rivative (0.025 mole) was placed in water, 0.025 mole of hydrochloric acid added, and made up to 50 ml. volume. Titrations were made with one normal sodium hydroxide so that 25 ml. of the base was equal to one equivalent of the group being titrated. This procedure gave curves for amino acids of approximately the correct *pK* values in the acid range but slightly low in the basic range because of the dilution factor.

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The Papain-catalyzed Synthesis of Acyl-D- and L-Phenylalanylphenylhydrazides from a Series of Enantiomorphic Pairs of Acylated Phenylalanines

BY WALTER HARRY SCHULLER AND CARL NIEMANN[1]

The first intimation that the papain-catalyzed synthesis of acylated α-amino acid phenylhydrazides[2] from the corresponding acylated α-amino acids and phenylhydrazine is not necessarily restricted to L-isomers was obtained by Bennett and Niemann[3] who showed that the reaction of carbobenzoxy-*o*-fluoro-DL-phenylalanine with phenylhydrazine in the presence of cysteine activated papain gave in addition to the expected carbobenzoxy-*o*-fluoro-L-phenylalanylphenylhydrazide, considerable amounts of the D-isomer. Further work by these investigators,[4] principally with acylated DL-phenylalanines, indicated that the nature of the acyl group is an important factor in determining the stereochemical specificity of the reaction. *E.g.*, with a series of five acylated DL-phenylalanines two, *i.e.*, acetyl- and benzoyl-, appeared to give only the L-phenylhydrazides, whereas the other three, *i.e.*, carbomethoxy-, carboethoxy- and carbobenzoxy-, gave both D- and L-phenylhydrazides. It should be noted that Milne and Stevens[5] have shown that carboallyloxy-DL-leucine reacts with phenylhydrazine in the presence of cysteine activated papain to give both D- and L-phenylhydrazides and that the D-phenylhydrazide is similarly formed from carboallyloxy-D-leucine and phenylhydrazine.

In this investigation a series of six enantiomorphic pairs of acylated phenylalanines were allowed to react individually at 40° and *p*H 4.6, with phenylhydrazine in the presence of cysteine activated papain and the extent of hydrazide formation determined by isolation of the corresponding phenylhydrazides. It will be seen from the data obtained in these experiments and summarized in Tables I and II that with the individual enantiomorphs the same general behavior is observable as with the corresponding DL-mixtures.[4] On the basis of information now at hand it appears reasonably certain that under the conditions specified cysteine activated papain is incapable of catalyzing the reaction between acetyl-D-phenylalanine and phenylhydrazine and that the reaction is restricted

TABLE I

PAPAIN-CATALYZED SYNTHESIS OF PHENYLHYDRAZIDES OF ACYLATED D- AND L-PHENYLALANINES[a]

Acyl group	Configuration	*M* Substrate, concn. Acid	Base	Yield of phenylhydrazide, % First fracn.	Second fracn.	Third fracn.	Total[j]
CH_3CO-	L	0.25	0.50	34[b]	21[f]	28[i]	83
CH_3CO-	D	.25	.50	...	...	...	0
C_6H_5CO-	L	.008	.016	82[b]	21[f]	...	103
C_6H_5CO-	D	.008	.016	4.5[c]	4.5[g]	...	9
C_2H_5OCO-	L	.025	.050	81[b]	12[f]	...	93
C_2H_5OCO-	D	.025	.050	61[d]	15[h]	...	76
$C_6H_5CH_2OCO-$	L	.002	.004	32.5[b]	24.5[f]	12[i]	70
$C_6H_5CH_2OCO-$	D	.002	.004	30[b]	2[f]	...	32
$C_6H_5SO_2-$	L	.004	.008	14[e]	...	...	14
$C_6H_5SO_2-$	D	.004	.008	...	...	...	0
C_6H_5NHCO-	L	.0025	.005	...	...	...	0
C_6H_5NHCO-	D	.0025	.005	...	...	...	0

