another vial containing a small amount of anhydrous Na_2SO_4. After shaking, this mixture was filtered into a third vial containing (*S*)-(−)-*N*-pentafluorobenzoylprolyl-1-imidazolide (6, 0.009 mmol). The vial was tightly stoppered and shaken with very gentle warming until the solid imidazolide dissolved. Glpc (conditions b) samples were taken directly from this vial. When the basic components of diazomethane treated lyophylizates were employed, the above procedure was followed except that the ratio of base to imidazolide was *ca.* 1:1. In the cases where the free amine was directly available, *ca.* 0.05 mmol was taken up in 2 ml of dry C_6H_6 and added directly to the imidazolide vial. The results of these analyses are discusssed in the text.

Mass determination on the *m/e* 486 peak of the amide reaction mixture from the collected metabolites gave an exact mass of 486.1583 (calcd for $C_{23}H_{23}F_5N_2O_4$, 486.1578).

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Ring D Bridged Steroid Analogs. 11.[1] The High Clauberg Activity of 19-Nor-14α,17α-ethano-4-pregnene-3,20-dione†

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3β-Acetoxy-14α,17α-etheno-5-pregnen-20-one (**1**) was converted to 14α,17α-ethano-4-pregnene-3,20-dione (**9**) and to 19-nor-14α,17α-ethano-4-pregnene-3,20-dione (**8**). On subcutaneous administration **9** was found to have 2.2 times the Clauberg activity of its 15′-dehydro analog **10**. **8**, which lacks the 10β-methyl of 9, showed a further 13.6-fold increase in Clauberg activity and is 17.6 times as active as progesterone. The possible significance of these results is discussed.

Recently, in order to account for a number of seemingly anomolous structure-activity effects, and especially to explain the surprisingly low Clauberg activity of 14α,17α-etheno-4-pregnene-3,20-dione (**10**), we proposed[2] an extension of Ringold's hypothesis[3] on the nature of binding of progesterone to its receptor. Essentially, our argument is that a two-atom bridge between the 14α and 17α positions of progesterone causes the 17-substituent (OH or acetyl side chain) to be deflected toward the α side of the molecule. Because the 17-substituent appears to interact strongly with the Clauberg receptor, the effect of this deformation is to increase the apparent bulk of all β substituents. However, the 10β-methyl group seems to be too large for optimum fit to the receptor, even in the case of progesterone. The result of any such deformation is therefore to decrease the binding affinity between the hormone analog and the Clauberg receptor. If our argument is correct, removal of the 10β-methyl group from a 14α,17α-etheno- (or ethano-) bridged progesterone analog should result in a larger than normal increase in Clauberg activity. We have already demonstrated this effect for 14α,17α-ethano-4-

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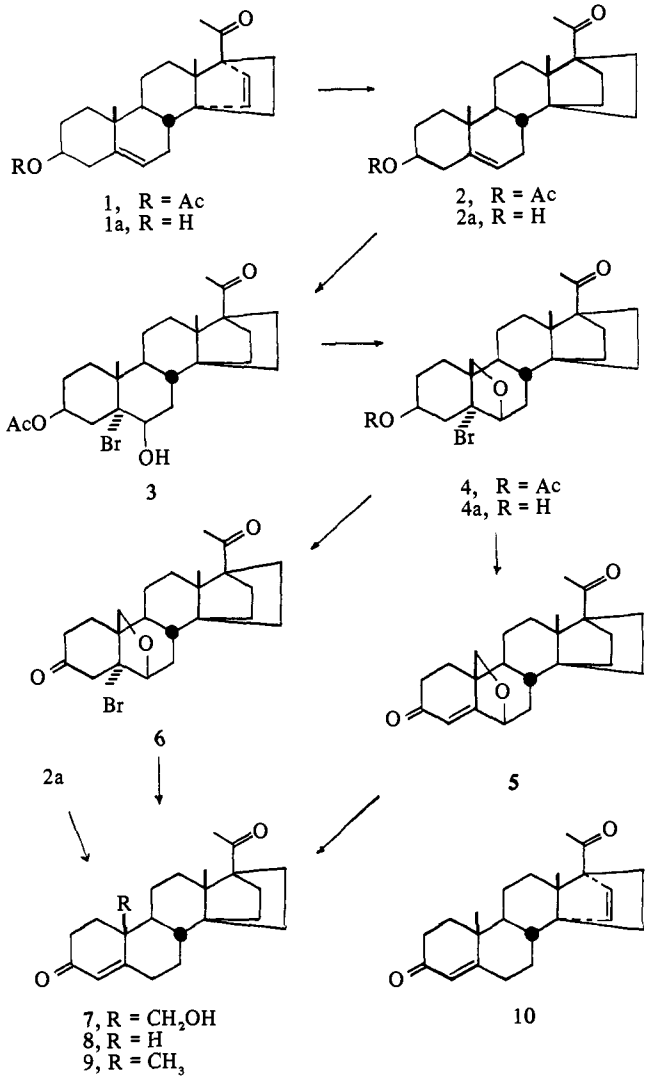
pregnene-3,20-diones which bear 16α-carbomethoxy groups,[4] but, because such ester groups strongly suppress Clauberg activity,[4] it seemed essential to attempt to confirm the effect in compounds lacking that functionality. We now report the results of such a study.

