rived from the two carboxyl groups, as expected.[15] It is reasonable to suppose that this decarboxylation occurs in two steps, in each of which a β-keto acid loses CO_2 to form an enol. The product of the first step would then be the enol of hydroxypyruvic acid. The fact that another molecule of CO_2 is lost shows that this enol does not ketonize to the relatively stable hydroxypyruvate but to hydroxymalonic semialdehyde which would be expected to undergo decarboxylation as observed.

Experimental

Material and Methods.—Potassium ferricyanide was recrystallized according to Folin.[16] DPNH was prepared as the salt of tris-(hydroxymethyl)-aminomethane, according to Loewus, *et al.*[17] A sample of the disodium salt of DPNH (Pabst) also was used. Lithium D,L-lactate was prepared by neutralizing boiling D,L-lactic acid (85%) with a saturated solution of lithium hydroxide and crystallizing from water–ethanol. The phenacyl derivative of the D,L-lactic acid was prepared according to Rather and Reid[18] and melted at 97–98°. Li-L-lactate and beef heart lactic dehydrogenase were the same as previously described.[4,19]

Lactic oxidase (cytochrome b_2) was prepared from dried baker's yeast according to the procedure of Boeri, *et al.*,[6] up through the last $(NH_4)_2SO_4$ fractionation. The preparation was dialyzed and lyophilized, with about 15% loss of activity. Protein was determined by the method of Lowry, *et al.*[20] The enzyme activity of the cruder fractions was determined by measuring the rate of methylene blue reduction.[21] The more purified fractions were assayed by measuring the rate of reduction of either ferricyanide (at 420 mμ) or of 2,6-dichlorophenol-indophenol (at 600 mμ). All measurements were made with a Beckman spectrophotometer. When measured in a cell of 1 cm. light path at 420 mμ, a solution containing 1 μmole of ferricyanide per ml. has an optical density (log I_0/I) of 1.03. The ferricyanide assay system was made up to contain 125 μmoles of phosphate buffer of *p*H 7.4, 2 μmoles of Versene (ethylenediaminetetraacetate), 400 μmoles of D,L-lactate, 2.0 μmoles of ferricyanide and water to make a total volume of 3.0 ml. The reaction was started by addition of enzyme. The reduction of ferricyanide was linear with time under these conditions. If 0.1 μmole of indophenol was substituted for the ferricyanide, the reduction was first order with respect to the indophenol. The lactic oxidase preparation employed in the experiments catalyzed the reduction of 1.1 μmoles of ferricyanide per minute per mg. protein at 20°. The half time for the reduction of 2,6-dichlorophenol-indophenol was 10 seconds per mg. protein (the first-order rate constant $K = 4.3$ min.$^{-1}$ mg.$^{-1}$).

Experiment 1.—The reaction mixture contained 120 μmoles of phosphate buffer of *p*H 7.0, 2 μmoles of Versene, 378 μmoles of lithium D,L-lactate, 77.2 μmoles of potassium ferricyanide and 9 mg. of yeast lactic oxidase in a total volume of 2.0 ml. of 99% D_2O. The *p*H was maintained at 7.0 by addition of small amounts of $NaHCO_3$ during the course of the reaction. After 32 minutes at room temperature, the ferricyanide was reduced completely. Then about 80 μmoles of DPNH was added, followed by 0.05 ml. of an $(NH_4)_2SO_4$ suspension of heart lactic dehydrogenase.[19] After 5 min. when the reduction of pyruvate was complete, the reaction mixture was heated for 1.5 minutes at 100° to inactivate the enzymes. The optical density at 340 mμ of suitable aliquots of the reaction mixture was determined before and after the lactic dehydrogenase reaction. From the change, the amount of DPNH oxidized was calculated[4,19] to be 38.7 μmoles. This is a measure of the amount of pyruvate formed in the lactic oxidase reaction, in agreement with the value of 38.6 μmoles calculated from the amount of ferricyanide reduced. It was essential that the ferricyanide be completely reduced in the first reaction, prior to addition of DPNH. If this was the case, the second reaction, *i.e.*, the reduction of pyruvate by DPNH, proceeded smoothly and could be measured accurately, as shown by the good agreement between the ferricyanide reduced initially by lactate, and the DPNH subsequently oxidized by pyruvate. If the ferricyanide was not completely reduced, it oxidized the DPNH directly both by a non-enzymatic reaction and by an enzymatic reaction catalyzed by a very active DPNH diaphorase in the lactic oxidase preparation. (As a further complication, there is a change of optical density at 340 mμ accompanying the reduction of ferricyanide to ferrocyanide.)

