TABLE III

INTERACTION OF HETEROLOGOUS ENZYMES WITH YEAST t-RNA FRACTIONS FROM COUNTERCURRENT DISTRIBUTION[a]

Substrate	Source of Enzyme		
	Ps. fluorescens	Liver	*E. coli* K_{12}
E. coli methyl deficient t-RNA	12,200	1800	10,200
Yeast t-RNA	1,440	650	1,120
Fractionated yeast t-RNA			
(a) Fraction 1	0	530	Not done
(b) Fraction 5	0	650	0
(c) Fraction 8	40	530	60
(d) Fraction 12	100	340	120
(e) Fraction 14	170	360	40
(f) Fraction 15	1,940	290	90

[a] The reaction mixture employed contained 1 mg of the indicated t-RNA, 100 μmoles of Tris buffer, *p*H 8.2, 10 μmoles of reduced glutathione, 10 μmoles of $MgCl_2$, 0.02 ml of *S*-adenosylmethionine-methyl-^{14}C, and 0.5 ml of enzyme extract in a total volume of 1.0 ml. The activity is expressed as counts per minute.

DNA, conferring upon it a species individuality. Such structural individuality might render difficult the integration of foreign DNA (from some infecting parasite) into the DNA of the host, and thus the species-specific methylation would serve as a guardian of DNA.

ACKNOWLEDGMENT

We are indebted to Miss Carol Sussman for her devoted assistance with these experiments. We also wish to acknowledge the generous help of Drs. Thomas Staudt and H. Boyd Woodruff of Merck and Co., Rahway, N. J., for growing large quantities of bacteria for us. We are grateful to Dr. R. W. Holley for gifts of specific t-RNA's.

ADDED IN PROOF

The tyrosyl t-RNA (fraction 15, Table III) which had been exposed to the methylating enzymes of *Ps. fluorescens* has now been examined for the pattern of methylation. Almost all of the radioactivity, and therefore methyl groups, was found in methyladenylic acid. It is noteworthy that a species of RNA which already contains eight methylated bases should be the recipient of another methyl group, in contrast to the valyl and alanyl t-RNA's which contain but a few methylated bases initially and receive no more even from the most efficient heterologous enzymes.

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Interactions of Purine with Proteins and Amino Acids*

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The effect of purine on the transition temperature (T_m) of bovine serum albumin, ribonuclease, and lysozyme has been studied by optical rotation at 589 mμ. It is found that purine decreases the T_m of these proteins in proportion to the activity of purine in solution. In appropriate concentration of purine (higher required for bovine serum albumin) the thermal denaturation of lysozyme and bovine serum albumin is instantly reversible, a phenomenon not observable in the absence of purine. In comparison to urea on activity basis, purine is 6- to 10-fold more effective in lowering the T_m of the proteins. Purine (0.3 molal) increases the solubility of tyrosine (300%), of tryptophane (250%), of phenylalanine (40%), and of histidine (15%), but does not increase the solubility of glycine and alanine. Secondary structures of proteins appear to be much less sensitive to the influence of purine than do those of nucleic acids. Purine may serve as a selective denaturant for nucleic acids in protein solution. A distinction between the mechanism of purine denaturation and urea denaturation has also been made.

Optical rotation measurements in the visible region have been successfully employed in the study of the interaction of nucleic acids with various ultraviolet-absorbing compounds of biological importance (Ts'o *et al.*, 1962a). This technique is very sensitive to changes in conformation of biopolymers, and optical activity versus temperature profiles of the biopolymers

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can be measured in this wavelength region without interference from certain chromophores. The data show that the helix-coil transition temperatures (T_m) of thymus DNA and helical poly-A are lowered by pyrimidines, purines, nucleosides, and various analogs and derivatives. The effectiveness of these compounds in lowering the T_m of nucleic acids was found to be much greater than that of urea. The order of effectiveness among them indicates that hydrophobic stacking interactions are of importance.

Understanding of the nature of interactions between nucleic acids (or nucleotides) and proteins (or amino acids) is of increasing biochemical importance. We therefore wish to use the same approach as outlined above to investigate the effect of purine on the secondary structure of proteins. The free base, rather than the purine nucleotide, has been chosen for this work because of its solubility, availability, and electrostatic neutrality. Also, as shown in a previous communication (Ts'o *et al.*, 1962a), the effect of purine on nucleic acids is the same as that of adenosine and guanosine. BSA,[1] lysozyme, and ribonuclease were chosen as representative proteins.

Since hydrophobic forces and base stacking appear to be chiefly responsible for the interactions between purine and nucleic acids, one might expect that the effect of purine on proteins would be principally upon the side chains, and especially on the aromatic residues. The secondary structure of proteins might be expected to be less sensitive to purine than that of nucleic acids. These two expectations will be shown to be consistent with the experimental data presented in later sections.

Various biochemical reactions, such as those between transfer RNA and amino acid–activating enzymes, involve both nucleic acid and protein in close association. In such reactions it is important to assess the role of the secondary structure of these biopolymers. Changing the environment, such as raising the temperature, usually affects both biopolymers indiscriminately and nonspecifically. We therefore need to search for compounds which preferentially affect the secondary structure of nucleic acid, leaving that of protein unattacked. Data presented here indicate that the secondary structure of protein is retained at purine concentration in which the nucleic acid is denatured. This system may allow one to control and investigate the contribution of secondary structure of nucleic acids in such biochemical reactions.

Experimental

Purine was purchased from Cyclo Chemical Corp., Los Angeles, Calif. For further purification, purine was dissolved in boiling absolute ethanol. Purified charcoal was added to the amount of 3% by weight of the purine. The mixture was boiled for 1–2 minutes, filtered, and distilled under reduced pressure to low volume. The purine crystallized out on cooling. The ribonuclease and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, Mo. Lysozyme was purchased from Worthington Biochemical Corp., Freehold, N.J. The amino acids and all other chemicals used were of reagent grade.