[a] Enzyme concentration, 8 g. per liter; L-cysteine concentration 0.067 *M*; buffer concentration, 0.5 *M* acetic acid–0.5 *M* sodium acetate except for experiments with acetyl derivatives where concentration of the buffer was doubled; all reactions at 40° and *p*H 4.6 with 0.001 mole of acid except for the benzenesulfonyl- and N-phenylcarbamyl-compounds where the acid was 0.002 *M*. [b] After 10.5 hours. [c] After 221 hours. [d] After 117 hours. [e] After 20 days. [f] After additional 13 hours. [g] After additional 16 days. [h] After additional 19 days. [i] After additional 93 hours. [j] After the collection of the final fractions the *p*H of the filtrate was again determined; in no case was a substantial change from the initial *p*H of 4.6 observed.

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TABLE II

PROPERTIES OF ACYL-D- AND L-PHENYLALANYLPHENYLHYDRAZIDES

Phenylalanylphenylhydrazide	M. p., °C. (cor.) Found	M. p., °C. (cor.) Lit.[4]	$[\alpha]^{25}D$ (in pyridine) Found	$[\alpha]^{25}D$ (in pyridine) Lit.[4]
CH_3CO-L-[a]	205–206	207–208	−35.8[d]	−34.6[j]
C_6H_5CO-L-[a]	215–216	215–217	−65.8[d]	−61.9[j]
C_6H_5CO-D-[b]	210–211	.....	+61.4[e]	.....
C_2H_5OCO-L-[a]	159.5–160	156.5–159.5	−25.0[f]	−22.2[j]
C_2H_5OCO-D-[a]	159.5–160	156–160.5	+24.4[g]	+23.4[j]
$C_6H_5CH_2OCO$-L-[a]	175–176	177–179	−24.2[h]	−24.6[j]
$C_6H_5CH_2OCO$-D-[a]	175–176	178–179	+24.2[h]	+24.4[k]
$C_6H_5SO_2$-L-[a,c]	163–164	.....	−27.4[i]	.....

[a] Recrystallized from aqueous ethanol. [b] Twice recrystallized from aqueous ethanol; *anal.* Calcd. for $C_{22}H_{21}O_2N_3$: C, 73.5; H, 5.9; N, 11.7. Found: C, 73.5; H, 5.8; N, 11.6. [c] *Anal.* Calcd. for $C_{21}H_{21}O_3N_3S$: C, 63.9; H, 5.3; N, 10.6; S, 8.1. Found: C, 63.9; H, 5.4; N, 10.7; S, 8.3. [d] *c*, 6.7. [e] *c*, 0.66; $[\alpha]^{25}D$ −64.3° (*c*, 0.66 in pyridine) for enantiomorph. [f] *c*, 7.6. [g] *c*, 7.4. [h] *c*, 6.8. [i] *c*, 1.4. [j] *c*, 8. [k] *c*, 7.

to the L-enantiomorph.[6] In contrast the cysteine activated papain-catalyzed formation of phenylhydrazides of carboethoxy- and carbobenzoxy-D- and L-phenylalanine is now well established.

In an earlier study[4] it was not clear as to whether cysteine-activated papain could or could not catalyze the formation of benzoyl-D-phenylalanylphenylhydrazide. It is now possible to state that the above reaction is feasible but that the rate is very much less than that observed for the L-isomer. Although it is difficult to make quantitative comparisons because of the fact that in the systems under consideration there is apparently a continuing inactivation of the enzyme both by heat[7] and by phenylhydrazine[8,9] it can be said that the difference in the rate of reaction for the D- and L-enantiomorphs is far greater for benzoylphenylalanine than it is for carboethoxy- or carbobenzoxyphenylalanine.