After the final heat inactivation of the enzymes, 475 μmoles of unlabelled lithium D,L-lactate was added, to give a total of 475 + 378 = 853 μmoles of lactate, out of which $^1/_{22}$ or 38.7 μmoles had been oxidized to pyruvate and rereduced to lactate. The extraction of the lactate with ether, conversion to the phenacyl derivative and analysis of the phenacyl lactate for D, were then carried out as previously described.[4,19] It is pertinent to note that the enzyme is specific for L-lactate but that D,L-lactate may be used as described in the experiment since the L-lactate oxidized by lactic oxidase is regenerated in the reduction catalyzed by lactic dehydrogenase, which is also specific for L-lactate. The final solution thus contains equal amounts of the D- and L-isomer, regardless of what proportion has been oxidized and rereduced in the enzymatic reactions.

Experiment 2.—The reaction mixture was similar to that of experiment 1 except that 254 μmoles of lithium L-lactate and 89.6 μmoles of ferricyanide were added. The results of the DPNH analyses showed that 45.7 μmoles of pyruvate was reduced in the lactic dehydrogenase reaction, in good agreement with the 89.6/2 = 44.8 μmoles calculated from the amount of ferricyanide reduced. The subsequent procedures were identical with those of experiment 1, except that L-lactate was used as diluent.

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[CONTRIBUTION NO. 2230 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

Interactions of Amino Acids with Deoxyribonucleic Acid (DNA)

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A study of the binding of various amino acids to DNA was conducted using the technique at equilibrium dialysis. Only the basic amino acids and peptides were found to interact with DNA in solutions of low ionic strength. An approximate binding constant was derived from the data after applying a correction for the Donnan effect.

The fact that nucleic acids are thought to be templates for protein synthesis stimulated the investigation of the interactions of (DNA) with various amino acids. These interactions may depend not only on the negative charge of the phos-

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phate groups but also on secondary forces which may render the binding more specific. Such specificity has been postulated by Gamow and Ycas[2] in the case of ribonucleic acid. Finally, work on the binding of various dyes to DNA indicates that secondary forces do play a role in this process.[3-6]

Experimental

The sodium salt of the DNA was prepared from calf thymus glands according to Kay, *et al.*[7] It was air-dried and stored in the solid state at −4°. Solutions of concentration approximately 0.2% by weight were prepared in ice-cold 10^{-2} *M* NaCl 12 to 24 hr. before use. Shaking by hand was sufficient for the preparation of homogeneous DNA solutions. Dilutions (1 to 10 with 10^{-2} *M* NaCl) were made for the determination of the ultraviolet spectrum in the Cary spectrophotometer and for that of phosphorus by the method of Allen.[8] The E_p was found to be 7500 and the ratio of 260/230 was 2.40. The phosphorus content of the solid DNA amounted to 6.6% by weight. The biuret and ninhydrin tests were negative. The molecular weight of this DNA preparation was higher than 1×10^6 as determined by the equilibrium sedimentation method in a density gradient of CsCl.[9]

