Reactions of *S*-Acylisothioureas. I. *S*- to *N*-Acyl Migrations in *S*-Benzoylisothiobiotin and Analogs*

R. F. Pratt† and Thomas C. Bruice‡

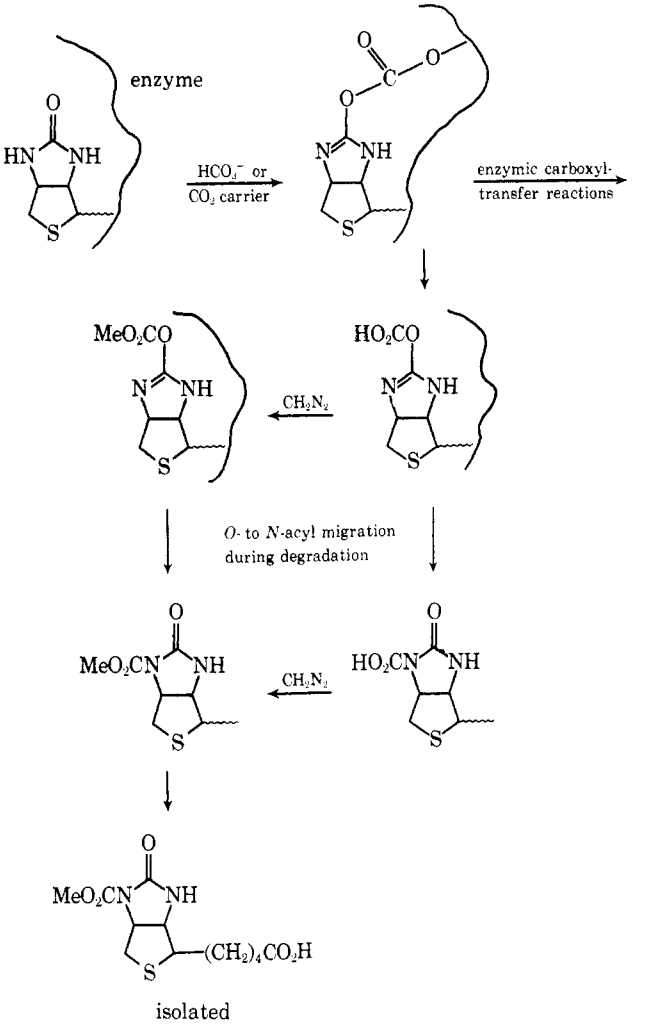
ABSTRACT: *S*-Benzoylisothioureas (**1**–**4**), including *S*-benzoylisothiobiotin, have been studied as models of *O*-carboxybiotin which has been recently proposed as the reactive intermediate in biotin-mediated enzymic carboxylations. In acidic and basic aqueous solutions **1**–**4** behave as thiol esters and hydrolyze to benzoic acid and the corresponding thiourea. The protonated benzoylisothioureas (pK_a's in the range 7–9) are extremely susceptible to nucleophilic attack and displacement at the ester carbonyl group. Around neutral pH a facile reaction competing with hydrolysis is a unimolecular rearrangement of the neutral *S*-benzoylisothioureas to *N*-benzoylthioureas. This type of reaction has been suggested to explain the isolation of 1′-*N*-carbomethoxybiotin from the trapping of enzyme·biotin·CO_2 complexes with diazomethane. Compounds **3** and **4** containing the isothiourea moiety as part of a five-membered ring are much (*ca.* 4000 times) less susceptible to benzoyl migration than the acyclic analog **1**. In terms of the proposed *O*-carboxybiotin intermediate the presence of such a five-membered cyclic urea structure in biotin can thus be seen as a desirable feature.

