separated by a 10-μ Teflon film. One membrane was saturated with the hemoglobin solution; the other, with water. By diffusing air at various pressures through such a system one determines the relative enhancement in transport from the oxygen-nitrogen ratio, and by placing such assemblies in the light path of a spectrophotometer (*2*, *4*) one measures the integrated oxygenation. The optical density for full oxygenation and full reduction was determined by having, respectively, air and nitrogen on either side.

In one series (Fig. 1, top) the hemoglobin membrane was on the input side. With full pressure of air (155 mm-Hg O_2) the ratio of O_2 to N_2 diffusing through was close to 56 percent, which is the ratio for zero enhancement given by methemoglobin or water. As soon as reduction appeared optically, when the air pressure was lowered, enhancement in transport developed (Fig. 1, center). In a second series the input membrane contained water, while the hemoglobin membrane faced the vacuum. Under such conditions there is always an oxygenation gradient in the hemoglobin, and transport was always enhanced (Fig. 1, bottom).

These experiments, like the back-pressure experiments (*3*), show that the hemoglobin does not aid the net transport of oxygen unless there is a hemoglobin-bound-O_2 gradient through the membrane. The results lend experimental support to the explanation formulated by Zilversmit (*5*) that the enhancement in the fully oxygenated part of a membrane is carried entirely by an increased oxygen-pressure gradient through the solvent. The reason for this steep gradient is, of course, the facilitated flux through the oxyhemoglobin gradient at the outlet. To what extent this "hemoglobin shuttle" may numerically fit the expedient but poorly defined concept of oxyhemoglobin diffusion must await determination of the diffusion characteristics of hemoglobin through a Millipore membrane.

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Temperature-Sensitive Repression of Staphylococcal Penicillinase

Abstract. *In eight highly inducible strains of* Staphylococcus aureus *repression of the formation of penicillinase was temperature-sensitive under conditions suggesting direct thermal inactivation of the repressor. Restoration of repression required protein synthesis. These strains were resistant to benzylpenicillin and to many other antibiotics. One auxotrophic mutant had greatly reduced temperature sensitivity but was still normally inducible. Six strains were relatively poorly inducible, exhibited a proportionately smaller increase in enzyme after exposure to elevated temperature, and were sensitive to antibiotics other than benzylpenicillin. Temperature sensitivity may be a useful character in studies of the physiology and genetics of the repression of staphylococcal penicillinase.*

Penicillinase, the enzyme which hydrolyzes the β-lactam bond of the penicillins, is found in all clinically pathogenic penicillin-resistant staphylococci in which it mediates resistance to penicillin treatment. Its usually low basal rate of formation can be increased manyfold by induction with penicillins, cephalosporins, or certain peptides (*1*). Increased synthesis, without specific inducers, may be achieved by cultivation of some inducible strains in highly acidic media (*2*).

In a search for other methods to analyze control mechanisms for synthesis of penicillinase in staphylococci we have investigated the property of temperature sensitivity, that is, the selective inactivation of biologically important macromolecules at moderately elevated temperature, an increasingly useful tool in the analysis of microbial biosynthetic mechanisms (*3*). In certain mutants of *Escherichia coli*, but not in wild-type organisms, the normal repression of synthesis of β-galactosidase or alkaline phosphatase is temperature-sensitive by either of two mechanisms. In one the proposed repressor itself appears to be inactivated at 43° to 45°C (*4*). In the other, the synthesis of repressor, but not its function, is progressively inhibited with rising temperatures of bacterial growth (*5*). In either case enzyme formation is repressed (inducible) during growth at low temperatures but is derepressed (constitutive) at higher temperatures.

We have found that repression of the formation of penicillinase is temperature-sensitive, apparently through the inactivation of preformed repressor, in many strains of wild-type *Staphylococcus aureus* and in one strain of wild-type *S. epidermidis*. In one auxotrophic mutant the repression system is relatively stable to elevated temperatures.