Instrumentation and Method of Analysis.—Optical rotation measurements were made with a Rudolph 200S polarimeter equipped with an oscillating polarizer and xenon and mercury-arc lamps (Ts'o *et al.*, 1962b). The 20-cm polarimeter utilized glass construction with water jacket and quartz window. The temperature of the polarimeter tube was read directly with a thermometer and held at any desired temperature ±0.1° by the flow of water through both compartments and the tube. Measurements of specific rotation were determined at protein concentrations of 8–10 mg/ml.

Measurements of *p*H were made with a Radiometer *p*H meter 22, Copenhagen, Denmark, to an accuracy of ±0.1 *p*H unit.

Protein solutions of known concentration were added to purine or urea solutions (except at the highest purine concentration, in which a weighed amount of protein was added directly) to bring the urea or purine solution to a known molar concentration (moles per liter). The molar concentrations of purine or urea were converted to molal concentration by means of the equation: $m = c/(d - 0.001cM)$ (Robinson and Stokes, 1959), where m = molality, c = concentration in moles/liter, M = molecular weight of solute, d = density of the solution, and units are in g/ml.

The density of different concentrations of purine and of urea were determined in water to obtain d in the above expression. The molal-activity coefficients determined by Ts'o *et al.* (1963) for purine and those determined by Scatchard *et al.* (1938) for urea were used to calculate the activity of the purine or urea in solution.

The extinction coefficients ($E^{1\%}_{1cm}$) of the proteins were based upon dry-weight determinations. A known weight of protein was dissolved in buffer (0.1 M K-phosphate, *p*H 6.54) and the concentration per ml was determined spectrophotometrically. A known volume of the protein solution was concentrated at 80° and then dried *in vacuo* at 80°. The weight of the buffer salt obtained by procedure similar to above was subtracted in each case. The $E^{1\%}$ obtained for ribonuclease is 7.6 (λ_{max} = 278 mμ), 6.7 for BSA (λ_{max} = 278 mμ), and 25.4 for lysozyme (λ_{max} = 281 mμ). Yang and Foster (1954) obtained $E^{1\%}_{max}$ = 6.7 for BSA (dry wt).

The refractive indices of the purine solutions were measured with a Zeiss Abbey refractometer. Refractive-index corrections were made for calculations of specific rotations for the protein-purine solutions. This correction is usually small even at the highest purine concentration. The refractive index of purine at 4 molal concentration (25°, 589 mμ) is 1.4080.

Solubility Measurements.—A known weight of amino acid together with 1 ml of solvent was sealed in a 10-ml glass ampule. The solvent was 0.1 M K-phosphate buffer, *p*H 6.5, containing purine of known activity. The sealed ampules were shaken in a 55° water bath for 15 minutes. Under these conditions the solution was supersaturated with reference to 25° at which the solubility was measured. A single determination of the solubility required four ampules. Two of these were shaken in the equilibration bath (25°) for 5 days. The other two ampules were stored in the refrigerator (4°) for 24 hours before being placed in the equilibration bath. The contents of the ampules were centrifuged for 1–2 minutes in a clinical centrifuge at room temperature (24–26°). The supernatant was carefully removed and centrifuged at about 3000 rpm for 5–10 minutes. The concentration of the amino acids was determined by the ninhydrin colorimetric method (Moore and Stein, 1954). All four measurements were always within ±2% of each other.

Results

For the sake of simplicity in discussion, the following terms will be defined: *Heating curve:* optical rotation–versus–temperature profile of protein measured during

[1] Abbreviation used in this work: BSA, bovine serum albumin.

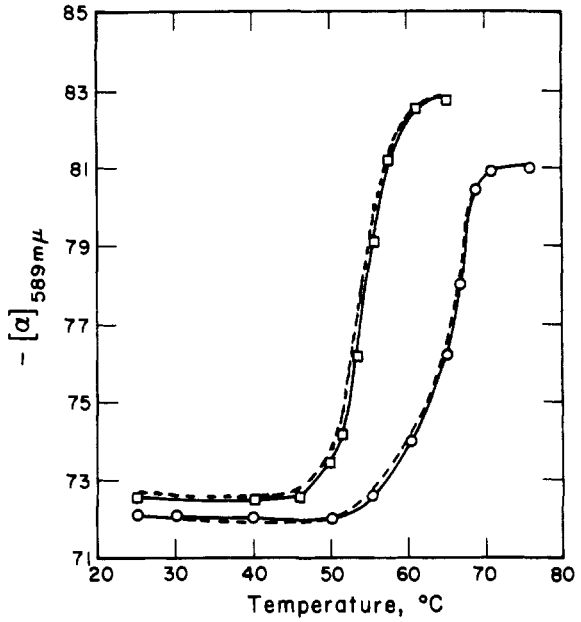

FIG. 1.—Profile of $-[\alpha]_{589}$ versus temperature of ribonuclease in 0.1 M K-phosphate, *p*H 6.54 (O—O) and in 1.1 *m* purine, 0.1 M K-phosphate, *p*H 6.54 (□—□). The dotted lines represent the cooling profiles.