Chemistry. Conversion of 3β-acetoxy-14α,17α-etheno-5-pregnen-20-one (1)[5] to 19-nor-14α,17α-ethano-4-pregnene-3,20-dione (8) was effected, *via* **2**–**7** (Scheme I), under

Scheme I



conditions similar to those developed[4] for the conversion of 3β-acetoxy-14α,17α-etheno-16α-carbomethoxy-5-pregnen-20-one to 14α,17α-ethano-16α-carbomethoxy-4-pregnene-3,20-dione. Selective catalytic hydrogenation of 3β-hydroxy-14α,17α-etheno-5-pregnen-20-one (**1a**) was followed by Oppenauer oxidation to afford the reference compound, 14α,17α-ethano-4-pregnene-3,20-dione (**9**).

Biological Activity. Compounds **8**, **9**, and **10** were assayed for Clauberg activity‡ with the results shown in Table I. When these compounds were administered by subcutaneous injection, they gave dose-response curves parallel to that of progesterone with their activities being respectively 17.6, 1.3, and 0.6 times that of progesterone.

In view of the high activity which the 19-nor compound **8** showed on subcutaneous injection, we felt that it would be of interest also to assay it for oral activity. The limited data available (Table I) indicate that on oral administration **8** is approximately 7.6 times as active as 17α-acetoxyprogesterone. The two compounds gave essentially parallel curves with a much shallower slope than was found on the curves discussed above.

Since removal of a 10β-methyl group from a progesterone analog typically causes a four-eightfold increase in Clauberg activity,[6] the finding that 19-nor compound **8** is 13.6 times as active as its homolog **9** lends some support to our hypothesis. Further support derives from a comparison of the Clauberg activity of **9** with that of its 15′-dehydro analog **10**. The tendency of the two-carbon atom bridge between 14α and 17α to cause the 17-acetyl group to be deflected toward the α side should be greater in **10** than in **9** because of the shorter C-14′,C-15′ bond distance caused by the presence of the double bond. Therefore, **9** should be more active than **10**, as is observed.

Models indicate that a 3-carbon atom bridge can be inserted between the 14α and 17α positions of progesterone without deforming the D ring. Since we attribute the low Clauberg activity of **10** to a ring-D deformation caused by the presence of the etheno bridge, we predict that 14α,17α-propano-4-pregnene-3,20-dione should have approximately the Clauberg activity of **8** rather than that of **10**. As a further test of our hypothesis, we therefore propose to synthesize and assay 14α,17α-propano-4-pregnene-3,20-dione.§

Experimental Section

Melting points were determined in capillary tubes on a Mel-Temp apparatus and are uncorrected. Ir spectra were determined on a Beckman IR-8 spectrophotometer. Nmr spectra were determined in $CDCl_3$ on a Varian A-60 spectrometer and are reported in parts per million downfield from a TMS internal standard. Elemental analyses were performed by Galbraith Microanalytical Laboratories, Knoxville, Tenn. Where analyses are indicated only by symbols of the elements, results obtained for those elements were within ±0.4% of theory.

3β-Hydroxy-14α,17α-ethano-5-pregnen-20-one 3-Acetate (2). A solution of 120 mg of **1**[5] in 110 ml of EtOH and 5 ml of H_2O was hydrogenated over 30 mg of 10% Pd/C under 3.18 kg/cm² of H_2 for 16 hr at ambient temperature. Standard work-up afforded **2** as long white needles from EtOH in a yield of 68 mg (56%), mp 140–141°. The nmr spectrum had singlets at δ 0.90 (C-18 H's), 1.03 (C-19 H's), 2.01 (acetate H's), and 2.08 (C-21 H's), and an unresolved multiplet at δ 5.40 (C-6 H). *Anal.* ($C_{25}H_{36}O_3$) C, H.