In typical experiments, cells of *S. aureus* strain 55C1, in the log phase of growth, grown at 37°C in tryptic digest broth (Baltimore Biological Laboratory, Inc.) with added 50 m*M* sodium phosphate buffer (*p*H 7.2), were washed once with buffer [11 m*M* KCl, 4 m*M* phosphate (Na), 1.4 m*M* $MgSO_4$, *p*H 7.2]. The cells were resuspended in the same buffer to a dilution where the optical density was 0.5, heated to 42°C for 10 minutes, and then diluted fourfold into broth or synthetic medium (*2*) at 37°C and grown while being shaken. Samples of cultures were collected into cold tubes with chloramphenicol (final concentration 100 μg/ml) and assayed iodometrically for penicillinase (*6*). A unit of penicillinase hydrolyzed 1 μmole of benzylpenicillin per hour at 30°C and *p*H 5.8. Specific activities are given in units per unit of optical density mea-

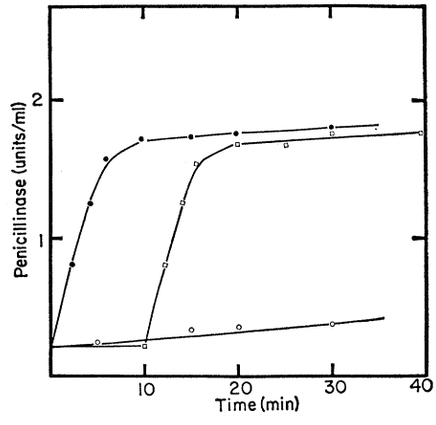

Fig. 1. Temperature-sensitive repression of staphylococcal penicillinase. Strain 55C1 was grown in broth, heated in buffer (with Mg^{++} reduced to 0.28 m*M*) at 42°C for 10 minutes; at zero time, the culture was added at 37°C to synthetic medium (closed circles), and to synthetic medium containing 500 μg/ml of puromycin (open squares). The culture with puromycin failed to grow, and, in separate experiments, its cells incorporated little or no carbon-14–labeled valine into protein. After 10 minutes, puromycin was removed by membrane filtration and washing. The cells, resuspended in synthetic medium, grew normally without lag. Open circles give results with an unheated culture.

Table 1. Induction of staphylococcal penicillinase by temperature elevation, benzylpenicillin, or cephalosporin C. Results are expressed as differential rates (units of penicillinase formed per unit increase in optical density of the cultures).

Basal	Temp. elevation*	Benzylpenicillin†	Cephalosporin C‡
	Strain 55C1		
3	58	138	410
	Strain B4		
2	13	7	70
	Strain B5		
2	15	7	60
	Strain 55 C1-P1		
2	5	188	410

* Differential rates were calculated for the period 0 to 6 minutes of growth in broth at 37°C after 10 minutes at 42°C in buffer. † Differential rates were calculated for the period 5 to 15 minutes after induction with benzylpenicillin (1 μg/ml) of a young culture in the log phase of growth at 37°C in broth. ‡ Assayed 3 hours after induction of a culture in the early log phase of growth at 37°C with cephalosporin C (50 μg/ml). At this time the differential rate was constant.

sured at 540 mμ, in round cuvets 19 mm in diameter, in the Coleman Jr. spectrophotometer. For strain 55C1, one optical density unit was equal to 550 μg of bacterial protein per milliliter.

After exposure to 42° to 44°C in buffer, staphylococci, when transferred to complete medium, resumed exponential growth without lag and exhibited a burst of penicillinase synthesis lasting about 10 minutes (Fig. 1). The differential rate of penicillinase synthesis varied rather widely between 40 to 100 units per optical density unit, the mean in 12 experiments being 58 units. This was 20 to 30 times the basal rate but was much less than the fully derepressed rate of about 400 units per optical density unit attained by induction with cephalosporin C (50 μg/ml), which is resistant to hydrolysis by penicillinase. The derepression by heating, in circumstances in which growth was impossible, resembled that reported by Horiuchi *et al.* (*4*) and assumed by them to reflect thermal instability of repressor.

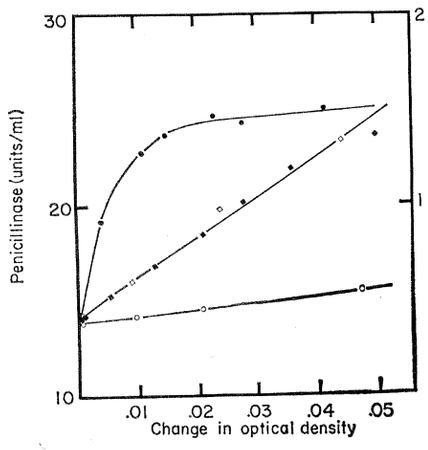

Fig. 2. Effect of exposure to elevated temperature on subsequent penicillinase formation in an inducible strain of *Staphylococcus aureus*, 8325 (αw), and its constructive derivative, 8325 (αi_T). Washed cells in the log phase of growth were heated in buffer at 42°C for 10 minutes. Penicillinase assays during subsequent growth in broth at 37°C are shown by circles for the inducible strain (right ordinate scale) and squares for the constitutive strain (left ordinate scale). Solid symbols give results after exposure to 42°C; open symbols are controls similarly treated at 37°C.