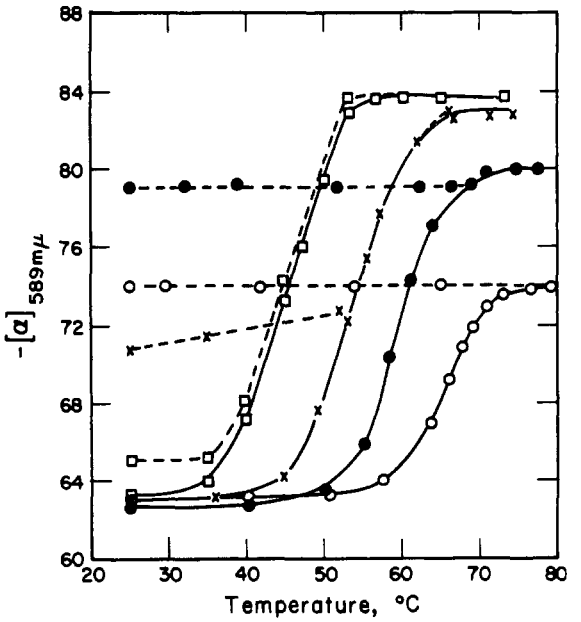

FIG. 2.—Profile of $-[\alpha]_{589}$ versus temperature of BSA in 0.1 M K-phosphate, *p*H 6.54 (O—O); in 0.8 *m* purine (●—●); in 1.7 *m* purine (×—×); and in 4 *m* purine (□—□). Purine solutions were in 0.1 M K-phosphate, *p*H 6.54. The corresponding dotted lines represent the cooling profiles.

the course of increasing temperature. *Cooling curve:* optical rotation–versus–temperature profile measured during the course of decreasing temperature. *Helix coil transition temperature* (T_m): the temperature which is at the mid-point of a heating curve, unless otherwise specified, between the temperature-insensitive zone of optical rotation in the lower temperature region (native state) and the temperature-insensitive zone of optical rotation in the higher temperature region (denatured state). This point usually coincides with the region in which the change in optical rotation is most sensitive to temperature. ΔT_m: the lowering of T_m at constant *p*H and ionic strength brought about by the presence of an interacting substance.

All rotation measurements were made at 589 mμ. At shorter wavelengths, such as 436 mμ, solutions of high concentrations of purine absorb appreciably. In addition, much of the earlier data on the optical rotation of these proteins were collected at this wavelength.

Effect of Purine on the Heating Curve and Denaturation.—Three observations have been made: (A) In the presence of purine the heating curves are shifted to lower temperature without noticeable change in shape. These shifts can therefore be characterized by their T_m (Figs. 1, 2, 3).

(B) The quantity ΔT_m is a linear function of purine activity up to an activity of about 0.25 *m* units (concentration = 1.1 *m*) (Fig. 4). As anticipated it is not a linear function against concentration without correction from activity coefficient. However, the activity-coefficient measurements of Ts'o *et al.* (1963) do not extend above 1.1 *m*. The points at higher purine concentration in Fig. 4 have been plotted with the activity coefficients at 1.1 molal. The meaning of the dotted lines beyond 0.25 *m* activity will be discussed below.

(C) The negative value of optical rotation at the temperature-insensitive region above *T*, i.e., the denatured state, is larger in the presence of purine than in the absence of purine. In other words, the curve reaches a higher plateau in the presence of purine than in the absence of purine (Figs. 1, 2, 3). The decrease in the negative rotation of the denatured state seems to be augmented with increase in purine concentration until a maximum is reached at certain concentrations of purine (above 3 *m*).

Effect of Purine on the Cooling Curve and Renaturation.—In the absence of an interactant, ribonuclease is the only protein of those tested which yields a cooling curve superimposable on the heating curve, an indication of rapid renaturation.

In the case of lysozyme there is very little decrease in negative rotation upon cooling from the denatured state at 85° (0.1 M K-phosphate, *p*H 6.5). Occasionally the solution becomes turbid at elevated temperatures. This occurrence of turbidity has been observed formerly by Foss (1960) at a temperature (67°) which is well below the denaturation temperature. He attributed this turbidity not to the precipitation of lysozyme but to impurities which can be eliminated by a short heat treatment. We found that a lysozyme solution which has been preheated in the presence of purine and cooled, and the purine removed by dialysis, developed no turbidity with further heating to 85°. However, it did sometimes become turbid on cooling to room temperature. Nevertheless in all these cooling curves, whether or not turbidity appears, the negative rotation was not at any time appreciably decreased from the value obtained at the highest temperature region (85°). Such irreversibility in rotation has been previously observed by Foss (1960). In the presence of purine, on the other hand, (molal concentration, 0.3–4.0) the cooling curve is superimposable on the heating curve (Fig. 3). This indicates that lysozyme can renature rapidly in the presence of purine. To test this conclusion, lysozyme was slowly heated to 80° in 1.7 *m* purine solution and then rapidly cooled to 25°. The purine was dialyzed out in the cold and the soluble fraction, which constituted about 90–95% of the original lysozyme solution, was reheated to determine T_m. As shown in Figure 3, the T_m of the lysozyme solution so treated is practically the same as that of the original solution.

Similarly with BSA, there is very little decrease in

TABLE I

THE EFFECTS OF UREA AND OF PURINE ON THE T_m OF THREE PROTEINS

Proteins	T_m in Buffer (°C)	Urea Activity[a] ($\gamma \times m$)	ΔT_m (°C) Due to Urea	Purine Activity[b] ($\gamma \times m$)	ΔT_m (°C) Due to Purine
Lysozyme	75.5	1.43	5.0	0.145	6.0
		2.78	8.5	0.231	10.5
		4.58	15.0	0.404	14.0
Ribonuclease	64.0	1.89	10.0	0.145	6.0
		2.8	14.0	0.231	9.0
				0.404	12.5
Bovine serum albumin (BSA)	66.0	0.925	5.0	0.231	6.0
		2.8	Partially denatured at 25°	0.404	11.0

[a] Scatchard *et al.* (1938). [b] Ts'o *et al.* (1963).