Recently the question of the mode of action of the vitamin biotin in enzymic transcarboxylation reactions has been reopened (Bruice and Hegarty, 1970). In particular the previously proposed structure of the biotin·CO_2 adduct has been criticized and an alternative structure suggested. The now classic experiments of Lynen, Knappe, and coworkers have suggested that in the enzyme·biotin·CO_2 intermediate complex of enzymes such as β-methylcrotonyl-CoA carboxylase (Knappe *et al.*, 1962, 1963), propionyl-CoA carboxylase (Lane and Lynen, 1963), transcarboxylase (Wood *et al.*, 1963), and acetyl-CoA carboxylase (Numa *et al.*, 1964) the CO_2 moiety is covalently bound to the 1′ position of biotin. This followed from the isolation of 1′-carbomethoxybiocytin and thence 1′-carbomethoxybiotin after the treatment of enzyme·biotin·CO_2 complexes with diazomethane and suitable proteinases and biotinidase. However, model studies of the *N*-carboxy- (and acyl- in general) imidazolidone system (Knappe, 1964; Caplow, 1965, 1968; Caplow and Yager, 1967) showed it to be an indifferent carboxyl- (and acyl-) transfer reagent.

Model studies by Hegarty and Bruice (1970a) suggest that the nucleophilic center of a neutral or anionic ureido nucleophile toward an acyl substrate with good leaving group is the oxygen atom rather than the nitrogen. These workers have proposed that the active complexes of the enzymes mentioned above have an OCO_2 rather than NCO_2 structure. The sequence of events leading to the product isolated by Lynen, Knappe, and others would then be as outlined in Scheme I.

The high acyl-transfer potential of the *O*-acylisourea system has been established by investigation of suitable model systems (Hegarty and Bruice, 1970b,c; Hegarty *et al.*, 1971). A further step with model systems is, of course, investigation of the *O*- to *N*-acyl-transfer mechanism of *O*-acylisoureas. However, *O*-acylisoureas where such transfer is not impossible on steric grounds are unknown due, presumably, to the fact that *O*- to *N*-acyl migration is most facile. In contrast suitable *S*-acylisothioureas can be prepared and in this paper we

SCHEME I



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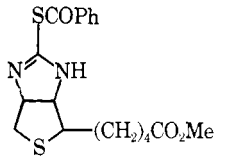
present the results of studies of the reactions in aqueous solution of some *S*-benzoylisothioureas and in particular *S*-benzoylisothiobiotin methyl ester.



Experimental Section

Materials. *S*-Benzoylisothiouronium halides were prepared from 1,3-dimethylthiourea, ethylenethiourea, and trimethylenethiourea in essentially quantitative yields from their reaction with equimolar quantities of benzoyl halides in acetone at room temperature as described by Dixon and Hawthorne (1907).

S-Benzoyl-1,3-dimethylisothiouronium Chloride (**1**). Recrystallization of this material from acetone–ether yielded a product of mp 118–120°. *Anal.* Calcd for $C_{10}H_{13}ClN_2OS$: C, 49.07; H, 5.35; Cl, 14.49; N, 11.45. Found: C, 48.97; H, 5.35; Cl, 14.47; N, 11.34.

S-Benzoyltrimethyleneisothiouronium Chloride (**2**). After recrystallization from acetone the product melted at 138–141°. *Anal.* Calcd for $C_{11}H_{13}ClN_2OS$: C, 51.45; H, 5.10; Cl, 13.81; N, 10.91. Found: C, 51.51; H, 5.11; Cl, 13.87; N, 11.06.

S-Benzoylethyleneisothiouronium Bromide (**3**). The melting point of the initially precipitated material was 148–150°. Analysis of this material suggested it to be a 2:1 complex of **3** and ethylenethiourea. *Anal.* Calcd for $C_{23}H_{28}Br_2N_6O_2S_3$: C, 40.84; H, 4.17; Br, 23.63; N, 12.42. Found: C, 40.77; H, 4.10; Br, 23.55; N, 12.41. The presence of such a mixture was verified by a nuclear magnetic resonance spectrum. Attempts to isolate **3** alone were not successful, recrystallization apparently leading to breakdown of the complex and decomposition of **3**.

The melting points of these compounds are not a good indication of purity since they are decomposition points and vary erratically over several degrees range with the rate of heating and with recrystallization. The acylisothiouronium structure is confirmed by spectral data. The infrared spectra typically show strong absorptions at 1690–1700 cm^{-1} (thio ester $\nu_{C=O}$) and at 1630–1660 cm^{-1} (N=C⁺=N), the ultraviolet spectra show $\lambda_{max}^{H_2O}$ 250–260 nm ($\epsilon \sim 10^4$), and the nuclear magnetic resonance spectra (DCl–D_2O) show that the *N*-methyl groups of **1** are equivalent as are the *N*-methylene groups of **2** and of **3**.

