D-Glucose obtained from the polysaccharide in each case still had much of the asymmetric labeling of the corresponding substrate. The smaller degree of isotope redistribution in the second experiment is presumably due to the shorter incubation time. The labeling pattern in the L-fucose closely parallels that of the D-glucose. With glucose-1-^{14}C as the sole carbon source the fucose is primarily labeled in C-1, whereas C-6 is predominantly labeled when glucose-6-^{14}C is the carbon source. In the second experiment, however, there was a somewhat greater redistribution of isotope in fucose than was observed in glucose.

The latter observation suggests the presence of a *minor* pathway for fucose biosynthesis from small fragments which can give rise to both the top and bottom halves of the molecule. However, the asymmetric labeling observed in L-fucose indicates that in the conversion of D-glucose to the deoxypentose either non-interconvertible small molecules derived from glucose act as intermediates or utilization of the intact hexose carbon-skeleton is involved.

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The inertness of the ammonium salt of N-acetyl-L-cysteic acid carboxamide in systems containing alpha-chymotrypsin

According to SANGER AND THOMPSON[1] α-chymotrypsin, or possibly another closely associated enzyme which is also inhibited by di*iso*propylfluorophosphate, causes the hydrolysis of three of the twenty peptide bonds present in the oxidized A-chain of bovine insulin, *i.e.*, Gly-Ileu-Val-Glu-Glu(NH_2)-$CySO_3H$-$CySO_3H$-Ala-Ser-Val-$CySO_3H$—Ser-Leu-Tyr—Glu(NH_2)-Leu-Glu-Asp(NH_2)-Tyr—$CySO_3H$-Asp(NH_2), when an aqueous solution of this substance and crystalline α-chymotrypsin, adjusted to pH 7.5 with ammonium hydroxide, is allowed to stand at 37° for 24 h. While a precedent for the hydrolytic cleavage of the two peptide bonds involving the carboxyl groups of the two tyrosine residues can be found among the many synthetic specific substrates of α-chymotrypsin which are simple peptides or amides derived from a variety of α-N-acylated L-tyrosines[2,3], no similar derivatives involving the carboxyl group of an N-acylated-L-cysteic acid have been examined for specific substrate activity. Therefore, it appeared desirable to prepare such a derivative and to examine its behavior with α-chymotrypsin.

The ammonium salt of N-acetyl-L-cysteic acid carboxamide was prepared. When aqueous solutions of this compound and crystalline α-chymotrypsin, adjusted to pH 6.2, 6.8, 7.3, 7.9 or 8.3 with aqueous NaOH, were allowed to stand at 25° for as long as 27.5 h, the extent of apparent hydrolysis was no more than would have been observed had the intended specific substrate been absent (Table I).

The absence of any significant hydrolysis raised the question whether the lack of reactivity of N-acetyl-L-cysteate carboxamide was due to an inability of this anion to combine with the catalytically active site of the enzyme or alternatively was due to combination with the active site in a mode or modes which did not lead to the subsequent formation of reaction products. To answer this question the ammonium salt of N-acetyl-L-cysteic acid carboxamide was examined with respect to its ability to function as a competitive inhibitor in the α-chymotrypsin-catalyzed hydrolysis of α-N-acetyl-L-tyrosinehydrazide[5] in aqueous solutions at 25° and pH 7.9 and 0.5 M in the THAM (tris(hydroxymethyl)aminomethane) component of a THAM–HCl buffer. It will be seen from the data given in Table II that no evidence was obtained for competitive inhibition by the N-acetyl-L-cysteate carboxamide anion and it may be concluded that, if this compound can so function, the value of K_I (the enzyme-inhibitor dissociation constant), is substantially greater than 0.1 M.

The inertness of the ammonium salt of N-acetyl-L-cysteic acid carboxamide in the above

TABLE I

ACTION OF α-CHYMOTRYPSIN UPON THE AMMONIUM SALT OF N-ACETYL-L-CYSTEIC ACID CARBOXAMIDE*

pH	% of expected titer**			
	2.5 h	8.5 h	22 h	27.5 h
6.2	0.05	0.55	0.55	0.55
6.8	0.8	1.1	1.4	1.1
7.3	0.5	0.5	1.5	1.5
7.95	0.8	1.3	2.5	3.0
8.3	1.0	1.7	3.0	3.3

* In aqueous solutions at 25° with 0.1444 mg chymotrypsin N/ml 0.2 *M* substrate.

** Based upon the formal titration described by HUANG AND NIEMANN[4] and corrected for a blank titer for $t = 0$.

TABLE II

INFLUENCE OF THE AMMONIUM SALT OF N-ACETYL-L-CYSTEIC ACID CARBOXAMIDE (I) UPON THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF α-N-ACETYL-L-TYROSINEHYDRAZIDE* (S)

$[S]_0$ $\times 10^3 M$	$[I]$ $\times 10^3 M$	v_0** $\times 10^5 M$ min	v_{0I}/v_{0S}
1.27	0.0	0.53	1.06
1.27	10.26	0.56	
1.69	0.0	0.69	0.88
1.69	10.26	0.61	
2.53	0.0	1.05	1.10
2.53	10.26	1.16	
4.23	0.0	1.79	0.98
4.23	10.26	1.76	
6.67	0.0	2.41	1.09
6.67	10.26	2.63	

* In aqueous solutions at 25° and pH 7.9 and 0.5 *M* in the THAM component of a THAM–HCl buffer with 0.1444 mg chymotrypsin N/ml.