3β,6β-Dihydroxy-14α,17α-ethano-5α-bromopregnan-20-one 3-Acetate (3). To a solution of 100 mg of **2** in 10 ml of dioxane was added 0.01 ml of 70% $HClO_4$ in 1 ml of H_2O. The mixture was cooled to 20° and 56 mg of NBA was added over 5 min. The mixture was stirred at 20° for 45 min. The yellow color was discharged by addition of aqueous $Na_2S_2O_3$. The mixture was poured into ice-water. The resulting white precipitate was collected by filtration, washed with H_2O, dissolved in CH_2Cl_2, dried ($MgSO_4$), and evaporated under reduced pressure to give a foam which crystallized from CH_2Cl_2–hexane to afford **3** as needles in a yield of 80 mg (65%): mp 180–181°; ν^{Nujol} 3400, 1735, 1672 cm⁻¹. The nmr had singlets at δ 0.90 (C-18 H's), 1.35 (C-19 H's), 2.03 (acetate H's), and 2.10 (C-21 H's). *Anal.* ($C_{25}H_{35}O_4Br$) C, H.

3β-Acetoxy-14α,17α-ethano-6β,19-oxido-5α-bromopregnan-20-one 3-Acetate (4). To a solution of 400 mg of **3** in 30 ml of anhydrous C_6H_6, 3 g of $Pb(OAc)_4$ (previously dried over KOH under reduced pressure for 4 hr) and 500 mg of I_2 were added. The mixture was refluxed over a 150-W incandescent lamp for 18 hr. The mixture was cooled and filtered. The residue was washed with C_6H_6. The combined organic layer was washed with H_2O, dried, and then chromatographed over 14 g of Woelm neutral Al_2O_3 (activity Grade III). PhH–EtOAc eluent afforded **4** as crystals in a yield of 276 mg

‡Clauberg assays were performed at the Endocrine Laboratory, Madison, Wis.

§Closely related 14α,17α-alkylidenedioxyprogesterone derivatives have recently been reported.[7]

Table I. Progestational Activity in a Modified Clauberg Assay[a]

Compd administered	Rte	Total dose, mg	No. of rabbits	Mean final body wt, g	Mean ovarian wt, mg	Mean uterine wt, g	Proliferation index	
							Range	Mean
Progesterone	sc	0.2	13	1376	49.5	1.24	0.5^+-2.0^+	$1.2^+ \pm 0.5$
	sc	0.5	11	1426	42.5	1.95	2.5^+-4.0^+	$3.2^+ \pm 0.4$
10	sc	0.1	2	1448	53.4	1.42	0	0
	sc	0.2	13	1493	45.0	1.09	0-2.0^+	$0.4^+ \pm 0.4$
	sc	0.5	11	1394	40.5	1.35	0.5^+-3.5^+	$1.6^+ \pm 0.6$
	sc	1.0	2	1307	83.2	2.64	4.0^+	4.0^+
9	sc	0.1	4	1271	35.7	1.11	0-0.5^+	$0.3^+ \pm 0.3$
	sc	0.2	6	1407	34.0	1.62	1.0^+-3.0^+	$1.8^+ \pm 0.6$
	sc	0.5	6	1236	30.9	2.35	3.0^+-4.0^+	$3.7^+ \pm 0.3$
8	sc	0.01	6	1294	35.7	1.18	0.5^+-1.0^+	$0.8^+ \pm 0.2$
	sc	0.02	6	1380	43.2	1.88	2.0^+-3.0^+	$2.8^+ \pm 0.3$
	sc	0.05	6	1268	30.3	2.56	3.5^+-4.0^+	$3.9^+ \pm 0.2$
17α-Acetoxy-progesterone	Oral	1.0	4	1278	37.7	1.57	0.5^+-1.0^+	$0.8^+ \pm 0.3$
	Oral	2.5	5	1306	33.2	1.64	0.5^+-2.5^+	$1.1^+ \pm 0.6$
8	Oral	0.01	2	1280	29.2	1.33	0	0
	Oral	0.2	4	1247	30.0	1.62	0.5^+-1.1^+	$0.9^+ \pm 0.4$
	Oral	0.5	3	1478	34.9	1.69	1.0^+-2.0^+	$1.3^+ \pm 0.4$

[a] Progestational proliferation is evaluated histologically according to the procedure of McPhail. For details, see T. Miyake in "Methods in Hormone Research," Vol. II, R. I. Dorfman, Ed., Academic Press, New York, N. Y., 1962, p 135.

