A Separate Antibiotic-binding Site in Xanthosine-5′-phosphate Aminase: Inhibitor- and Substrate-binding Studies*

T. T. FUKUYAMA† AND H. S. MOYED

From the Department of Microbiology, University of Southern California, School of Medicine, and the Los Angeles County General Hospital, Los Angeles, Calif.

Received June 29, 1964

A direct examination has been made of the interaction between xanthosine-5′-phosphate aminase, its inhibitor, the antibiotic psicofuranine (6-amino-9-D-psicofuranosylpurine), and its substrates. Psicofuranine binding by the aminase, like psicofuranine inhibition, is greatly stimulated by a substrate, xanthosine-5′-phosphate, in cooperation with one of the products, inorganic pyrophosphate. The inhibited complex contains equimolar amounts of xanthosine-5′-phosphate, pyrophosphate, and psicofuranine. Elevated levels of ATP or NH_3, the amino donor for the aminase, reduce psicofuranine's inhibitory effect but not its binding to the enzyme. It is highly likely that the antagonistic effect of these substrates is indirect, the result of more rapid depletion of xanthosine-5′-phosphate, a compound necessary for full expression of psicofuranine's inhibitory action. In agreement with earlier kinetic analyses, the binding studies show that the primary interaction of the aminase with psicofuranine is a noncompetitive process. This suggests that psicofuranine is recognized by a special site or area of the aminase whose normal function is the recognition of a metabolic regulator.

The antibiotic psicofuranine (6-amino-9-D-psicofuranosylpurine) is a highly specific inhibitor of bacterial xanthosine-5′-phosphate (XMP) aminase (Slechta, 1960a), an enzyme essential for the biosynthesis of guanylic acid (Moyed and Magasanik, 1957). This particular site of action and specificity raises a problem about the mechanism of the inhibition. If psicofuranine were a simple competitive inhibitor of ATP because of the structural similarities between the two compounds, it might be expected that other ATP-driven reactions would also be inhibited. However, the complete neutralization of the bacteriostatic effects of large excesses of psicofuranine by guanine (Slechta, 1960b) strongly suggests that no other important reaction involving ATP is affected. The possibility of a simple competitive relationship between the other purine-containing substrate, XMP, also seems unlikely on structural grounds and, more significantly, because of the failure of xanthine and its congenors to overcome bacteriostasis by the drug. The problem of the relationship between psicofuranine and the substrates of XMP aminase was explored by kinetic analysis (Udaka and Moyed, 1963) which revealed that the inhibition of the parental form of XMP aminase from *Escherichia coli* is dependent on one of the substrates, XMP, and on a product, inorganic pyrophosphate. Because of the progressive nature of the inhibition of the parental aminase, it was not possible to determine convincingly by kinetic analysis whether psicofuranine is competitive with any of the substrates. In contrast, inhibition of a mutant form of the aminase was found to be constant with time, and therefore susceptible to such analysis which showed that psicofuranine is a readily reversible noncompetitive inhibitor. To account for this unusual type of inhibition, it was suggested that the aminase possesses a separate site for binding the antibiotic. In order to substantiate this suggestion, and to examine further the dependence of psicofuranine inhibition on XMP and pyrophosphate, as is the case with the parental aminase, a direct study has been made on the interaction between psicofuranine, the substrates, and the aminase.

* This investigation was supported in part by the Hastings Foundation Fund of the University of Southern California, School of Medicine and by research grants (GM-11269 and AI-03874) from the National Institutes of Health, U.S. Public Health Service. Presented in part at the meeting of the American Society of Biological Chemistry, Chicago, Ill., April, 1964.

† Postdoctoral Fellow of the National Institutes of Health, U.S. Public Health Service.

Methods and Materials

Bacterium and Growth Conditions.—A guanine-requiring mutant of *E. coli*, strain B-24-1, blocked at IMP dehydrogenase was obtained from Dr. S. Udaka. Its XMP aminase was found to have wild-type sensitivity to psicofuranine. Growth conditions were similar to those described by Udaka and Moyed (1963) with the following modifications for the production of large amounts of cells with derepressed levels of XMP aminase. Forty liters of minimal medium supplemented with 7 μg of guanine per ml were inoculated with 1 liter of an overnight culture. After 16 hours of incubation with aeration at 37°, additional sterile glucose was added to the culture to a final concentration of 0.2%. Then 8 mg of guanine was added every 15 minutes over a 7- to 8-hour period. The yield of cells was approximately 85 g wet wt. For later preparations, a New Brunswick continuous fermentor was employed. The cells were pregrown for 15 hours at 37° under vigorous aeration in a fermentor assembly containing 10 liters of minimal medium with 7 μg/ml of guanine. Subsequent growth was limited by the addition of guanine in minimal medium (7 μg/ml) at a rate varying between 1.8 and 2.5 liters/hour. The yield of cells was approximately 1.2 g wet wt per liter of culture medium.