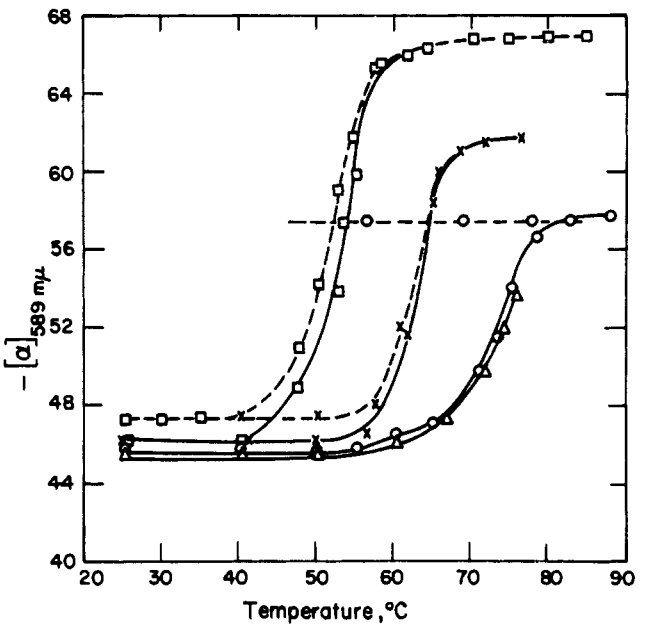

FIG. 3.—Profiles of $-[\alpha]_{589}$ versus temperature of lysozyme before addition of purine (Δ—Δ); in 0.8 *m* purine (×—×); and in 4 *m* purine (□—□); all buffered with 0.1 M K-phosphate, *p*H 6.54. (○—○) represents the profile for a sample previously heated with purine and the purine removed by dialysis at 4°. The corresponding dotted lines represent the cooling profiles.

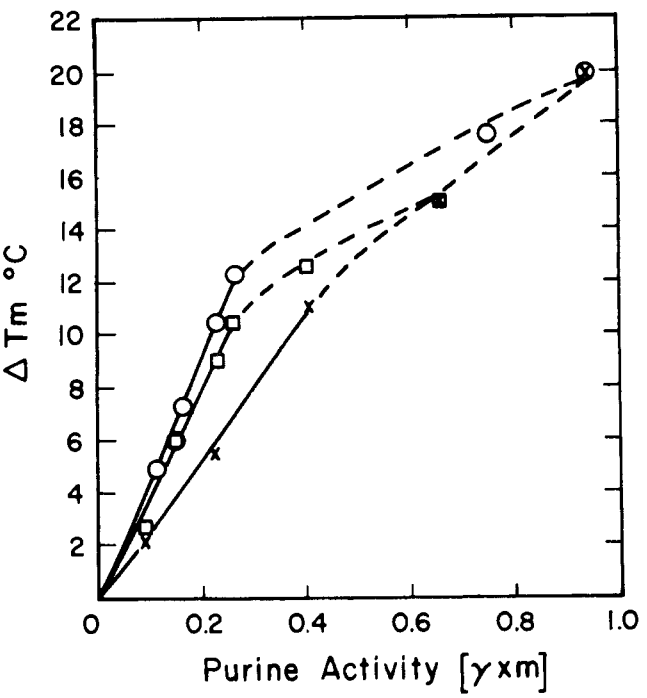

FIG. 4.—Plot of change in the transition temperature (ΔT_m) versus the activity ($\gamma \times m$) of purine (0.1 M K-phosphate, *p*H 6.54) for lysozyme (○), ribonuclease (□), and BSA (×).

negative rotation upon cooling from the denatured regions at 85° in 0.1 M K-phosphate *p*H 6.5 buffer in the absence of purine. This indicates irreversible denaturation as a result of heating above the T_m. In the presence of 3 M purine the cooling curve is superimposable on the heating curve. This is indicative of rapid renaturation. Interestingly, as the concentration of purine decreases, say to 1.7 *m*, only the upper part of the cooling curve simulates the heating curve. The lower part of the cooling curve deviates from the heating curve and exhibits a much higher negative rotation. This observation suggests that BSA renatures only partially in 1.7 *m* purine. In the presence of 0.8 *m* purine the renaturation of BSA becomes hardly noticeable. These results indicate that a higher concentration of purine is required for BSA than for lysozyme for the rapid renaturation process.

Comparison of the Effect of Purine and Urea.—A detailed study of urea denaturation of these proteins is clearly beyond the scope of this paper. Our intention here is merely to compare the effects of purine and urea under a comparable set of conditions, and as revealed by heating and cooling curves.

Again as with purine, urea causes no significant difference in the shapes of the heating curves of lysozyme and ribonuclease though it lowers the T_m of these two proteins (Fig. 5). Therefore a comparison of effects of urea and purine on ΔT_m can be made and is given in Table I. It can be seen that the purine is usually 6–12 times more effective than urea based on the same activity. The kinetics of the denaturation of BSA in urea is complex, and thus no simple comparison can be made. In 1 *m* urea, however, the rotation of BSA remains essentially constant up to 49° and the shape of the transition is similar to that of BSA in the absence of urea starting from 76°. On cooling to 25° a slight but noticeable negative increase over the value obtained at the highest temperature (76°) can be observed. In urea concentration of 2.2 *m* this additional increase in negative rotation becomes more noticeable. The same phenom-