S-Benzoylisothiobiotin Hydrobromide (**4**). Thiobiotin was prepared from biotin by the method of Green (1966) and converted into its methyl ester by refluxing it overnight in methanol with a drop of concentrated H_2SO_4. The melting point of the ester was 215–215.5°. Jansen and Stokes (1962) report its melting point as 214–215°. It was treated with benzoyl bromide in acetone in the usual way but no precipitation of the required product occurred. The solid remaining after removal of the acetone was suspended in dry ether and isolated by filtration. This material which melted over the range 90–100° was clearly not pure. It was partially soluble in water, the insoluble portion being the starting material, thiobiotin methyl ester. An infrared spectrum showed strong absorptions at 1740 cm^{-1} (methyl ester $\nu_{C=O}$) and 1705 cm^{-1} (thiol ester $\nu_{C=O}$) and the ultraviolet spectrum $\lambda_{max}^{H_2O}$ 261 nm. A nuclear magnetic resonance spectrum (HCO_2H, sodium 3-(trimethylsilyl)-1-propanesulfonate) of the water-soluble material was essentially the same as that of thiobiotin methyl ester but with the absorptions of the bridgehead protons (complex multiplet) shifted downfield from τ 5.2 to 4.85 and with the addition of an aromatic multiplet τ 2.2. No further purification of this material was possible but the spectral data along with evidence from the hydrolysis, both qualitative and quantitative, described below, are sufficient to identify the water-soluble fraction as the desired *S*-benzoylisothiobiotin methyl ester hydrobromide (**4**).

1-Benzoylethylenethiourea. Equimolar amounts of ethylenethiourea and benzoyl chloride were refluxed together in benzene for 20 hr. During this period the solution turned yellow and a yellow solid separated. After the mixture had been cooled this material was filtered off and recrystallized from acetic acid. Its properties included mp 231–232°, ν_{max}^{KBr} 1675 cm^{-1} (no absorption above 3050 cm^{-1}), and nuclear magnetic resonance spectrum ($CDCl_3$, Me_4Si) multiplet τ 2.7, broad singlet τ 5.43 (relative areas 2.5:1). *Anal.* Calcd for $C_{17}H_{14}N_2O_2S$: C, 65.78; H, 4.55; N, 9.03; S, 10.33. Found: C, 65.63; H, 4.61; N, 8.93; S, 10.07. The compound is thus 1,3-dibenzoylethylenethiourea.

Evaporation of the original benzene solution to dryness yielded a pale yellow solid. Most of this was insoluble in chloroform and shown to be ethylenethiourea. The chloroform-soluble material was chromatographed on silica gel with chloroform as eluent. After elution of the dibenzoyl compound a fraction was obtained containing a small amount of a white crystalline material. It had mp 146–147° (from chloroform–hexane), ν_{max}^{KBr} 3150 cm^{-1} (ν_{NH}) and 1660 cm^{-1} ($\nu_{C=O}$), and nuclear magnetic resonance spectrum which contained 3 H complex triplets ($J \sim 8$ Hz) τ 6.43, 5.77, 5 H multiplet τ 2.5, and 1 H broad singlet τ 2.1. *Anal.* Calcd for $C_{10}H_{10}N_2OS$: C, 58.22; H, 4.89; N, 13.58; S, 15.55. Found: C, 58.07; H, 4.80; N, 13.40; S, 15.64. This material must be 1-benzoylethylenethiourea. The yield was *ca.* 5%.