Binding Measurements.—Binding of [^{3}H]psicofuranine, $^{32}P^{32}P_i$, [8-^{14}C]ATP, and [8-^{14}C]XMP to the aminase was determined by ultracentrifugation procedures. Procedure A: One ml of a reaction mixture containing enzyme, 100 μmoles Tris buffer, *p*H 8.5, 12 μmoles $MgCl_2$, 3.3 μmoles NaF, and additions as indicated was incubated at 25° for 15–20 minutes. The mixture was layered over 4.0 ml of carbon tetrachloride in tubes of 5-ml capacity, and then centrifuged at 120,000 × *g* for 15 hours in the SW 39L rotor of the Model L Beckman ultracentrifuge. Two 0.4-ml portions of the upper layer and the 0.2-ml portion of the lower layer of the aqueous phase were removed and assayed for protein and radioactivity. All of the protein was found in the lower one-fifth of the aqueous phase. The actual distribution of the radioactivity found in the

upper and lower fractions was compared to that calculated for equal distribution. Differences between these values represent radioactivity bound by the protein fraction. In all such experiments, the additional amount of radioactivity associated with the protein fraction corresponded closely to the amount removed from the upper parts of the tube. Procedure B: For binding studies requiring greater precision, 0.50 ml of the reaction mixture containing 165 μg of *p*-aminobenzoic acid was centrifuged in tubes 4.8 × 16 mm. The precise volumes of the upper and lower fractions were determined from the concentration of *p*-aminobenzoic acid as measured by the method of Bratton and Marshall (1939) after dilution of the fractions to known volumes. The lower fraction containing all of the protein was generally 0.1 the total volume. Sedimentation of the labeled compounds in the absence of protein or, in the case of [^{3}H]psicofuranine, with a nonspecific protein, either bovine serum albumin or an aminase-free protein fraction from *E. coli*, was too small to require correction of the binding data.

Enzyme Assay.—XMP aminase activity was determined by measuring the increased absorption at 290 mμ owing to the formation of GMP from XMP after the reaction mixture had been acidified with 3.5% perchloric acid (method A, Moyed and Magasanik, 1957). A unit of activity is equivalent to the production of 1 μmole of GMP per minute.

Determination of Protein and Radioactivity.—Protein was determined by the spectrophotometric procedures of Warburg and Christian (1941) or by the method of Lowry *et al.* (1951). Radioactivity was measured after direct plating using a Nuclear-Chicago low-background, thin-window counter, or by scintillation counting in a Nuclear-Chicago 725 liquid scintillation system. The channel-ratio procedure was used for quench correction.

Chemicals.—Psicofuranine and tritium-labeled psicofuranine (8.56 mc/g) were gifts of the Upjohn Co. $^{32}P^{32}P_i$ was prepared from 20 μc of carrier-free $H_3{}^{32}PO_4$ diluted with 20 μmoles of Na_2HPO_4 by the method of Bergmann (1962). [8-^{14}C]XMP was prepared by the deamination of [8-^{14}C]GMP (30.8 mc/mmole, Schwarz BioResearch, Inc.) with nitrous acid. [8-^{14}C]ATP (4 mc/mmole) was purchased from Schwarz BioResearch, Inc.

Results

Purification of XMP Aminase.—The purification of XMP aminase from *E. coli* strain B-24-1 is summarized in Table I. All manipulations were carried out at 4°.