** Initial velocity calculated from a least squares fit of 9 points observed from $t = 2$ to $t = 18$ min under conditions where $\ln [S]_t$ *vs* t, appeared to be linear, *i.e.*, for a total extent of reaction of from 6 to 9%. While these values are adequate for the comparison made in this study, their use for the evaluation of K_S and k_3 is questionable because of the low initial substrate concentration.

systems and the reported cleavage of a peptide bond involving the carboxyl group of a cysteic acid residue present in the oxidized A-chain of bovine insulin[1] may be explained in several ways.

The fact that only one of the four peptide bonds involving the carboxyl groups of the four cysteic acid residues present in the polypeptide was reported to be hydrolyzed by crystalline α-chymotrypsin[1] and that this particular peptide bond also involved the α-amino group of an adjacent seryl residue[1] suggests the possibility that the reported cleavage actually involved an ester type of bond which could have arisen, at least in part, by a N- to O-acyl migration[6] during the preparation of the peptide. Since the observations of SANGER AND THOMPSON[1] were qualitative in nature and since it is known[2] that acylated α-amino acid esters are much more susceptible to an α-chymotrypsin-catalyzed hydrolysis than are the corresponding amides, it is possible that the anomaly noted above is more apparent than real. Alternatively, it is possible that there are, in α-chymotrypsin, accessory combining sites at loci removed from the catalytically active site of the enzyme. Thus, concepts of specificity, which are based upon the interaction or lack of interaction of relatively small specific substrate molecules with the catalytically active site of the enzyme, but which can not subtend such accessory combining sites, can not be extended to the larger specific substrate molecules which in addition can interact with one or more of the postulated accessory combining sites.

Experimental. L-Cystine dimethyl ester dihydrochloride was prepared in 89% yield from L-cystine. The diester dihydrochloride, 0.1 mole, was suspended in 2 l dry tetrahydrofuran, the suspension cooled to −5°, and 1.0 mole dry redistilled triethylamine added to the suspension

followed by the slow addition of 0.22 mole freshly distilled acetyl chloride. After 4 h, the precipitated triethylammonium chloride was removed by filtration and the filtrate evaporated to give 71 % of crude N,N'-diacetyl-L-cystine dimethyl ester. 0.05 mole of this product was dissolved in a mixture of 250 ml distilled water and 60 ml conc. HCl and to this solution Br_2 was added, slowly and with stirring, until a slight excess was present. Approximately 0.25 mole was required. The resulting solution was concentrated *in vacuo* to give 75 % of crude N-acetyl-L-cysteic acid carboxymethyl ester, m.p. 186° with decomp. A solution of 0.04 mole of the crude ester in 1 l dry methanol was saturated with anhydrous ammonia and the reaction mixture maintained at 25° in a sealed vessel for 5 days. The reaction mixture was then evaporated to dryness and the residue recrystallized from anhydrous ethanol to give the ammonium salt of N-acetyl-L-cysteic acid carboxamide, m.p. 201–202°, $[\alpha]_D^{25} = -9.3°$ (in water). Yield from L-cystine, 18 %.

Anal. Calcd. for $C_5H_{13}O_5N_3S$ (227): C, 26.4; H, 5.7; N, 18.5; S, 14.1. Found: C, 26.4; H, 5.8; N, 18.5; S, 14.0.

The conditions employed in examining the action of α-chymotrypsin on the ammonium salt of N-acetyl-L-cysteic acid carboxamide are summarized in Table I. The α-chymotrypsin was an Armour preparation, lot No. 00592.

The procedure employed for observing the effect of added ammonium N-acetyl-L-cysteate carboxamide upon the α-chymotrypsin-catalyzed hydrolysis of α-N-acetyl-L-tyrosinehydrazide (Table II) was identical to that described previously[4] and again the enzyme preparation was crystalline α-chymotrypsin, Armour lot No. 00592.

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3-Methoxy-4-hydroxy-D-mandelic acid, a urinary metabolite of norepinephrine*

One of the phenolic acids in human urine, compound 10[1], appears to be of endogenous origin, since its excretion is not affected by dietary changes. Many of the qualitative reactions of this substance were observed to be similar to those of some compounds containing the 3-methoxy-4-hydroxyphenyl group. This fact, along with the recent observation that homoprotocatechuic acid undergoes biological methylation to homovanillic acid[2], and the solubility characteristics of the unknown substance as indicated by its chromatographic behavior were suggestive that it might be 3-methoxy-4-hydroxymandelic acid (I). (I) might be expected to be formed by the action of amine oxidase upon norepinephrine or epinephrine[3], followed by methylation of the resulting 3,4-dihydroxymandelic acid. The following results indicate that (I) is, indeed, an important urinary metabolite of norepinephrine: (1) The parenteral administration of norepinephrine leads to an increased amount of (I) in the urine**; (2) orally ingested 3,4-dihydroxy-DL-mandelic acid gives rise to an increased excretion of (I); and (3) three patients with pheochromocytomas excreted greatly increased amounts of (I) preoperatively and normal amounts postoperatively***.

Authentic DL-(I) was prepared by the method of GARDNER AND HIBBERT[4]; m.p. 129–130° dec.§. L- and D-(I) were prepared by fractional crystallization of the cinchonine salt of DL-(I). The

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§ All melting points were made in open capillary tubes and are uncorrected.