1′-Benzoylthiobiotin Methyl Ester. Thiobiotin methyl ester (0.5 g, 0.00182 mole) was refluxed in toluene with 0.39 g (0.00278 mole) of benzoyl chloride for 24 hr. After removal of the toluene chromatography of the residue on silica gel with chloroform yielded firstly an oil whose yellow color and infrared spectrum, ν_{max}^{KBr} 1735 and 1680 cm^{-1} (no absorption above 3050 cm^{-1}), suggested 1′,3′-dibenzoylthiobiotin methyl ester by analogy with ethylenethiourea. A second colorless fraction yielded *ca.* 0.1 g of a white crystalline solid which melted at 158–159° after recrystallization from benzene–hexane. The infrared spectrum, ν_{max}^{KBr} 3100, 1735, and 1670 cm^{-1}, of this compound suggested a monobenzoylthiobiotin methyl ester, as did its analysis. *Anal.* Calcd for $C_{18}H_{22}N_2O_3S_2$: C, 57.45; H, 5.81; N, 7.34; S, 16.81. Found: C, 57.29; H, 5.76; N, 7.16; S, 16.69. The position of the benzoyl group (1′ or 3′) was indicated by the nuclear magnetic resonance spectrum ($CDCl_3$, Me_4Si). The spectrum of thiobiotin methyl ester is very similar to that reported for biotin by Glasel (1966) with the exception of a 3 H singlet (ester methoxyl) at τ 6.44, and an expected downfield shift of the NH protons (to τ 1.87) and the C_3 and C_4 bridgehead protons (to τ 5.60). The spectrum of the monobenzoyl compound above is again similar with the addition of a 5 H multiplet τ 2.5 and the disappearance of one NH. More importantly the bridgehead protons are now separated. One, appearing as a double triplet ($J = 8, 3$ Hz) moved downfield to τ 4.55 and the other, appearing as a broad double doublet ($J = 8, 4$ Hz) remained at τ 5.6. The double triplet identifies the low-field proton as the one on C_4 and its downfield position indicates that it is ad-

jacent to the *N*-benzoyl group. The upfield double doublet must result from the proton on C_3 adjacent to the NH. Shaking the sample for 5 min with D_2O removed the NH absorption from the spectrum and turned the broad double doublet into a sharp double doublet without affecting the double triplet. Coupling between the 3′-NH and the C_3H is *ca.* 1 Hz. The product then must be 1′-benzoylthiobiotin methyl ester.

3′-Benzoylthiobiotin Methyl Ester. Thiobiotin methyl ester (0.5 g, 0.00182 mole) was converted into 1′,3′-dibenzoylthiobiotin methyl ester by refluxing a suspension of it in benzene with excess (5 g, 0.027 mole) benzoyl bromide for 24 hr. The benzene was then removed and the residual oil stirred with aqueous sodium bicarbonate solution for several hours to destroy the excess benzoyl bromide. The insoluble material was taken up into chloroform and isolated by evaporation of the chloroform after the solution had been dried. The yellow oil thus obtained was dissolved in *ca.* 1 l. of 50% aqueous ethanol, and the pH of the stirred solution adjusted to 11.5 and kept there for 45 min. The pH was then reduced to 7 and an equal volume of water was added to the solution before extraction with chloroform. The chloroform extracts were dried, the chloroform removed, and the residual colorless oil chromatographed (silica gel, chloroform). This procedure yielded *ca.* 0.3 g of a colorless glass whose infrared spectrum, ν_{max}^{KBr} 3200, 1735, and 1670 cm^{-1}, also suggested a monobenzoylthiobiotin methyl ester. The nuclear magnetic resonance spectrum was very similar to that of the 1′ isomer except that the downfield bridgehead proton (τ 4.60) was in this case a double doublet (J = 8.5, 5 Hz) and the upfield proton (τ 5.45) a double triplet (J = 8.5, 4 Hz). *Anal.* Calcd for $C_{18}H_{22}N_2O_3S_2$: C, 57.45; H, 5.81; N, 7.34; S, 16.81. Found: C, 57.03; H, 5.91; N, 7.20; S, 16.69. This evidence indicates that the product is 3′-benzoylthiobiotin methyl ester.

Kinetic Measurements. Kinetic studies were carried out in deionized, glass-distilled water. Reagent grade potassium chloride, potassium bicarbonate, potassium hydroxide, potassium acetate, sodium formate, and Tris were used without further purification. Ethoxyacetic acid was distilled before use, chloroacetic acid was recrystallized from benzene, and imidazole recrystallized twice from ethyl acetate.