The cell paste from 40 liters of culture medium was suspended in 200 ml of 0.0075 M potassium phosphate buffer, *p*H 7.4, and the cells were disrupted by subjecting 35-ml batches to sonic vibration in a 10-kc oscillator (Raytheon) for 6 minutes. The extract was clarified by centrifugation at 30,900 × *g* for 30 minutes. It was then dialyzed against 8 liters of the potassium phosphate buffer for 15 hours. Nucleic acids were removed from the dialyzed extract by the slow addition, with stirring, of streptomycin sulfate to a final concentration of 1.0%. After 30 minutes of additional stirring, the suspension was centrifuged and the precipitate was discarded. The streptomycin-treated extract was further fractionated by the addition of a saturated solution of ammonium sulfate adjusted to *p*H 7.4 with NH_4OH and containing 1 × 10^{-4} M EDTA. The protein precipitating between 51 and 63% of saturation was dissolved in 15 ml. of 0.02 M Tris buffer, *p*H 7.4, and dialyzed against 2.5 liters of the Tris buffer for 20 hours with a change of buffer after 8 hours. The dialyzed ammonium sulfate fraction was applied to a 4 × 40-cm column of DEAE-Sephadex, A-50, which had been previously equilibrated with 0.02 M Tris buffer, *p*H 7.4. Elution of the protein was accomplished by use of a linear gradient of potassium chloride from 0.2 to 0.42 M in 0.02 M Tris buffer, *p*H 7.4. The total volume of the gradient was 1000 ml. The flow rate was approximately 30 ml/hour. Fractions of 6.5 ml were collected. Peak tubes containing aminase activity were pooled and dialyzed against 8 liters of the Tris buffer for 24 hours with a change of buffer after 6 hours. The dialyzed preparation was applied to a second column, 2.5 × 30 cm, of DEAE-Sephadex, A-50. The protein was eluted with a linear gradient of potassium chloride from 0.25 to 0.38 M in 0.02 M Tris buffer, *p*H 7.4. Total volume of the gradient was 400 ml. Fractions which contained enzyme with specific activities greater than 2.65 units/mg protein were pooled and concentrated by dialysis against an amount of a saturated solution of ammonium sulfate calculated to give a final saturation of 0.65. The resulting precipitate was collected by centrifugation and dissolved in 10 ml of 0.02 M Tris buffer, *p*H 7.8; the enzyme preparation was then dialyzed against several changes of the Tris buffer for 24 hours. Small amounts of inactive protein precipitated during dialysis resulting in a slight increase in specific activity. The final preparation was stored at −20°.

TABLE I

PURIFICATION OF XMP AMINASE FROM *Escherichia coli*, STRAIN B-24-1

Fraction	Volume (ml)	Protein (mg)	Activity (units)	Specific Activity (units/mg protein)
Crude extract	205	6900	1325	0.19
Streptomycin treatment	240	5000	1125	0.22
Ammonium sulfate 51–63% saturation	27.3	1390	917	0.66
First DEAE-Sephadex	185	290	500	1.72
Second DEAE-Sephadex	6.5	92	314	3.41

Purity of the Enzyme Preparation.—The purification procedures described in Table I resulted in an 18-fold increase in specific activity. Since the specific activity of the crude extract from derepressed cells was 50- to 60-fold greater than that from wild-type cultures, the final enzyme preparation represents a purification of approximately 1000-fold.

Chromatography on DEAE-Sephadex of an enzyme preparation with a specific activity of 2.80 revealed a single peak containing enzyme with constant specific activity throughout the peak tubes. Examination of such a purified preparation in a Spinco Model E analytical ultracentrifuge revealed a single major boundary (Fig. 1). Slight impurities are noticeable, both trailing and in advance of the main component. Separate sedimentations in sucrose gradients showed that the aminase activity coincided with the single detectable protein peak. A sedimentation coefficient of 3.5 S was calculated for the major component.

Aided Binding of Psicofuranine.—Inhibition of XMP aminase by psicofuranine was found to be dependent on XMP and PP_i (Udaka and Moyed, 1963). An examination of these requirements in terms of psicofuranine binding is shown in Table II. The binding of psicofuranine, like its inhibitory effect, was found to be

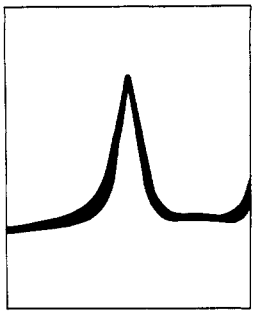

FIG. 1.—Ultracentrifuge schlieren pattern of XMP aminase. Before centrifugation the enzyme preparation (specific activity of 2.92 units/mg of protein) was dialyzed against 0.01 M potassium phosphate buffer, *p*H 7.4. The protein concentration was 12 mg/ml. Tracing is from a photograph obtained 96 minutes after attaining a speed of 59,780 rpm in a Spinco Model E ultracentrifuge at 20°.