It has been reported previously[10] that cysteine-activated papain will catalyze the formation of benzenesulfonyl- and N-phenylcarbamylglycinanilide and benzenesulfonylglycylphenylhydrazide. It is not obvious as to why no phenylhydrazide formation was observed in the case of N-phenylcarbamyl-L-phenylalanine and in view of the slow rate of reaction observed with benzenesulfonyl-L-phenylalanine it should not be concluded that under no circumstance will the phenylhydrazide of benzenesulfonyl-D-phenylalanine be formed. It appears that in this as in other instances[11] a systematic study of the effect of *p*H, and other

TABLE III

PREPARATION OF ACYLATED PHENYLALANINES

Acyl group	Configuration	Method of prepn.	Recrystd. from	Yield, %
CH_3CO-	L	$(CH_3CO)_2O$; NaOH	H_2O	96
CH_3CO-	D	Directly from resolution	CH_3OH-H_2O	..
C_6H_5CO-	L	C_6H_5COCl; NaOH	H_2O	94
C_6H_5CO-	D	C_6H_5COCl; NaOH	H_2O	88
C_2H_5OCO-	L	C_2H_5OCOCl; NaOH	H_2O	95
C_2H_5OCO-	D	C_2H_5OCOCl; NaOH	H_2O	66
$C_6H_5CH_2OCO$-	L	$C_6H_5CH_2OCOCl$; NaOH	$C_2H_5OH-H_2O$	61
$C_6H_5CH_2OCO$-	D	$C_6H_5CH_2OCOCl$; NaOH	$C_2H_5OH-H_2O$	85
$C_6H_5SO_2$-	L	$C_6H_5SO_2Cl$; NaOH	H_2O	44
$C_6H_5SO_2$-	D	$C_6H_5SO_2Cl$; NaOH	H_2O	80
C_6H_5NHCO-	L	C_6H_5NCO; NaOH	$C_2H_5OH-H_2O$	98
C_6H_5NHCO-	D	C_6H_5NCO; NaOH	$C_2H_5OH-H_2O$	96

TABLE IV

PROPERTIES OF ACYLATED PHENYLALANINES

Compound	M. p., °C.	$[\alpha]D$	Analyses, % Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found	Nitrogen Calcd.	Nitrogen Found
CH_3CO-L-[a]	170–171	+48.2[h]	..	..	...	...	...	...
CH_3CO-D-[b]	170–171	−49.3[i]	..	..	...	...	...	...
C_6H_5CO-L-[c]	142–143	+19.8[j]	71.4	71.6	5.6	5.5	5.2	5.3
C_6H_5CO-D-[d]	142–143	−19.8[k]	71.4	71.3	5.6	5.8	5.2	5.2
C_2H_5OCO-L-	85	+48.7[l]	60.7	60.7	6.4	6.4	5.9	5.9
C_2H_5OCO-D-	85	−47.7[m]	60.7	60.8	6.4	6.3	5.9	6.0
$C_6H_5CH_2OCO$-L-[e,f]	125–126	+32.9[n]	66.2	66.4	5.9	5.9	4.5	4.5
$C_6H_5CH_2OCO$-D-[e]	124–125	−33.4[o]	66.2	66.5	5.9	5.9	4.5	4.5
$C_6H_5SO_2$-L-[g]	133	− 7.0[p]	59.0	59.1	5,0	5.0	4.6	4.5
$C_6H_5SO_2$-D-[g]	133	+ 6.7[q]	59.0	59.2	5.0	5.0	4.6	4.5
C_6H_5NHCO-L-[r]	171	+51.5[s]	67.6	67.8	5.7	5.6	9.9	9.7
C_6H_5NHCO-D-	171	−52.2[t]	67.6	67.6	5.7	5.7	9.9	9.8