(60%), mp 176-177°. Repeated recrystallization from Et_2O gave an analytical sample: mp 179-181°; ν^{Nujol} 1730, 1690 cm^{-1}. The nmr spectrum had singlets at δ 0.90 (C-18 H's), 2.03 (acetate H's), 2.08 (C-21 H's), and a pair of doublets at δ 3.88 and 4.07 (C-19 H's). *Anal.* ($C_{25}H_{33}O_4Br$) C, H.

3β-Hydroxy-14α,17α-ethano-6β,19-oxido-5α-bromopregnan-20-one (4a). A solution of 317 mg of 4 and 75 mg of KOH in 20 ml of MeOH and 1.5 ml of H_2O was stirred overnight at room temperature. The resulting mixture was taken to dryness under reduced pressure and then partitioned between $CHCl_3$ and H_2O. After being dried ($MgSO_4$) the $CHCl_3$ solution was concentrated, and the residue was chromatographed over 12 g of Woelm neutral Al_2O_3. Elution with C_6H_6-EtOAc afforded 4a as rods in a yield of 290 mg (90%), mp 215-216°. Repeated crystallization from Me_2CO gave an analytical sample: mp 219-220°; ν^{CHCl_3} 3450, 1685 cm^{-1}. The nmr spectrum had singlets at δ 0.91 (C-18 H's), 2.10 (C-21 H's), and a pair of doublets at δ 3.85 and 4.07 (C-19 H's). *Anal.* ($C_{23}H_{31}O_3Br$) C, H.

14α,17α-Ethano-6β,19-oxido-4-pregnene-3,20-dione (5). To a solution of 65 mg of 4a in 10 ml of Me_2CO was added 0.1 ml of 8 *N* chromic acid with stirring and cooling (0°). After 1 hr several drops of EtOH were added to the mixture which was then filtered. The residue was washed with Me_2CO and the combined organic layer was evaporated to dryness to give a foam. The foam crystallized from CH_2Cl_2 to give 5 as plates in a yield of 45 mg (80%): mp 140-142°; ν^{Nujol} 1680, 1665 cm^{-1}. The nmr spectrum showed singlets at δ 1.00 (C-18 H's) and 2.12 (C-21 H's); a pair of doublets at δ 3.55 and 4.30 (C-19 H's), and a multiplet at δ 5.90 (C-4 H). *Anal.* ($C_{23}H_{30}O_3$) C, H.

14α,17α-Ethano-6β,19-oxido-5α-bromopregnane-3,20-dione (6). To a solution of 700 ml of 4a in 12 ml of glacial AcOH was added 225 mg of CrO_3. The mixture was stirred for 10 min at ambient temperature (≥35°) and then for 15 min at 60°. The mixture was then poured into ice-water. The precipitate was filtered, washed with H_2O, and dried under vacuum for 4 hr to afford crude 6 which was not characterized but rather was used directly in the following experiment.

14α,17α-Ethano-19-hydroxy-4-pregnene-3,20-dione (7). A. The crude 6, reported in the experiment above, was dissolved in 50 ml of *i*-PrOH and 4 g of Zn dust was added. The mixture was refluxed for 24 hr and then filtered while hot. The residue was washed with MeOH and the combined organic phase was distilled to dryness. The residue was dissolved in $CHCl_3$, washed with aqueous HCl and then with H_2O, dried ($MgSO_4$), and concentrated under reduced pressure to give 400 mg of foam. Treatment with Me_2CO caused 200 mg of 7 to separate as crystals. The mother liquors were chromatographed over 10 g of Woelm neutral Al_2O_3 to afford a further 30 mg of 7 and 150 mg (26%) of 5, identical in properties with that reported above. The combined fractions of 7 (41%) crystallized from Me_2CO as needles, mp 210-212°. Repeated crystallization gave as analytical sample: mp 212-214°; ν^{Nujol} 3370, 1690, 1668 cm^{-1}. The nmr spectrum had singlets at δ 0.91 (C-18 H's), 2.07 (C-21 H's), a broad peak at δ 4.05 (C-19 H's), and a peak at δ 5.93 (C-4 H). *Anal.* ($C_{23}H_{32}O_3$) C, H.