The amino acids used are listed in Table I.[10] Solutions of arginine and lysine of final concentrations varying from about 1.5×10^{-3} to 7×10^{-4} *M* were prepared in sodium phosphate buffer 1.33×10^{-3} *M* *p*H 7.0 ± 0.1. The interactions with DNA were studied by the method of equilibrium dialysis using final DNA–P concentrations of from 4×10^{-4} to 7×10^{-4} *M*. Into Visking dialyzing bags which had been washed in distilled water 18 ml. of the amino acid and 2 ml. of the DNA solutions were pipetted. The dialyzing bags were then placed in test-tubes containing 20 ml. of amino acid solution. The tubes were covered with parafilm paper and shaken in a rotating basket inside an incubator maintained at 37 ± 1°. Appropriate blanks containing all constituents except the amino acid were included. The amino acid concentration inside and outside the bag was determined by the ninhydrin method of Moore and Stein,[11] after correcting for the slight color of the blank solution. The equilibration was essentially complete after 4 hr. since no difference in the colorimeter readings was seen after 4 or 24 hr. of incubation.

TABLE I

THE BINDING OF AMINO ACIDS TO DNA[a]

Amino acid	Binding[b] (moles/mole DNA–P)
l-Arginine·HCl	0.037 ± 0.002
l-Lysine·HCl	0.039 ± 0.004
l-Threonine	—
dl-Glutamic acid	—
dl-Histidine	—
l-Isoleucine	—
l-Leucine	—
dl-Valine	—
dl-Tyrosine	—
dl-Phenylalanine	—
dl-Tryptophan	—
l-Gly-glycine	—
l-Gly-ly-glycine·HCl	0.030

[a] Amino acid concentration 1.5×10^{-4} *M*, NaDNA–P from about 4 to 7×10^{-4} *M*, phosphate buffer 1.33×10^{-3} *M* *p*H 7.0 ± 0.1 and NaCl 5×10^{-4} *M*. Equilibrated for 4 hr. at 37 ± 1°. ± values are standard errors of the mean obtained from five independent experiments. The dash lines indicate that the difference in the colorimeter reading of the solutions inside and outside the dialysis bag lies within 0.02 absorbancy unit, ±0.01 unit being the range of variation in the determination. [b] Not corrected for the Donnan effect which in this case amounts to 13% of the value given in the table.

Results and Discussion

It is seen from Table I that only the basic amino acids arginine and lysine as well as the tripeptide gly-ly-gly are found to interact with DNA under the conditions specified. The amino acid concentration used in this case was such that the difference in the absorbancies inside and outside the bag was close to the maximum at least for the basic amino acids. It is possible, however, that some of the neutral amino acids may interact with DNA at *p*H's lower than 7 since a decrease in the fraction of ionized carboxyl groups will minimize the electrostatic repulsion between the negatively charged DNA and amino acid.

The results on the binding of arginine and lysine at four different concentrations are shown on Fig. 1 and 2. On the basis of the assumption that the interaction is governed by a single binding constant the following equation can be used[12]

$$r(1 + k[A]) = nk[A]$$

where r = moles/liter of bound amino acid/moles/liter of DNA–P, [A] = moles/liter of amino acid at equilibrium, n = the fraction of the DNA phosphate groups serving as binding sites and k the binding constant. As pointed out by Schellman, *et al.*,[13] the form of this equation applies also in the case of heterogeneous binding sites at the very low substrate concentrations frequently used in studies with proteins. It is noted that the points for both arginine and lysine fall on a fairly good straight line when either $r/[A]$ *vs.* r (Fig. 1) or $1/r$ *vs.* $1/[A]$ (Fig. 2) are plotted.

At the ionic strength and DNA concentration employed in these experiments the difference in the amino acid concentration inside and outside the dialysis bag is greatly affected by the Donnan equilibrium as seen from Fig. 1 and 2. A maximum correction to the data was applied which was based on the assumption that all of the DNA phosphate groups carry a single negative charge at *p*H 7. The parameter λ which is the ratio of the concentration inside and outside the bag for any cation in an ideal system was derived as shown previously.[14] The equation shown below is however an approximation of that which was derived rigorously. This was accomplished by considering the value of the concentration of the material transferred at constant volume due to the Donnan effect to be negligible compared to that of the DNA–P and the