All reactions studied kinetically were carried out at an ionic strength of 1.0 (adjusted with potassium chloride) at a temperature of 30.0 ± 0.1° and followed spectrophotometrically at 260 nm (disappearance of substrate). Reactions whose half-lives fell between 10 sec and 5 hr were carried out in the absence of buffer in a Radiometer pH-Stat assembly specifically designed for a Cary 15 spectrophotometer (Maley and Bruice, 1970). Faster reactions and those in buffered solution were carried out in a Durrum-Gibson Model 13001 stopped-flow spectrophotometer or a Gilford 2000 spectrophotometer. Reactions were initiated in all cases by the addition of a solution of the substrate in 10^{-3} M hydrochloric acid (1 M KCl) to give final concentrations of substrate between 10^{-5} and 10^{-4} M. The stopped-flow rate measurements on **1** and **2** at pH's above 7 were carried out in phosphate, Tris, and carbonate buffers which at the concentrations used ($\leq$ 0.2 M) did not affect the observed rates.

Product Analyses. These were carried out both on a preparative scale (isolation of products or identification by thin-layer chromatography) and spectrophotometrically from the final spectra after kinetic runs.

Results

Qualitative Hydrolyses. Dixon and coworkers (1903, 1906, 1907, 1912, 1920) have examined the reactions of several *S*-acylisothiouronium salts under a variety of conditions. They established that many such compound could be rearranged under conditions of heat or treatment with various bases to the corresponding *N*-acylthioureas. The required $S \rightarrow N$ shift has thus been established. The experiments described below probe the reactions of **1**–**4** in aqueous solution rather more systematically.

Samples (0.1–0.2 g) of **1**–**4** were dissolved in 10^{-3} M HCl at room temperature and added with stirring to the following solutions.

0.2 M HCl. In all cases precipitation of white crystalline solids occurred over periods ranging from minutes (**3**, **4**) to hours (**1**, **2**). These were found to be benzoic acid or a mixture of benzoic acid and the parent thiourea. Thin-layer chromatography of the aqueous filtrate suggested that no other products were obtained. The reaction in 0.2 M HCl solution was also followed spectrally (substrate concentration $\sim 5 \times 10^{-5}$ M). In all cases the spectrum changed smoothly from that of the starting material to that of a mixture of benzoic acid and the thiourea.

pH 7–9 Buffer (0.1 M Phosphate or Carbonate). Compound **1** yielded a white crystalline solid which after recrystallization from aqueous ethanol had mp 79–80°, ν_{max}^{KBr} 1650 cm^{-1}, and nuclear magnetic resonance spectrum ($CDCl_3$, Me_4Si) 3 H doublet τ 6.88 (J = 5.0 Hz), 3 H singlet τ 6.54, 5 H singlet τ 2.74, and 1 H broad singlet τ −1.0. These data indicate that the product is 1-benzoyl-1,3-dimethylthiourea. Brown and Phillips (1970) report its melting point as 78–80°.

A similar reaction occurred with **2** where the product, after recrystallization from chloroform–petroleum ether (bp 30–60°), had mp 170–172° dec, ν_{max}^{KBr} 1690 cm^{-1}, and nuclear magnetic resonance spectrum ($CDCl_3$, Me_4Si) 2 H quintet τ 7.90 (J = 6 Hz), 2 H double triplet τ 6.70 (J = 6, 3 Hz), 2 H triplet τ 6.30 (J = 6 Hz), 5 H multiplet τ 2.6, and 1 H broad singlet τ 2.25. *Anal.* Calcd for $C_{11}H_{12}N_2OS$: C, 59.97; H, 5.49; N, 12.72; S, 14.56. Found: C, 59.76; H, 5.42; N, 12.54; S, 14.52. The product is thus 1-benzoyltrimethylenethiourea.

At spectral concentrations the reaction of **1** and **2** in the above buffers also yielded the above products.

Addition of **3** to these buffers yielded a mixture of benzoic acid, ethylenethiourea, and 1,3-dibenzoylethylenethiourea. Thin-layer chromatography (SiO_2–$CHCl_3$) showed no trace of 1-benzoylethylenethiourea. At spectral concentrations, however, the final spectrum was consistent with a mixture of benzoic acid, ethylenethiourea, and 1-benzoylethylenethiourea. Identification of the latter was confirmed by comparison of the rate of hydrolysis of this product to that of authentic 1-benzoylethylenethiourea. At pH's 11.5 and 12.0 pseudo-first-order rate constants for hydrolysis of 1-benzoylethylenethiourea (to ethylenethiourea and benzoate, followed spectrophotometrically at 260 nm) were 2.70×10^{-3} and 4.17×10^{-3} sec^{-1}, respectively; at the same pH's the rates of hydrolysis of the product from **3** were 2.76×10^{-3} and 4.23×10^{-3} sec^{-1}, respectively.