[a] Lit.,[12] m.p. 170–171°, $[\alpha]^{25}D$ +47.5° (in ethanol). [b] Lit.,[13] m.p. 172°, $[\alpha]^{26}D$ −51° (in ethanol). [c] Hemihydrate obtained from water, m.p. 141–142°, *anal.* Calcd. for $C_{16}H_{15}O_3N\cdot{}^1/_2H_2O$: C, 69.1; H, 5.8; N, 5.0. Found: C, 69.1; H, 5.8; N, 5.2; loss on drying to constant weight at 100°: calcd. 3.2; found: 3.4; properties in table are for anhydrous compound. [d] As for L-isomer properties in table are for anhydrous compound: lit.,[3] m.p. 139.5–140.5°, $[\alpha]^{25}D$ −18° (*c*, 8 in 0.4 *N* NaOH). [e] Hemihydrate; *anal.* calcd. for $C_{17}H_{17}O_4N\cdot{}^1/_2H_2O$. [f] Lit.,[14] m.p. 126–128°, $[\alpha]^{21}D$ +4.9° (in glacial acetic acid). [g] *Anal.* Calcd. for $C_{15}H_{15}NO_4S$; S, 10.5. Found: S, 10.4. [h] *t*° 25.5°; *c*, 1.68% in ethanol. [i] *t*° 24.5°; *c*, 1.66 in ethanol. [j] *t*° 24°; *c*, 8.9 in 0.4 *N* NaOH. [k] *t*° 24.5°; *c*, 8.8 in 0.4 *N* NaOH. [l] *t*° 23.2°; *c*, 1.94 in chloroform. [m] *t*° 23.7°; *c*, 2.02 in chloroform. [n] *t*° 24.5°; *c*, 2.95 in chloroform. [o] *t*° 25.5°; *c*, 2.8 in chloroform. [p] *t*° 25.5°; *c*, 5.3 in chloroform. [q] *t*° 20°; *c*, 5.5 in chloroform. [r] Lit.,[15,16] m.p. 180–181°; $[\alpha]^{20}D$ −61.3° (in alkali). [s] *t*° 25°; *c*, 5.19 in *N* aqueous sodium hydroxide. [t] *t*° 25.5°; *c*, 5.11 in *N* aqueous sodium hydroxide; $[\alpha]^{23.5}D$ −50.7 (*c*, 8.08 in *N* aqueous potassium hydroxide; $[\alpha]^{25.5}D$ −51.0° (*c*, 9.21 in *N* aqueous sodium hydroxide).

(6) It should be noted that when the enzyme concentration was raised to 25 g. per liter the same results were obtained.

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variables, will be required before a generally consistent account can be given.

Experimental[17,18]

Substrates.—D-Phenylalanine was obtained in a 60% yield and L-phenylalanine in a 73% yield from DL-phenylalanine (Dow) by conversion of acetyl-DL-phenylalanine into acetyl-L-phenylalanine-*p*-toluidide with the aid of cysteine-activated papain and subsequent hydrolysis of the above toluidide and the residual acetyl-D-phenylalanine to the corresponding amino acids.[19] The acylated D- and L-phenylalanines used in this study were prepared as described in Table III and their properties are given in Table IV. The phenylhydrazine hydrochloride was a recrystallized product.

Enzyme Experiments.—The experiments summarized in Table I were conducted in the following manner. The various acylated phenylalanines were weighed into stoppered flasks, the indicated amounts of phenylhydrazine hydrochloride and L-cysteine hydrochloride added, sufficient 0.5 *M* acetic acid–0.5 *M* sodium acetate buffer added at 40° to effect complete solution of the solids in each flask, the *p*H of each solution adjusted to 4.60 by the addition of 4.5 *N* aqueous sodium hydroxide, the required amount of enzyme solution[20] added, the solution adjusted to volume with the buffer, and immediately incubated at 40°. All experiments reported in Table I were started simultaneously. At the stated time intervals (*cf.* Table I) the precipitates in each flask were collected, on sintered glass filters, washed with water, dried and weighed. The precipitates were then extracted with boiling ethanol until no significant amount of ethanol-soluble material remained on the filters and the loss in weight upon ethanol extraction taken as the yield of phenylhydrazide. The ethanol extracts were concentrated and the phenylhydrazides recovered, the properties of which are given in Table II. From the precipitates collected after the fourth day of incubation an ethanol insoluble substance was recovered which was identified as L-cystine, both by decomposition point and specific rotation. After 24 days incubation at 40° and after all insoluble fractions had been collected the solutions were acidified with aqueous hydrochloric acid and the residual acylated phenylalanines recovered *via* a continuous liquid-liquid ether extraction. The results of these operations are summarized in Table V.