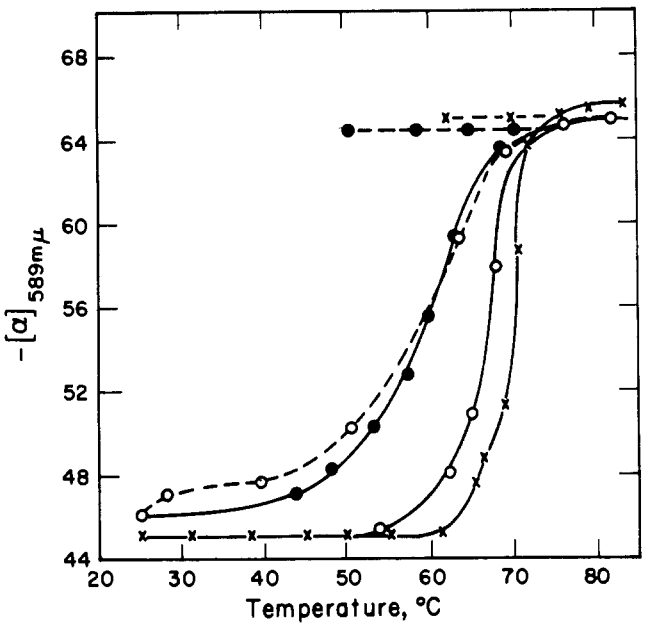

FIG. 5.—Profile of $-[\alpha]_{589}$ versus temperature for lysozyme in 1.6 *m* urea (×—×); first heating profile in 3.5 *m* urea (–O–O); second heating cycle in 3.5 *m* urea waited 12 hours later at 25° (–●–●–). The corresponding dotted lines represent the cooling profiles of each cycle.

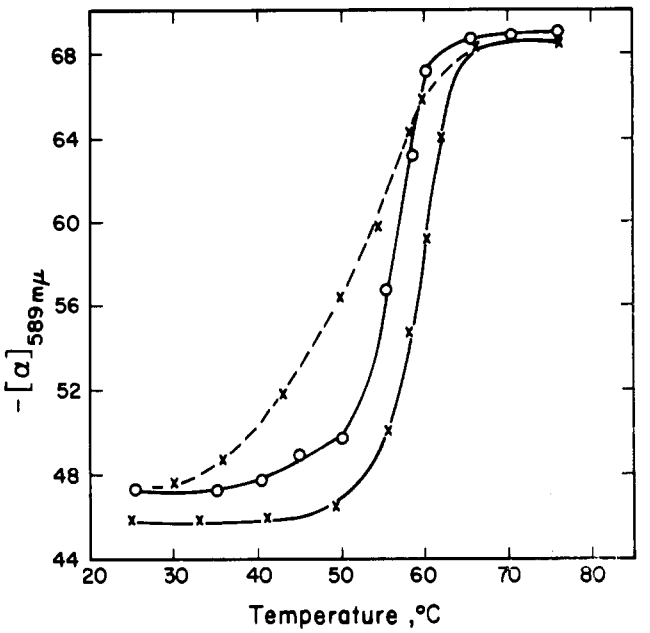

FIG. 6.—Profile of $-[\alpha]_{589}$ versus temperature for lysozyme in 5 M urea, 0.1 M K-phosphate, *p*H 6.5 (×—×), and in the same 5 M urea solution on second heating cycle waited 12 hours later (O—O). The dotted lines represent the cooling profile for the heating curve.

enon, i.e., a gradual time-dependent increase in negative rotation, can be observed if the BSA solution is left for a considerable length of time before cooling. These complications are typical of BSA denaturation by urea. The ΔT_m reported at 1 and 2.2 *m* urea concentration for BSA (Table I), however, refers to the middle of the transition obtained immediately after the plateau has been reached at elevated temperature. It does not take the time-dependent increase in negative rotation into consideration. Urea also tends to increase the negative value of rotation at the plateau region of high temperature. Kauzman and Simpson (1953) obtained a negative rotation of −74° for BSA after it had been allowed to stand for 120 minutes at 60°, and a value of −105° for the same protein in 6.67 M urea after 120 minutes. Foss (1960) observed the same irreversible denaturation in 3 M and 5 M urea for lysozyme.

Lysozyme solution becomes turbid in 1.6 *m* urea. In 3.5 *m* concentration of urea the cooling curve has a lower T_m than the heating curve, but is essentially the same as the reheating curve carried out after 12 hours at 25° (Fig. 5). After the second heating the lysozyme solution becomes turbid on cooling. In 6.5 *m* concentration of urea again the cooling curve has a lower T_m than the heating curve and the shape of the profile is broadened. After 12 hours of waiting at 26° the shape of the reheating curve appears to be sharpened, with the secondary T_m lying between the primary heating and cooling curves (Fig. 6). Therefore, in respect to promotion of rapid renaturation, in no case does urea have a similar effect as purine.

Increase of Solubility of Amino Acids in the Presence of Purine.—Interactions of amino acids with purine were investigated in an attempt to locate the site of interaction between proteins and purine. Solubility of amino acids (glycine, alanine, leucine, histidine, tyrosine, phenylalanine, and tryptophane) in the absence and presence of various concentrations of purine was measured (Table II). The solubilities of glycine and alanine are slightly decreased by 0.53 *m* purine, and the solubilities of leucine and histidine are increased by about 10–20% (Fig. 7). The effect on the solubility of phenylalanine is larger by about 40%. The effects on tryptophane and on tyrosine are much larger, up to 300–400% in 0.53 *m* purine (activity = 0.19). All changes in solubility are linearly related to activity of purine except for tryptophane. Interestingly enough, in 0.53 *m* purine the solubility of tryptophane is lower than in 0.4 *m* purine. In 0.63 *m* purine the solubility is still further decreased (Fig. 7). No conclusive explanation can be given to this observation. It can, however, be suggested that the purine-tryptophane mixture probably begins to form a more insoluble complex at higher purine concentrations.