B. A solution of 100 mg of 5 in 10 ml of glacial AcOH was stirred overnight at room temperature with 2.5 g of Zn dust. The mixture was filtered, and the residue was washed with MeOH. The organic phase was concentrated to dryness, partitioned between CH_2Cl_2 and H_2O, dried, and taken to dryness to give a foam which crystallized from Me_2CO to give 7, identical in properties with that reported in A, in a yield of 25%.

19-Nor-14α,17α-ethano-4-pregnene-3,20-dione (8). To a solution of 230 mg of 7 in 50 ml of Me_2CO was added 0.45 ml of 8 *N* chromic acid dropwise with stirring and cooling to 0°. After the addition (1 hr), several drops of EtOH were added. The mixture was filtered and the residue was washed with Me_2CO. The Me_2CO solution was concentrated to dryness, and the residue was partitioned between CH_2Cl_2 and H_2O. The CH_2Cl_2 was distilled and the residue was refluxed for 2 hr in a mixture of 20 ml of MeOH, 0.5 ml of concentrated HCl, and 0.5 ml of H_2O. The mixture was then taken to dryness. The residue was dissolved in CH_2Cl_2 and washed to neutrality with H_2O. The solution was dried ($MgSO_4$), taken to dryness, and subjected to preparative tlc to afford 8 in a yield of 80 mg and 70 mg of the intermediate 10-carboxaldehyde: ν^{Nujol} 1711, 1695, and 1675 cm^{-1}; δ 0.87 (C-18 H's), 2.08 (C-21 H's), 9.83 (C-19 H).

The above aldehyde was reoxidized with 0.12 ml of 8 *N* chromic acid and worked up as above. Preparative tlc afforded an additional 35 mg of 8 to give a total yield of 115 mg (54%). The substance crystallized from Me_2CO-hexane as needles: mp 145-146°; ν^{Nujol} 1680, 1662, 1615 cm^{-1}. The nmr had singlets at δ 0.97 (C-18 H's), 2.13 (C-21 H's), and a multiplet at δ 5.83 (C-4 H). *Anal.* ($C_{22}H_{28}O_2$) C, H.

3β-Hydroxy-14α,17α-ethano-5-pregnen-20-one (2a). To a solution of 50 mg of 1a[5] in 15 ml of MeOH was added 10 mg of 10% Pd/C. The mixture was hydrogenated at 3.52 kg/cm^2 for 3.5 hr at ambient temperature. 2a was obtained in quantitative yield as small needles from MeOH: mp 241-244°; ν^{Nujol} 3485, 1670 cm^{-1}; δ 0.91 (C-18 H's), 1.03 (C-19 H's), 2.10 (C-21 H's), 5.36 (C-6 H). *Anal.* ($C_{23}H_{34}O_2$) C, H.

14α,17α-Ethano-4-pregnene-3,20-dione (9). A solution of 600 mg of 2a in 5 ml of cyclohexanone and 100 ml of toluene was azeotroped under a Dean-Stark head for 4 hr. Then 0.65 g of $Al(O\text{-}i\text{-}Pr)_3$ was added and reflux was continued for 2 hr. Standard work-up afforded 9 in a yield of 330 mg (53%) as opaque white crystals from ether: mp 130-131°; ν^{Nujol} 1690 (sh), 1675 cm^{-1}; δ 0.94 (C-18 H's), 1.22 (C-19 H's), 2.11 (C-21 H's), and 5.76 (C-4 H). *Anal.* ($C_{23}H_{32}O_2$) C, H.

Acknowledgments. The synthesis of 2 was originally achieved by Dr. Baldev Singh.[8]

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Tyrosyl Transfer Ribonucleic Acid Synthetase from *Escherichia coli* B. Analysis of Tyrosine and Adenosine 5′-Triphosphate Binding Sites†

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Structural and stereochemical requirements for substrate binding to tyrosyl-tRNA synthetase from *Escherichia coli* B have been investigated using analogs of L-tyrosine and ATP. The two major binding loci for the amino acid have been shown to be the phenol and amine moieties. The phenolic hydroxyl is bound as its neutral form and does not act as a hydrogen bond acceptor. It is the primary site of recognition and its omission results in at least a 10,000-fold loss in binding. The amino group of the substrate binds as its protonated form to an area of the enzyme which is probably best represented as anionic. The carboxylate moiety does not appear to be a contact point and may be substituted by disparate groups with little effect on binding. Adjacent to the carboxylate binding site lies a hydrophobic region and a group capable of interaction with negatively charged substituents. The stereospecificity of the enzyme is not exact and D enantiomers complex with only small losses in affinity. These losses may be attributed to the energy required for rotation about the C_α-C_β bond of D-tyrosine and its analogs or an analogous conformational change of the enzyme, which is necessary to accommodate the major binding loci. Binding of ATP requires interactions of the intact triphosphate moiety. Analogs not possessing this moiety bind as weak, noncompetitive inhibitors and may interact as dimers at a site remote from that which binds to ATP.