TABLE II
REQUIREMENTS FOR PSICOFURANINE BINDING BY XMP AMINASE[a]

Reaction Mixtures	Psicofuranine Bound (mμmoles)	Inhibition of XMP Aminase Activity[b] (%)
[^{3}H]Psicofuranine + XMP + PP_i	8.74	83
[^{3}H]Psicofuranine + XMP	0.61	0
[^{3}H]Psicofuranine + PP_i	0.81	3.2

[a] [^{3}H]Psicofuranine binding was determined by procedure B. The reaction mixtures contained 5×10^{-5} M [^{3}H]-psicofuranine, 5×10^{-4} M XMP, 5×10^{-4} M PP_i, as indicated, and 4.80 units of enzyme (1.88 mg protein). [b] A sample of each reaction mixture was diluted 1000-fold for the measurement of XMP aminase activity. Psicofuranine at this low concentration has no significant effect on the aminase. Thus a loss in activity as measured in the diluted sample is an index of the nearly irreversible formation of inhibited enzyme in the more concentrated reaction mixture used for the binding studies.

dependent on both XMP and PP_i. Subsequent experiments revealed that the small amount of psicofuranine bound in the absence of one of these compounds is nearly the same as that bound in the absence of both compounds. This is referred to as *unaided binding.*

Binding of PP_i *and XMP.*—The requirements for maximal binding of psicofuranine by XMP aminase raise the possibility that the inhibited enzyme complex contains, in addition to psicofuranine, XMP and/or PP_i. The association of these compounds with the inhibited enzyme was therefore examined. When equimolar amounts of $^{32}P^{32}P_i$, XMP, and psicofuranine were incubated with the aminase, PP_i binding was readily measurable (Table III). Omission of either

TABLE III
PYROPHOSPHATE BINDING BY INHIBITED XMP AMINASE[a]

Reaction Mixtures	PP_i Bound (mμmoles)
$^{32}P^{32}P_i$ + psicofuranine + XMP	4.23
$^{32}P^{32}P_i$ + XMP	0.28
$^{32}P^{32}P_i$ + psicofuranine	0.62

[a] $^{32}P^{32}P_i$ binding was determined by procedure A. The inhibited system contained 3×10^{-5} M $^{32}P^{32}P_i$, 3×10^{-5} M XMP, 3×10^{-5} M psicofuranine, and 3.38 units of enzyme (1.26 mg protein). Inhibition of XMP aminase activity was 60%.

XMP or psicofuranine reduced the binding of PP_i to a low level. As in the case of psicofuranine binding, appreciable amounts of PP_i were attached to the enzyme only if the other compounds necessary for inhibition were available.

The binding of XMP by the aminase is shown in Table IV. In sharp contrast to the situation with psicofuranine and PP_i, XMP binding was readily

TABLE IV
XMP BINDING BY XMP AMINASE[a]

Reaction Mixtures	XMP Bound (mμmoles)
[8-^{14}C]XMP + psicofuranine + PP_i	9.27
[8-^{14}C]XMP + psicofuranine	4.03
[8-^{14}C]XMP + PP_i	4.25

[a] [8-^{14}C]XMP binding was determined by procedure A. The inhibited system contained 2.5×10^{-5} M [8-^{14}C]XMP, 8.3×10^{-5} M psicofuranine, 8.3×10^{-5} M PP_i, and 5.80 units of enzyme (2.90 mg protein). Inhibition of XMP aminase activity was 80%.

measurable in the absence of either of the two additional compounds required for inhibition. Such extensive binding of the substrate is predictable on the basis of its low K_m value, 2.9×10^{-5} M (Udaka and Moyed, 1963). When psicofuranine and PP_i were both initially present in the reaction mixture, XMP binding was increased, the increase being limited by the amount of enzyme used in the experiment. The mutually enhanced binding of XMP, PP_i, and psicofuranine establishes that these compounds are intimately associated with the inhibited state of the enzyme.