Compound **4** yielded a mixture of thiobiotin methyl ester, benzoylthiobiotin methyl ester, and 1′,3′-dibenzoylthiobiotin methyl ester. 1′- and 3′-benzoylthiobiotin methyl esters could not be satisfactorily separated by column chromatography although on thin layer some separation was possible, *e.g.*, silica gel, $CHCl_3$–1% MeOH. The monobenzoylated material was, therefore, separated from the other products by column chromatography (silica gel, $CHCl_3$) and its rate of hydrolysis at pH 11.5 followed spectrophotometrically at 260 nm. The OD *vs.* time curve obtained suggested the presence of two

components, one hydrolyzing some ten times faster than the other. Given the presence of such a mixture rate constants could be derived for the two components (after approximately 20 min the OD *vs.* time curve described only the pseudo-first-order hydrolysis of the slower hydrolyzing material and from this the rate constant could be derived; the other rate constant could then be obtained from the initial part of the curve). At pH 11.5 the pseudo-first-order rate constants for the slower and faster hydrolyzing components of the mixture from **4** were $(3.21 \pm 0.15) \times 10^{-4}$ and $(4.3 \pm 0.5) \times 10^{-3}$ sec^{-1}, respectively. At the same pH the rate constants for hydrolysis of 3′- and 1′-benzoylthiobiotin methyl esters were 3.12×10^{-4} and 4.30×10^{-3} sec^{-1}, respectively.

From the measured changes in extinction coefficients on hydrolysis of the two isomers at 260 nm (1.07×10^4 and 1.04×10^4 for the 1′ and 3′ isomers, respectively) it was possible to calculate the relative amounts of the two isomers present in the reaction mixture from **4**. Slow evaporation of a solution of the isomer mixture in benzene–hexane yielded crystalline 1′-benzoylthiobiotin methyl ester identical (infrared spectrum, melting point, and mixture melting point) with the authentic material. There seems sufficient evidence then to conclude that the reaction of **4** under these conditions yields both 1′- and 3′-benzoylthiobiotin methyl esters. At spectral concentrations the products were thiobiotin methyl ester and the two monobenzoyl derivatives, the latter two distinguished by quantitative hydrolysis at pH 11.5 as described above.

0.2 M *Potassium Hydroxide.* Compounds **1** and **2** yielded their respective *N*-benzoylthioureas as at pH 7–9. Compounds **3** and **4** yielded ethylenethiourea and thiobiotin methyl ester, respectively. Spectrally the same products were observed.

Action of Heat on 3′-Benzoylthiobiotin Methyl Ester. A sample of this compound was heated at 140° and analyzed at suitable times by thin-layer chromatography (silica gel, $CHCl_3$–1% MeOH) and by hydrolysis followed spectrally as described above. Some thiobiotin methyl ester and 1′,3′-dibenzoylthiobiotin methyl ester were produced but more significantly the composition of the monobenzoylated material changed with time from that of the pure 3′ isomer to a mixture of the 1′ and 3′ isomers. Half-time for this conversion was about 1 hr and after about 8 hr no further change in composition of the monobenzoyl fraction seemed to occur; its final composition was (77 ± 1)% 1′-benzoylthiobiotin methyl ester.

Kinetics. Plots of logarithms of observed pseudo-first-order rate constants (log k_{obsd}) *vs.* pH for the disappearance of **1**–**4** from reaction in aqueous solution are shown in Figure 1. The points on these plots are experimental and the lines theoretical, having been derived from the empirical eq 1. Empirical constants fitting this equation for **1**–**4** are given in Table I.

$$k_{obsd} = \frac{k_0 a_H + k_a K_a + k_b K_a K_w / a_H}{a_H + K_a} \quad (1)$$

From the previously described qualitative product analyses it would be reasonable to assume that the reaction path described by k_0 leads to hydrolysis, k_a to the *S*- to *N*-acyl-transfer product, and k_b again to hydrolysis. Final spectra after kinetic runs suggested this assumption was correct. Confirmation came through a more quantitative approach to the final spectra.