In experiments of this kind, the rate and duration of derepressed synthesis depended, among other factors, on the physiologic age of the culture, the temperature and time of heating, and the composition of the medium in which cells were heated and then grown. In particular, strain 55C1, when heated in complete medium at 42°C, was only slightly derepressed whether tested by continuing growth at that temperature or at 37°C. Thus, in contrast to the situation with *Escherichia coli* mutants, the enzyme did not appear to be constitutive in cells grown continuously at 42°C. At 48°C, however, in complete medium, penicillinase was formed at a differential rate of 40 units per optical density unit for the 20 to 30 minutes that the cells continued to grow.

The increase in enzymatic activity after heating was the result of synthesis of new enzyme, for it did not occur if the cells were incubated for 10 minutes at 37°C in phosphate buffer plus glucose or in complete medium containing puromycin or chloramphenicol. The effect of the heating persisted, nevertheless, for if the medium was then made suitable for growth, either by addition of nutrients or removal of the inhibitors by filtration, enzyme was synthesized rapidly (Fig. 1). Thus, in line with current opinion that repressors are proteins, protein synthesis would appear to be essential for restoration of repression.

Novick (*7*) derived mutants constitutive for penicillinase from inducible strains of staphylococci by apparently single-step mutations. Comparison of such a pair afforded additional evidence that elevated temperature exerted its effect upon the repression system. The inducible parental strain, 8325 (αw), responded like 55C1 with a burst of penicillinase synthesis, but its constitutive derivative, 8325 (αi_T), was unaffected by the same treatment (Fig. 2).

In seven strains of *Staphylococcus aureus* and in one of *S. epidermidis* the rate of synthesis of penicillinase after exposure to elevated temperature was similar to that of 55C1. Also, like 55C1, these strains formed the enzyme at a high rate after induction with benzylpenicillin or cephalosporin C and were resistant to one or more antibiotics other than benzylpenicillin (tetracycline, chloramphenicol, erythromycin, or streptomycin) by the conventional disc-plate method. Thus, these strains appear to fall into the group of staphylococci characterized by multiple antibiotic resistance and inducibility to high levels of penicillinase (*8*).

In six strains of *S. aureus* the rate of penicillinase formation after exposure to elevated temperature was about 25 percent as high as in 55C1 (Table 1). These strains were virtually uninducible with benzylpenicillin (1 μg/ml), but with cephalosporin C (50 μg/ml) they formed penicillinase to a level of activity about 15 percent as high as that of 55C1. Thus the rate of penicillinase synthesis after derepression by heating, relative to that after full induction with cephalosporin C, was roughly similar in both groups of staphylococci. Growth of organisms of the second group was inhibited by antibiotics other than benzylpenicillin. Accordingly, they resemble the staphylococci characterized by antibiotic sensitivity and relatively poor inducibility of penicillinase (*8, 9*).

Temperature sensitivity of staphylococcal penicillinase repression appears to be subject to modification by mutation. Strain 55C1 was treated with ultraviolet light and mutants were selected for purine or pyrimidine auxotrophy (*10*). One mutant strain (55C1-P1) required both adenine and guanine as supplements for growth in a synthetic medium, an auxotrophic pattern that has been related to a deficiency in adenylosuccinase (*11*). The basal rate of penicillinase formation in this mutant was comparable to the parental strain, but was virtually unchanged after heating at 42° to 46°C for 10 minutes (Table 1). Unlike the naturally occurring strains with a weak response to heat, strain 55C1-P1 was readily inducible with benzylpenicillin, and, after full induction with cephalo-

sporin C, produced as much enzyme as the parental strain. We have no evidence for any causal relation between the purine auxotrophy of this mutant and the reduced-temperature sensitivity of its penicillinase-repression system. Five other mutants requiring either a purine or pyrimidine base responded to heat like the parental strain.

The properties of the penicillinase system described in this report should be useful in the further study of the physiology and genetics of the regulation of this enzyme (*12*).