Difficulties have been encountered in the qualitative measurements of the solubilities of the polypeptides (polyalanine, polyphenylalanine, and polytryptophane) and the amino acid *N*-acetylamide derivatives (*N*-acetyl-L-tryptophane and *N*-acetyl-L-phenylalanine) due to their low solubility in water. However, gravimetric measurements of the insoluble residue left when a known weight of the polymer or amino acid derivative is dissolved in a large volume of solvent consistently showed increased solubility for the aromatic amino acid derivatives and the polypeptides in 0.53 *m* purine solution.

DISCUSSION

There are three salient features of these experiments: (A) The plot of ΔT_m versus purine activity is linear up to 0.25–0.4 activity (1–1.5 *m* concentration). The slope $\Delta(\Delta T_m)/\Delta$ activity can be defined as the unit of effectiveness of purine in lowering the T_m of the proteins. The slopes for both ribonuclease and lysozyme are essentially the same, but slightly higher than that for BSA. Above 1.5 *m* purine the lines are no longer linear (the dotted lines in Fig. 4). The activity in this region was calculated with the molality activity coefficient determined at 1 *m* (Ts'o *et al.*, 1963). If the nonlinear portion of the curve

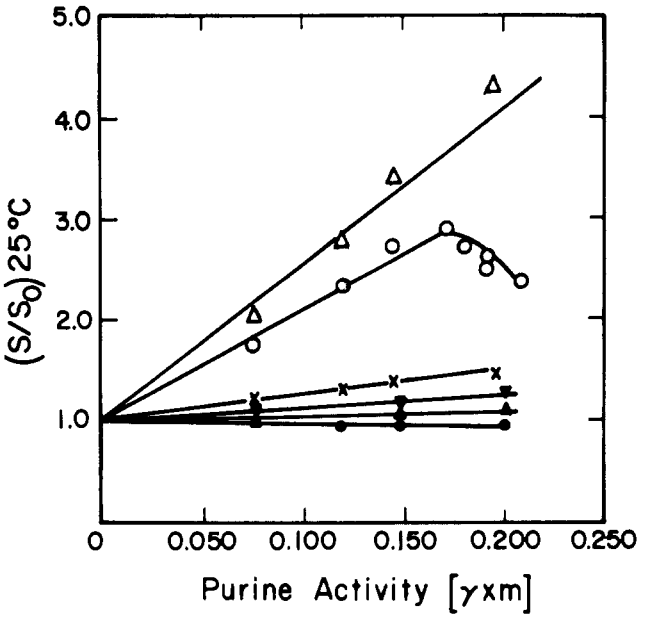

FIG. 7.—Plot of solubility in purine/solubility in buffer (S/S_0) at 25°, 0.1 M K-phosphate, *p*H 6.54, versus the activity of purine ($\gamma \times m$) for tyrosine (Δ), tryptophan (O), phenylalanine (×), glycine and alanine (●), histidine (▼), and leucine (▲).

is entirely due to the use of incorrect activity coefficient, the ratio of $\Delta(\Delta T_m)/\Delta$ activity at the linear portion to $\Delta(\Delta T_m)/\Delta$ activity at the nonlinear portion should be equal for all three curves. Such a calculation gives a value of 1.69 for lysozyme, 1.73 for ribonuclease, and 1.13 for BSA at the same linear and nonlinear concentrations of purine for the three proteins. In other words, in changing from the linear to the nonlinear region the slope has been lowered by about 70% for the lysozyme and ribonuclease, but only 13% for BSA. Thus, the deviation from linearity at higher purine concentrations must be attributed to some other phenomenon beside the use of incorrect activity coefficients. From the shapes of the curves and from solubility data it appears that at least part of the lowering of effectiveness of purine at concentrations higher than 1.5 *m* may be owing to the saturation of sites on the protein molecules.

(B) The solubility data gave information about the groups of the protein side chains which are likely to be the reactive sites with purine. The data showed that aromatic groups such as tyrosine, tryptophan, phenylalanine, and perhaps histidine are much more susceptible to the influence of purine than the side chains of glycine, alanine, and leucine. This finding is consistent with other observations about the properties of purine. The hydrophobic and stacking interaction of purine has been observed from studies of its osmotic and nuclear magnetic resonance properties in water (Ts'o *et al.*, 1963; Chan *et al.*, 1963; Schweizer *et al.*, 1964). A similar observation has been made from studies of its interactions with adenine and thymine (Ts'o *et al.*, 1963), polycyclic aromatic compounds (Booth and Boyland, 1953; Booth *et al.*, 1954), and nucleic acids (Ts'o *et al.*, 1962a). It is interesting to compare the effectiveness of purine to that of urea in enhancing the solubility of several amino acids (Whitney and Tanford, 1962). On per activity basis, purine is over 40-fold more effective than urea in solubilizing tyrosine, twelve times as effective in solubilizing phenylalanine, and over 6-fold more effective in solubilizing leucine.