Effect of ATP on the Binding of Psicofuranine.—Psicofuranine had been shown to be a noncompetitive inhibitor of the mutant aminase (Udaka and Moyed, 1963) and to have little or no effect on ATP binding by the parental aminase (Table V). Therefore, the

TABLE V
EFFECT OF PSICOFURANINE ON ATP BINDING[a]

Psicofuranine (M)	ATP Bound (mμmoles)
0	2.95
5×10^{-5}	3.24
1×10^{-3}	3.16

[a] [8-^{14}C]ATP binding was determined by procedure B. The reaction mixture contained 5×10^{-5} M [8-^{14}C]ATP psicofuranine as indicated and 6.1 units of enzyme (2.03 mg protein).

antagonism between ATP and psicofuranine for the parental aminase (Udaka and Moyed, 1963) appeared to be paradoxical. Further examination of this relationship revealed that neither the unaided binding of psicofuranine (Table VI) nor the XMP-PP_i stimulated binding of the antibiotic (Table VII, expt 1) was reduced by ATP. However, when a less exhaustively dialyzed preparation of the aminase was used, ATP caused a marked reduction in psicofuranine binding as well as inhibition (Table VII, expt 2). This result can be attributed to the presence of a trace amount of NH_3 in the enzyme preparation which together with ATP allows XMP to be depleted by conversion to GMP. Thus depletion of XMP, one of the compounds necessary for optimal binding of psicofuranine, would appear to account for the antagonism by ATP of the inhibitory effect of the antibiotic on the parental en-

TABLE VI
EFFECT OF ATP ON THE UNAIDED BINDING OF PSICOFURANINE[a]

Reaction Mixtures	Ratio Psicofuranine/ ATP	Psicofuranine Bound (mμmoles)
[^{3}H]Psicofuranine	1/0	1.36
[^{3}H]Psicofuranine + ATP	1/10	1.33

[a] [^{3}H]Psicofuranine binding was determined by procedure B. The reaction mixture contained 5×10^{-5} M [^{3}H]psicofuranine, 5×10^{-4} M ATP, and 11.1 units of enzyme (4.71 mg protein).

TABLE VII
EFFECT OF ATP ON THE AIDED BINDING OF PSICOFURANINE BY AMMONIA-FREE AND AMMONIA-CONTAMINATED AMINASES

Experiment[a]	ATP (M)	Psicofuranine Bound (mμmoles)	Inhibition of XMP Aminase Activity (%)
1	0	5.40	72
(Aminase free	4.5×10^{-5}	5.55	72
of NH_3)[b]	9.1×10^{-4}	4.25	63
2	0	8.31	86
(Aminase with	1.5×10^{-4}	4.94	46
trace of	4.5×10^{-4}	1.23	16
NH_3)[c]			

[a] Experiment 1: Procedure B was used to determine [^{3}H]psicofuranine binding. The reaction mixture contained 4.5×10^{-5} M [^{3}H]psicofuranine, 4.5×10^{-5} M XMP, 4.5×10^{-5} M PP_i, ATP as indicated, and 3.34 units of NH_3-free enzyme (1.13 mg protein). Experiment 2: [^{3}H]Psicofuranine binding was determined by procedure A. The reaction mixture contained 2.5×10^{-5} M [^{3}H]psicofuranine, 1.2×10^{-4} M XMP, 1.2×10^{-4} M PP_i, ATP as indicated, and 4.35 units of enzyme (3.80 mg protein). The enzyme was preincubated with XMP, PP_i, and ATP for 10 minutes prior to the addition of psicofuranine. [b] The aminase preparation used had been dialyzed exhaustively and contained little or no NH_3, as shown by its failure to convert [8-^{14}C]XMP to [8-^{14}C]GMP in the absence of added ammonia. [c] The aminase preparation of this stage of purification contained sufficient NH_3 to convert 10.7 mμmoles of [8-^{14}C]XMP to [8-^{14}C]GMP.

zyme observed in earlier kinetic analyses (Udaka and Moyed, 1963).

Effect of Ammonia on Psicofuranine Binding.— NH_3 had also been found to be an antagonist of psicofuranine inhibition (Moyed, 1961), although its effect is less marked than that of ATP (Udaka and Moyed, 1963). Since NH_3 was unable to reduce the binding of psicofuranine (Table VIII) its effect on psicofuranine inhibition of the functioning aminase may also be attributable to its ability to reduce the XMP concentration.