From the measured extinction coefficients of the products of hydrolysis and *S*- to *N*-acyl transfer, product distributions at any pH could be calculated. If the plateau in the pH–rate profile does describe the pH dependence of the acyl-transfer reaction then the fraction of *N*-benzoylthiourea produced at any pH (f) is given by eq 2, where f_0 is the fraction of product

$$f = \frac{f_0 k_a K_a}{k_0 a_H + k_a K_a + k_b K_a K_w / a_H} \quad (2)$$

arising *via* k_a representing *N*-benzoylthiourea. For **1** a plot of f *vs.* pH is shown in Figure 2. The points are experimental, calculated from final spectra, and the line is calculated using eq 2, the constants of Table I and $f_0 = 0.97$. pK_{app}, where $f/f_0 = 0.5$ is 3.52 (this is, of course, given by $k_a K_a / k_0$ if $k_b K_a K_w / a_H \ll k_0 a_H + k_a K_a$). The coincidence of the kinetic and product analysis data here confirms assignment of k_a to the acyl-transfer reaction whose pH dependence is described in terms of K_a. Similar results were obtained for **2**–**4** whose f_0 values were 0.90, 0.27, and 0.40, respectively, and pK_{app} values 4.65, 6.91,

TABLE I: Empirical Rate Constants Used to Define pH–Rate Profiles of **1**–**4**.

Compound	$k_0 \times 10^4$ (sec^{-1})	k_a (sec^{-1})	pK_a	k_b (sec^{-1} M^{-1})
1	2.8	21	8.40	293
2	2.05	3.6	8.90	*a*
3	16.4	0.018	7.95	83
4	35	0.0125	7.20	81

[a] pH not taken high enough to obtain this constant.

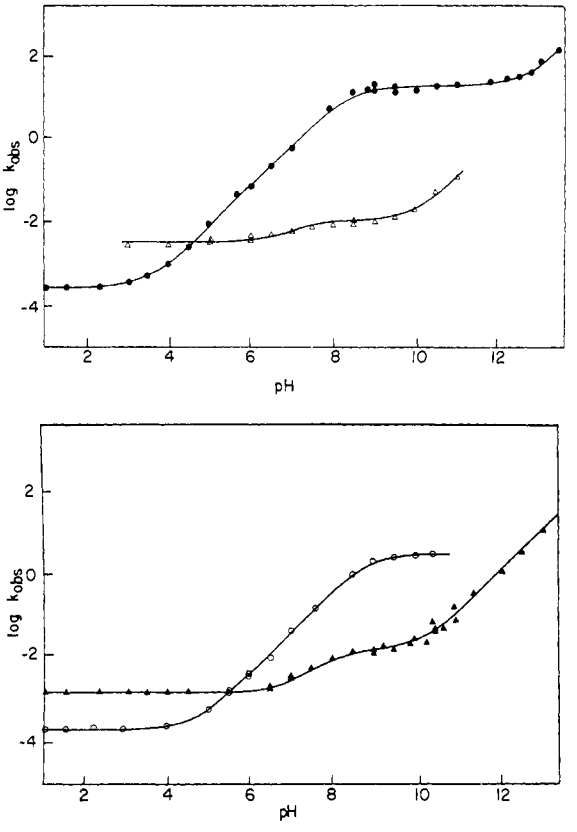


FIGURE 1: Plots of log k_{obsd} (k_{obsd} in sec^{-1}) *vs.* pH for the reactions of **1** (●), **2** (○), **3** (▲), and **4** (△) in water (μ = 1.0, 30°). Points are experimental and the curves generated by eq 1.