TABLE II
SOLUBILITY OF AMINO ACIDS IN PURINE

	Solubility (g/100 ml solvent, 25°)	S/S_0 (at 25°)	Purine Activity ($\gamma \times m$)
Tyrosine			
Buffer	0.0445	1.00	
0.1 *m* purine	0.0912	2.05	0.073
0.2 *m* purine	0.121	2.72	0.115
0.3 *m* purine	0.149	3.35	0.144
0.5 *m* purine	0.192	4.31	0.188
Tryptophan			
Buffer	1.201	1.00	
0.1 *m* purine	2.090	1.74	0.073
0.2 *m* purine	2.822	2.35	0.115
0.3 *m* purine	3.311	2.75	0.144
0.4 *m* purine	3.483	2.90	0.167
0.45 *m* purine	3.180	2.65	0.177
0.5 *m* purine	3.003	2.50	0.188
0.63 *m* purine	2.942	2.45	0.198
Phenylalanine			
Buffer	2.895	1.00	
0.1 *m* purine	3.474	1.20	0.073
0.2 *m* purine	3.735	1.29	0.115
0.3 *m* purine	3.995	1.38	0.144
0.5 *m* purine	4.111	1.42	0.188
Glycine			
Buffer	25.1	1.00	
0.2 *m* purine	24.1	0.96	0.115
0.3 *m* purine	23.9	0.95	0.144
0.5 *m* purine	23.9	0.95	0.188
Alanine			
Buffer	16.6	1.00	
0.1 *m* purine	16.3	0.98	0.073
0.2 *m* purine	16.1	0.97	0.115
0.3 *m* purine	15.9	0.96	0.144
0.5 *m* purine	15.8	0.95	0.188
Leucine			
Buffer	2.51	1.00	
0.1 *m* purine	2.61	1.04	0.073
0.3 *m* purine	2.72	1.08	0.144
0.5 *m* purine	2.82	1.12	0.188
Histidine			
Buffer	4.05	1.00	
0.1 *m* purine	4.29	1.06	0.073
0.3 *m* purine	4.74	1.17	0.144
0.5 *m* purine	5.10	1.25	0.188

(C) The thermal denaturation of proteins in purine solution has an interesting property. Besides lowering their T_m, purine prevents the proteins in the unfolded stage at higher temperature from undergoing an irreversible conformational change such as is observed in the absence of purine. For instance, when lysozyme is heated to 80° it is irreversibly denatured in the absence of purine but not in the presence of purine. Thus, on the one hand purine facilitates the unfolding of the protein molecule, as evidenced by the lowering of its T_m; on the other hand, purine keeps the unfolding molecule from undergoing irreversible reactions and therefore keeps it instantly renaturable upon cooling.

What then is a likely mechanism for the interaction of purine with these representative proteins—a mechanism that may serve as a working hypothesis for future experimentation? The lowering of the helix-coil-transition temperature of a polymer in the presence of an interactant can be treated formally as if the interactant prefers to bind with the polymer in the coil, or unfolded state, over the polymer in the helical or folded state. The binding of the interactant thus

leads to a preferential stabilization of the unfolded state and facilitates the unfolding of the polymer. This proposal was first made by Peller (1959) on theoretical grounds. It has been well substantiated by the work in our laboratories on the interaction of purine and nucleosides with nucleic acids (Ts'o *et al.*, 1962a; Ts'o and Lu, 1964). Thus the problem of reduction of T_m can be examined on the basis of interaction through binding. Purine compared to urea, on activity basis, is 6- to 12-fold more effective in lowering the T_m of the protein (Table I). Urea is probably one of the best hydrogen bond-forming agents. It is very unlikely that the superiority of purine over urea in reducing the T_m of the protein is due entirely to the more superior ability of purine to form hydrogen bonding with proteins in aqueous solution. Furthermore, recent investigations indicate that the free energy of hydrogen bonding of urea is very low in aqueous solution (Whitney and Tanford, 1962; Schellman, 1955; Klotz and Frazen, 1960; Levy and Magoulas, 1962). It has been suggested that urea breaks hydrophobic bonds by disrupting the hydration layer around the hydrocarbon side chains of protein molecules (Klotz and Stryker, 1960; Kauzman, 1959).

Therefore hydrogen bonding is not likely to be the predominant force for the binding of purine and proteins.

As mentioned, purine interacts preferably with the aromatic side chains over the short aliphatic side chains. Furthermore, there appears to be a correlation between the superiority of purine over urea in its solubilizing influence on the aromatic amino acids and its reduction of the T_m of these proteins.

These facts strongly indicate that hydrophobic and stacking interaction is the most likely mechanism for the interaction between purine and protein. This idea is certainly supported by what is known about the properties of purine in solution and its interaction with polycyclic compounds, nucleosides, and nucleic acids.

The following picture of the interaction of purine and proteins starts to emerge from the synthesis of all the hypotheses so far discussed. Purine binds to proteins presumably through hydrophobic and stacking interaction, and preferably with the aromatic side chains. This binding weakens the hydrophobic forces which hold the protein in its native conformation. The binding of purine, however, also provides shielding for the reactive groups exposed in the unfolded molecules. For instance, the bound purine may prevent the approach of water molecules to form hydrogen bonds with the exposed sites on the peptide chain. The bound purine may hinder the interaction between the exposed groups of two unfolded polypeptides. Without the protection of the bound purine, the subsequent reaction of the exposed groups of these polypeptide chains can lead to aggregation or other irreversible changes which prevent rapid renaturation.

Proceeding from this deduction, clear distinction appears to emerge between the mechanism for purine denaturation and the mechanism for urea denaturation of proteins. All the evidence aforementioned points to the idea that purine combines *directly* with the side chains through hydrophobic stacking interactions, and thus weakens the hydrophobic forces of the polymers by its immediate action. On the other hand, all the current findings and concepts (see references cited above) on urea denaturation point to the hypothesis that urea interacts with the water layer around the polymer. Our guess is that the interaction with the hydration layer involves hydrogen bonding with the water, resulting, therefore, in the weakening of the hydrophobic forces of the polymer through its *indirect* action. It is therefore not suprising that purine is much more effective than urea in reducing the T_m of both proteins and nucleic acids.