TABLE VIII
EFFECT OF AMMONIA ON THE AIDED BINDING OF PSICOFURANINE[a]

$(NH_4)_2SO_4$ (M)	[^{3}H]Psicofuranine Bound (mμmoles)
0	14.5
5×10^{-4}	14.1
1×10^{-3}	13.6

[a] [^{3}H]Psicofuranine binding was measured by procedure B. The reaction mixture contained 5×10^{-5} M [^{3}H]psicofuranine, 5×10^{-4} M XMP, 5×10^{-4} M PP_i, $(NH_4)_2SO_4$ as indicated, and 6 units of enzyme (3.6 mg protein).

DISCUSSION

The direct examination of the interaction between XMP aminase, its powerful inhibitor, psicofuranine, and its substrates, XMP, ATP, and NH_3, confirm the earlier conclusion based on kinetic analysis (Udaka and Moyed, 1963) that psicofuranine is neither a simple nor a usual kind of enzyme inhibitor. Inhibition of the parental form of the aminase by this antibiotic is highly dependent on XMP and a product of the reaction, PP_i. Similarly, binding of psicofuranine by the aminase is stimulated several orders of magnitude by XMP in cooperation with PP_i. ATP, which blocks inhibition by psicofuranine, does not directly affect binding of the antibiotic by the aminase. Similarly, NH_3, an improbable analog of psicofuranine, but nevertheless a weak antagonist of its inhibitory effect on the parental aminase, does not displace the antibiotic from the enzyme. It is highly probable that the decrease in sensitivity to psicofuranine observed in the presence of elevated levels of NH_3 or of ATP results from the greater depletion of XMP, a compound necessary for the full expression of sensitivity.

Thus the present binding studies, together with the earlier kinetic analysis of psicofuranine inhibition of a mutant aminase, show that the primary interaction of the enzyme and the drug is a noncompetitive process. Therefore it is suggested that the aminase has a separate site for recognition of psicofuranine. This suggestion is supported by the recent finding that the aminase can be made insensitive to psicofuranine by exposure to several reagents, including urea, mercaptoethanol, and ethylene glycol (Kuramitsu amd Moyed, 1964). It seems unlikely that *E. coli* would produce and preserve such a site fortuitously or for the purpose of being sensitive to bacteriostasis by psicofuranine. Accordingly, it is likely that the psicofuranine site has a more appropriate function. The obvious resemblance to end product–sensitive or allosteric sites (Gerhart and Pardee, 1962; Monod *et al.*, 1963) suggests that its *in vivo* function might be recognition of a normal regulatory substance.

Considerable amounts of PP_i and XMP, as well as psicofuranine, are bound by the inhibited enzyme. Examination of the data obtained in separate experiments, in which enzyme preparations of differing activities and different concentrations of the reactants were used, revealed that similar amounts of XMP, PP_i, and psicofuranine are bound per unit of aminase (Table IX). When these values are corrected for the degree of inhibition obtained in the separate experiments and expressed per unit of aminase activity inhibited, it is clear that equimolar amounts of psicofuranine, XMP, and PP_i are associated with the inhibited enzyme. If it is assumed that a mole of the enzyme binds only one

TABLE IX
STOICHIOMETRY OF BINDING

Compound	Amount Bound mμmoles per Enzyme Unit	Amount Bound mμmoles per Inhibited Enzyme Unit
Psicofuranine	1.81	2.18
PP_i	1.25	2.09
XMP	1.60	2.00

mole of each of these compounds, a molecular weight of 137,000 can be calculated using the protein content of the most active aminase preparations. This compares with a molecular weight of 140,000 calculated from the sedimentation velocity and the diffusion constant as roughly estimated by boundary spreading.

The mutual enhancement by XMP, PP_i, and psicofuranine of one another's attachment to the aminase might be the consequence of bond formation among themselves as well as with the aminase. However, failure to detect adjuncts of psicofuranine and either XMP or PP_i in ethanol-water extracts of the inhibited complex indicates that such bonds, if they are formed, could not be particularly stable. An alternative explanation might be that these compounds cause a cooperative distortion of the aminase which improves its ability to bind each of them. This latter explanation is particularly attractive since it can also be invoked in answer to the question of how binding of psicofuranine, XMP, and PP_i causes inhibition of the aminase's action. However, such configurational changes remain to be demonstrated.

References

Bergmann, F. H. (1962), *Methods Enzymol. 5*, 108.