(17) The authors wish to express their indebtedness to Dr. A. Elek for all microanalyses reported in this communication.

(18) All melting points are corrected.

(19) The details of this procedure will be described shortly in another communication from these laboratories.

(20) The enzyme solution was prepared by dissolving sufficient freshly purified papain[4] in 0.5 *M* acetic acid–0.5 *M* sodium acetate buffer to give an enzyme concentration of 0.1 g. per ml.

TABLE V

ISOLATION OF UNREACTED SUBSTRATES

Phenylalanine	Quantity (g.) Present	Quantity (g.) Isolated	Recovery, %	M. p., °C. Crude	M. p., °C. Recryst.[a]	M. p., °C. Orig.[b]
CH_3CO–D–[c]	0.35	0.28	80	163–166	167–168	170–171
C_6H_5CO–D–	.27	.23	91[d]	134–136	142	142–143
$C_6H_5SO_2$–D–	.71	.66	93	123–126	133	133
C_6H_5NHCO–L–	.57	.45	79	159–163	166–167	171
C_6H_5NHCO–D–	.57	.48	84	159–161	166–167	171

[a] One recrystallization from solvents indicated in Table III. [b] *Cf.* Table IV. [c] From experiment with an enzyme concentration of 25 g. per liter. [d] Corrected for the 9% yield of phenylhydrazide isolated.

Summary

The papain-catalyzed synthesis of phenylhydrazides of carboethoxy- and carbobenzoxy-D-phenylalanine has been confirmed and it has been established that under the same conditions the above reaction does not proceed with acetyl-D-phenylalanine. Benzoyl-D-phenylalanine was shown to react to a limited extent.

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[CONTRIBUTION NO. 1237 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

A Reinvestigation of the Kinetics of the Urease–Catalyzed Hydrolysis of Urea. I. The Activity of Urease in the Presence of Sodium and Potassium Phosphate

BY GERALD D. FASMAN AND CARL NIEMANN[1]

In an investigation of the factors operative in the urease-catalyzed hydrolysis of urea in aqueous solutions buffered at *p*H 7.0 with sodium or potassium phosphate it has been found that both of the buffer components participate in the hydrolytic reaction and that the buffer anion apparently functions as an activator and the buffer cation as an inhibitor.

In an earlier communication from these laboratories[2] it was concluded from a study of the urease-catalyzed hydrolysis of urea in the presence of potassium phosphate buffers of *p*H 7.0 that the hydrolytic reaction was competitively inhibited by phosphate ion. It is now known that this conclusion is incorrect and that an error was made in assuming that potassium ion was incapable of interaction either with the enzyme or the enzyme-substrate complex. It is the purpose of this communication to show that in the urease-catalyzed hydrolysis of urea at 25° and *p*H 7.0 in the presence of a sodium or potassium phosphate buffer, in addition to their action as buffers, phosphate ion apparently functions as an activator and sodium or potassium ion as an inhibitor.

From the data given in Figs. 1 and 2 it will be seen that at equivalent buffer concentrations and with all other factors held constant the activity of urease is greater in a potassium phosphate buffer than in a sodium phosphate buffer. Thus it appears that sodium ion is a more effective inhibitor of the phosphate–urease–urea system than is potassium ion. Furthermore if sodium chloride is added to a system containing urease, urea and sodium phosphate, or potassium chloride to a system containing urease, urea and potassium phosphate a significant diminution in the activity of the urease is observed (*cf.* Fig. 3). It should not be assumed that chloride ion is without effect upon the above system for it will be shown in a subsequent communication that chloride ion, in common with a number of other anions, can function as an activator of urease. However, at equiv-

(1) To whom inquiries regarding this article should be sent.

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