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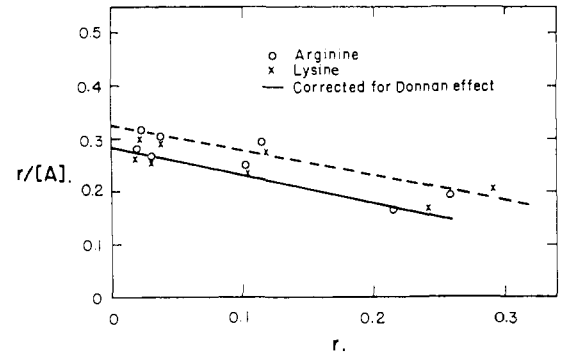

Fig. 1.—Each point represents the mean of five independent experiments. The letters r and [A] are defined in the text.

singly and doubly charged groups of the phosphate buffer

$$\lambda = 1 + \frac{[\text{DNA-P}]}{\sqrt{[PO_4^{=}] + 2[A] + 2[NaCl] + 2[PO_4^{-}] + 2[PO_4^{=}]}}$$

where the brackets denote the initial concentrations of the various constituents.

The concentration of the bound amino acid corrected for the Donnan effect was obtained by subtracting from the measured concentration inside the bag the quantity $\lambda[A]_0$ where the subscript denotes the concentration outside. Percentagewise this effect is most pronounced at the higher amino acid concentrations and of the order of 20% of the measured difference in the concentration inside and outside the bag.

From the slope and intercept of the line going through the points which have been corrected for the Donnan effect (Fig. 2), one can obtain an estimate of an apparent binding constant and of the fraction of binding sites, k and n: these were found to be 400 and 0.66, respectively. Both values for k and n may be considered to be at best orders of magnitude, however, since a maximum but approximate correction for the Donnan effect in addition to the relatively large error in the determination of the difference in amino acid concentration inside and outside the dialysis bag were included in the calculation. The fact that there is no difference in the binding of arginine and lysine provides evidence that secondary forces, if any, must be operating almost identically for both amino acids. It is noted that the value for the binding constant is lower than those reported for DNA interactions with various dyes by at least 2 to 3 orders of magnitude.

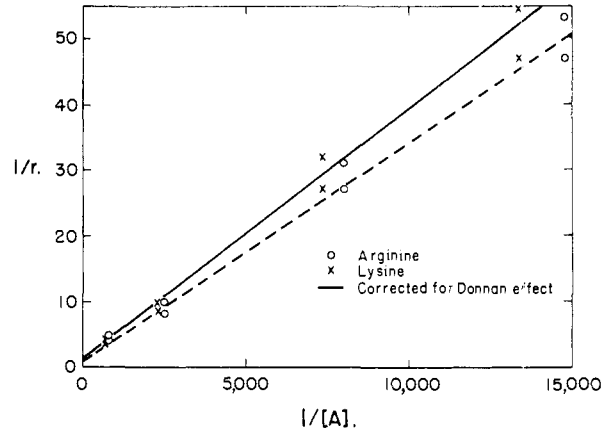

Fig. 2.—A different plot of the data shown in Fig. 1.

It may at first sight be surprising that histidine which is also a basic amino acid does not bind to the DNA. However, one ought to recall that the isoelectric point of this amino acid is 7.6 and therefore at pH 7 only a small fraction of the molecules will possess a net positive charge. It is to be expected that at higher histidine concentrations binding might be detected after correcting for the Donnan effect. The latter was shown to be quite pronounced at the low ionic strength of the solutions used. Increasing the ionic strength by the addition of sodium chloride on the other hand completely obliterates the binding of both arginine and lysine to DNA.

It would be interesting to investigate the interaction of DNA with various small peptides of known sequence for possible specificity in the binding. The results in one experiment with gly-ly-gly (Table I) look encouraging. This study unfortunately was not pursued any further because of the unavailability of this and other peptides containing lysine and/or arginine.

It may be concluded from this study that it is primarily the electrostatic interaction associated with the extra basic group in the case of arginine, lysine and gly-ly-gly which accounts for the binding to DNA.

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