Note added in proof. Recent work indicates that the special properties of the 55C1-P1 mutant are related to its auxotrophy. Penicillinase formation in this strain is derepressed by heat in cells grown in nutritionally optimal, but not in suboptimal, concentrations of adenosine and guanosine. Failure to obtain derepression in cells grown in tryptic digest broth (Table 1) is due to this mechanism, as shown by increased growth rate and derepression in cells grown in broth supplemented with adenosine and guanosine.

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Biosynthesis of Vitamin A with Rat Intestinal Enzymes

Abstract. *Vitamin A is synthesized from β-carotene in cell-free homogenates of rat intestinal mucosa, the biosynthetic enzymatic activity being present in the soluble protein fraction of the homogenate. Also required are a heat-stable factor in the particulate fraction, molecular oxygen, and bile salts. The reaction is stimulated by glutathione. The product, obtained in yields of up to 50 percent, has been identified as vitamin A aldehyde (retinal) by way of its semicarbazone derivative. The reaction mechanism involves the central cleavage of β-carotene into two molecules of retinal.*

The conversion of β-carotene to vitamin A was first demonstrated by Moore, who fed β-carotene to vitamin A–deficient rats (*1*). Since then it has been established that the intestine is the major site of this conversion. Thus, retinyl ester (*2*), but not β-carotene, appears in the mesenteric or thoracic-duct lymph of rats and pigs given test meals of β-carotene (*3, 4*). Retinyl ester predominates in the thoracic-duct lymph of human subjects fed test meals of labeled β-carotene (*5*). The conversion of labeled β-carotene into retinyl ester has also been demonstrated during experiments with isolated rat intestinal loops in vivo and during studies in vitro with rat intestinal slices (*6*).

Studies on the mechanism of vitamin A biosynthesis have been greatly limited by the lack of a cell-free system capable of mediating the conversion of β-carotene to vitamin A. Information available on the reaction mechanism and other details of retinol formation has been reviewed (*7*). The two major hypotheses have continued to be: (i) central fission of β-carotene resulting in two molecules of vitamin A, and (ii) oxidative attack at a site other than the central double bond of β-carotene, with the eventual formation of one molecule of vitamin A per molecule of carotene.

We now report the active conversion of β-carotene to retinal with cell-free homogenate fractions from rat intestinal mucosa. Mucosal scrapings of rat duodenum and jejunum were homogenized, in a Potter-Elvehjem homogenizer with a loose-fitting teflon pestle, in 0.1*M* potassium phosphate buffer, *p*H 7.7, containing 30 m*M* nicotinamide and 4 m*M* $MgCl_2$. After centrifugation of the homogenate at 2000*g* for 20 minutes, the supernatant fraction (designated S-2) showed enzymatic activity for the conversion of β-carotene to retinal. Further centrifugation of the S-2 at 104,000*g* for 60 minutes resulted in the separation of soluble and particulate fractions, both of which were almost completely inactive when tested individually (*8*). The combination of soluble plus particulate fractions, however, was fully as active as the unfractionated S-2.

The activity of the combined soluble and particulate fractions was only slightly reduced after heating the particulate fraction at 95° to 100°C for 20 minutes. There was complete loss of enzymatic activity after heating the soluble fraction at 64°C for 55 seconds. The soluble fraction contains the enzyme (or enzymes) responsible for retinal biosynthesis, and this enzyme has been partially purified by precipitation with ammonium sulfate between 25 and 45 percent saturation. The particulate fraction appears to provide a necessary factor which is not an enzyme. This factor can be extracted from the particulate fraction with 90 percent acetone; it has not yet been identified.

The enzyme preparations were incubated with C^{14}-β-carotene, produced biosynthetically from C^{14}-acetate by the fungus *Phycomyces blakesleeanus*, as substrate. The reaction was allowed to proceed in the dark at 37°C with room air as the gas phase. At the end of 1 to 2 hours the incubation mixtures were extracted with 20 volumes of chloroform-methanol (2:1, by volume), and then 5 volumes of 0.01*N* H_2SO_4 were added. The lower chloroform phase was collected and evaporated, and the total lipid extract so obtained was chromatographed on columns of alumina (Woelm, activity grade III). Five fractions were collected, containing, respectively, β-carotene, retinyl esters, retinal, retinol, and more polar compounds including retinoic acid (*4, 9*).

With the complete system as described in Table 1, β-carotene can be converted to retinal with a usual yield of 30 to 50 percent. There was an absolute requirement for molecular oxygen and an almost absolute require-