There are two broad implications of the above findings and discussion. First, from both experimental and theoretical grounds it is clear that the secondary structure of nucleic acids is much more sensitive to the influence of purine than of proteins. The advantage of this difference has been magnified by the recent finding (Sander and Ts'o, unpublished data) that nucleic acids in the coil form are much more sensitive to the effect of purine than nucleic acids in helical form. After heating DNA or RNA in the presence of 0.5 M purine to temperatures above its T_m, optical rotation studies indicate that there is no secondary structure left in the nucleic acids upon cooling. In other words, once a nucleic acid has been exposed to high temperature in presence of purine, it will not regain its structure on cooling. The purine is so dilute in this solution (0.5 M) that it has no effect on the protein subsequently dissolved in it. It is possible, therefore, to obtain an aqueous solution of protein and nucleic acid in which the secondary structure of the protein is intact while the secondary structure of the nucleic acid is reduced to a minimum, such as will be obtained for the nucleic acid at elevated temperatures (95–100°). This experimental setup opens many possibilities.

Second, the hydrophobic and stacking interaction of purine with the aromatic side chains of proteins may prove to be of importance in the specific interaction between nucleic acids and proteins. The hydrogen-bonding sites in the peptide backbone are not likely to offer any specificity in this interaction since they represent a common property of proteins, and probably in most cases remain unexposed for such interactions. It is hoped that these experiments will bring more understanding to this area of research.

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Chromatography of Mixed Oligonucleotides on DEAE-Sephadex

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Oligonucleotides as large as the octamer of different purine-pyrimidine ratios were separated according to their chain length by column chromatography on DEAE-Sephadex A-25 in 7 M urea–0.02 M Tris-Cl, *p*H 7.6, and increasing gradients of NaCl. Mixed tetranucleotides and pentanucleotides obtained in this manner were further subfractionated at *p*H 2.7 and found to be eluted according to their net charge. Potential applications of these procedures are discussed.

The fractionation of oligonucleotides from enzymatic digests of ribonucleic acid (RNA) is of importance for the elucidation of nucleic acid sequences. Tomlinson and Tener (1962, 1963) found that 7 M urea at neutral *p*H greatly facilitated DEAE-cellulose chromatography of mixed polynucleotides by resolving them according to their net charge or chain length. Subsequent investigations by Bartos *et al.* (1963) showed that such compounds were eluted in the same chromatographic position not only because of equal chain length but also because of identical purine-pyrimidine ratios. Thus, the trinucleotides ApApUp and GpGpCp (from pancreatic ribonuclease digests of RNA) emerged in one peak, while ApApGp and CpCpGp (from ribonuclease T_1 digest of RNA) did not.

It was also reported that tri- and tetranucleotides from pancreatic ribonuclease digests of RNA could be subfractionated according to their net charge at *p*H 3 by chromatography on DEAE-cellulose in 7 M urea and 0.1 M formic acid (Rushizky and Sober, 1964). However, this subfractionation procedure also failed with oligonucleotides of different purine-pyrimidine ratios.

As described here, the use of DEAE-Sephadex A-25 in 7 M urea at two different *p*H values permits the resolution of mixed oligonucleotides first according to chain length and subsequently by base composition as follows. At *p*H 7, where the dissociation of the amino groups of purines and pyrimidines is completely repressed and each of the four nucleotide residues has the same negative charge due to the phosphate groups, separation occurs as a function of chain length. However, at *p*H 2.7 where there are differences in the degree of dissociation of the amino groups of the nucleotides and the secondary phosphate groups are uncharged, separation of mixed oligonucleotides of equal chain length depends on the variation in net charge brought about by base composition. Applications of these procedures are discussed.

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Experimental Procedure

Reagents and Materials.—All spectrophotometric measurements were made in silica cells with a 1-cm light path in a Beckman DU spectrophotometer and are expressed as absorbancy (A). A Leeds and Northrup meter equipped with microelectrodes was used for *p*H determinations. Reagent-grade urea (Baker) was used without purification since the A_{260} of a 7 M solution was usually less than 0.05. Whatman No. 3MM paper was employed for descending paper chromatography with a solvent containing 40 g of ammonium sulfate per 100 ml of 0.1 M sodium phosphate, *p*H 7.0 (solvent A).

Enzymes.—Ribonuclease T_1, T_2, and *B. subtilis* ribonuclease were prepared as described (Rushizky and Sober, 1962a, 1963; Hartley *et al.*, 1963).

RNA.—High-molecular-weight RNA from yeast was isolated by the method of Crestfield *et al.* (1955). Bacteriophage MS 2 was grown on *E. coli* C-3000 and isolated as described by Strauss and Sinsheimer (1963). The sedimentation coefficient $s_{20,w}$ of a 0.01-ml sample of $A_{260} = 2.0$ was found to be 79.5 in 0.04 M potassium phosphate, *p*H 7, by the method of Vinograd *et al.* (1963). RNA of MS 2 was obtained as described by Strauss and Sinsheimer (1963); the A_{260} for 1 mg/ml at neutral *p*H in water was taken as 25.0.

Enzymatic Digests of RNA.—The preparation of ribonuclease T_1-digests of yeast RNA has been published (Bartos *et al.*, 1963). RNA from MS 2 was similarly digested except that 1 M ammonium carbonate (*p*H 7.6) was employed as the buffer. After hydrolysis (at 37°) the enzyme was removed with phenol and remaining traces of phenol were extracted with ether. The solution was then lyophilized to remove the volatile buffer. The *B. subtilis* ribonuclease digest was obtained by treating yeast RNA in 0.1 M ammonium carbonate, *p*H 8.6, for 6 hours at 37° with 8 units of enzyme per mg of RNA. Under these conditions, the mono- and oligonucleotides in the digest terminate in the 2′,3′-cyclic-terminal-phosphate form (Rushizky *et al.*, 1963). Enzyme and buffer were then removed as described above.