Bratton, A. C., and Marshall, E. K., Jr. (1939), *J. Biol. Chem. 128*, 537.

Gerhart, J. C., and Pardee, A. B. (1962), *J. Biol. Chem. 237*, 891.

Kuramitsu, H. K., and Moyed, H. S. (1964), *Biochim. Biophys. Acta 65*, 504.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Monod, J., Changeux, J. P., and Jacob, F. (1963), *J. Mol. Biol. 6*, 306.

Moyed, H. S. (1961), *Cold Spring Harbor Symp. Quant. Biol. 26*, 323.

Moyed, H. S., and Magasanik, B. (1957), *J. Biol. Chem. 226*, 351.

Slechta, L. (1960a), *Biochem. Pharmacol. 5*, 96.

Slechta, L. (1960b), *Biochem. Biophys. Res. Commun. 3*, 596.

Udaka, S., and Moyed, H. S. (1963), *J. Biol. Chem. 238*, 2797.

Warburg, O., and Christian, W. (1941), *Biochem. Z. 310*, 384.

Participation of Ribosomes in the Biosynthesis of Gramicidins and Tyrocidines*

JAMES W. BODLEY,† ISAMU UEMURA,‡ P. RADHAKANTHA ADIGA, KIYOSHI OKUDA,‡ AND THEODORE WINNICK

From the Department of Biochemistry and Biophysics, University of Hawaii, Honolulu

Received May 25, 1964

The role of ribosomes in the biosynthesis of gramicidins, tyrocidines, and protein by cell-free preparations of *Bacillus brevis* has been investigated. With extracts of the Dubos strain of this organism, prepared under a variety of conditions, the major ribosomal component had a sedimentation coefficient ($s^\circ_{20,w}$) of 50 S. This particle, separated on sucrose gradients, was active in promoting incorporation of isotopic amino acids into both groups of polypeptides and protein. A small 32 S peak was also consistently observed, which participated only in peptide formation. Ribosomes sedimenting in the region of 70 and 100 S were detected in minor amounts under certain circumstances. Under conditions of low Mg^{2+} and high buffer concentration, two small subunits with sedimentation coefficients of approximately 18 and 27 S appeared on the sucrose gradient. Newly synthesized peptide remained bound to both these particles, while [^{14}C]protein was associated only with the larger subunit. The smaller particle had approximately the same total peptide-synthesizing activity as did the 32 S ribosome. Neither subunit was active alone in protein formation, but a recombination of the particles restored protein and peptide synthesis to their original levels, presumably by the reconstitution of the 50 S ribosome. A cellular fraction sedimenting at 40,000 $\times$ *g* was found capable of accepting the polypeptides newly synthesized by the ribosomes. Experiments with another strain of *B. brevis*, which synthesized gramicidin S, indicated the existence of the usual 30-50-70 S ribosomal pattern. When extracts were prepared in 0.1 M Tris and 5×10^{-3} M Mg^{2+}, the ribosomes appeared in the 30 and 50 S form, with biosynthetic activity residing in the 50 S unit. It was concluded that the 70 S ribosome was probably the physiological form in the *B. brevis* organism.

Ribosomes are well known to serve as the site of protein synthesis in bacterial, plant, and mammalian systems. Until recently, however, there had been no demonstration of the participation of ribosomes in the biogenesis of naturally occurring polypeptides. In fact, the synthesis of uridine nucleotide peptide (Strominger, 1962), and glutamyl polypeptide (Leonard and Housewright, 1963) seems to require only soluble enzymes.

Last year Uemura *et al.* (1963) described a cell-free system from *Bacillus brevis* which catalyzed the net synthesis of the gramicidin and tyrocidine polypeptides, as well as the incorporation of [^{14}C]amino acids into protein. An absolute requirement for ribosomes by both processes was demonstrated. Subsequent work (Okuda *et al.*, 1964a,b), has indicated a close relation-

* This research was supported by a grant (RG-9335) from the National Institutes of Health. Contribution No. 35 from the Pacific Biomedical Research Center, University of Hawaii.

† Research performed in partial fulfillment of requirements for the degree of Doctor of Philosophy in Biochemistry, University of Hawaii. Present address, Department of Biochemistry, University of Washington School of Medicine, Seattle.

‡ On leave from the Osaka City University Medical School, Osaka, Japan.