Each of the aa-tRNA‡ synthetases is responsible for the attachment of an amino acid to the tRNA's which recognize the codons for that amino acid. Should an uncorrected mistake occur at this stage, the amino acid would be incorporated into an incorrect position of the protein.[1] The overall reaction catalyzed by these enzymes appears to involve (1) activation of a particular amino acid to form an aminoacyl adenylate intermediate and (2) transfer of the activated amino acid to its cognate tRNA.

$$\text{aa} + \text{ATP} \rightleftharpoons \text{aa-AMP} + \text{PP}_i \quad (1)$$

$$\text{aa-AMP} + \text{tRNA} \rightleftharpoons \text{aa-tRNA} + \text{AMP} \quad (2)$$

An understanding of the molecular basis for the specificity of these enzymes requires a knowledge of the intermolecular forces which lead to substrate recognition and binding, as well as differences in the binding sites among the various synthetases.

A number of studies have recently been reported in which competitive inhibitors have been utilized to map the active sites of these enzymes[2-5] in an attempt to answer pertinent questions regarding their specificity. Investigations of this type also provide fundamental information necessary for the design of potent and specific inhibitors of these enzymes which should be useful for a variety of biological investigations.

In the present work, analogs of L-tyrosine and ATP are utilized to probe the topography and localized environment of the substrate binding sites of tyrosyl-tRNA synthetase from *Escherichia coli* B. Comparisons with other activating enzymes from the same source have permitted the assignment of a number of differences and similarities in substrate binding and recognition sites.

Materials and Methods

A 550-fold purified preparation of TRS was isolated from *E. coli* B harvested in the late log phase (General Biochemicals) by the method of Calendar and Berg[6] with the exception that C-γ gel fractionation was omitted. Under standard assay conditions with saturating amounts of substrates, this preparation catalyzed the exchange of 55 μmol of $^{32}PP_i$ into ATP per minute per milligram of protein. Inhibition constants (K_i) were obtained by double-reciprocal plots[7] varying L-tyrosine or ATP·Mg in equal increments from 30 to 3.3 nmol/ml or 1.25 to 0.14 μmol/ml, respectively; all other components were held constant at the concentrations given above. Values for K_i for noncompetitive inhibitors were calculated using equations presented by Dixon and Webb.[8] The $MgCl_2$ concentration used falls on the broad optimum (5-15 m*M*) where the exchange rate is insensitive to small variations. When inhibitors known to complex with Mg^{2+} were tested, an equivalent amount of $MgCl_2$ was added to ensure against depletion. Protein concentration was determined spectrophotometrically by the method of Warburg and Christian[9] or Groves, *et al.*[10] One unit of enzyme is that amount incorporating 1 μmol of $^{32}PP_i$ into ATP in 1 min in the standard assay. The ATP-PP_i exchange assay was carried out as described by Calendar and Berg.[6] The standard assay reaction mixture (1 ml) contained 100 m*M* Na cacodylate (pH 7.0), 5 m*M* $MgCl_2$, 2 m*M* ATP, 2 m*M* $Na_4P_2O_7$ (*ca.* 10^5 cpm/μmol), 10 m*M* 2-mercaptoethanol, 0.1 mg of BSA, 0.1 m*M* tyrosine, and enzyme (0.005-0.007 unit). The assay mixture was incubated at 37° and, at appropriate

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‡Abbreviations used are: TRS, tyrosyl-tRNA synthetase (E.C. 6.1.1.1) of *Escherichia coli* B; PRS, phenylalanyl-tRNA synthetase (E.C. 6.1.1.4) of *Escherichia coli* B, $tRNA^{Tyr}$, tRNA specific for tyrosine acceptance; aa-tRNA, aminoacyl-tRNA; aa-AMP, aminoacyl adenylate; α,β-CH_2-ATP, α,β-methylene ATP.