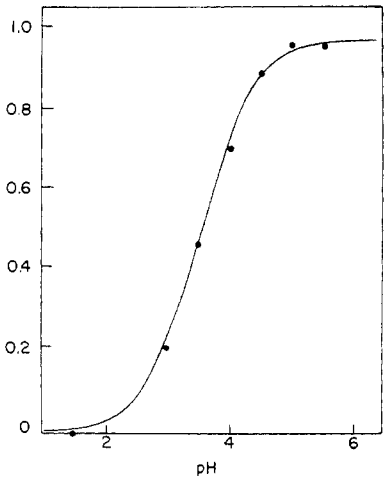


FIGURE 2: A plot of the fraction (f) of 1-benzoyl-1,3-dimethylthiourea produced from reaction of **1** in water as a function of pH. The points are experimental and the curves generated by eq 2.

and 6.65, respectively. For **4** ratios of 1′- to 3′-benzoylthiobiotin methyl esters were obtained as described in the previous section. Values obtained were 0.26 ± 0.01 from three separately prepared samples of **4**.

The results reported above are best interpreted in terms of Scheme II from which eq 3 can be derived.

$$k_{obsd} = \frac{k_1 a_H + (k_2 K_w + k_3 K_1 + k_5 K_1) + k_4 K_1 K_w / a_H}{a_H + K_1} \quad (3)$$

In terms of the empirical eq 1 we then have

$$k_0 = k_1$$

$$k_a = k_2 K_w / K_1 + k_3 + k_5$$

$$K_a = K_1$$

$$k_b = k_4$$

Furthermore, using the product analysis data: $f_0 = k_5/k_a$. Hence the rate constants of Scheme II can be obtained and are as given in Table II.

Kinetics in Buffered Solutions. At pH < pK_{app} the rate of disappearance of **1**–**4** in aqueous solution was markedly accelerated by the presence of buffer species. At higher pH's, buffers, *e.g.*, phosphate, Tris, and carbonate, had little or no

SCHEME II

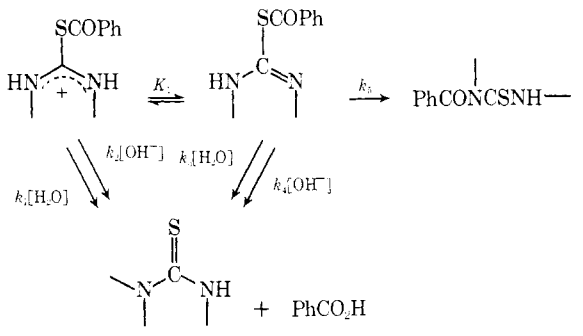
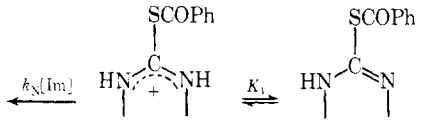


TABLE II: Derived Constants for Reaction of **1**–**4** in Aqueous Solution.

Compound	k_5 (sec^{-1})	K_1	$k_2K_w/K_1 + k_3$ (sec^{-1})
1	20.4	8.40	0.6
2	3.24	8.90	0.26
3	4.85×10^{-3}	7.95	1.31×10^{-2}
4	4.95×10^{-3}	7.20	7.55×10^{-3}

effect on the measured rates of reaction of **1** and **2**. This suggests that buffer species attack the substrate directly or catalyze the hydrolysis but do not catalyze the acyl-transfer reaction.

The results of experiments in imidazole buffers with **3** are given in Figure 3 where a plot of $(k_{obsd} - k_0)/[Im_T]$ *vs.* pH is presented. Here k_{obsd} is the observed pseudo-first-order rate constant for disappearance of **3** in imidazole buffers, k_0 is the background rate calculated from eq 1, and $[Im_T]$ is the total imidazole concentration. A curve of the type obtained is best interpreted in terms of eq 4, where K_2 is the dissociation con-



$$\frac{(k_{obsd} - k_0)}{[Im_T]} = \frac{k_N K_2 a_H}{a_H^2 + (K_1 + K_2)a_H + K_1 K_2} \quad (4)$$

stant of the imidazolium ion, measured under the prevailing conditions as 7.20. Using this value of K_2, the value of K_1 from Table II and $k_N = 140$ sec^{-1} M^{-1} the solid line of Figure 3 is obtained from eq 4 which is clearly a good fit to the experimental points.

The product of the reaction of **3** with imidazole absorbed strongly at 250 nm and in acid solution (pH 1.0) disappeared with a pseudo-first-order rate constant of 3.89×10^{-2} sec^{-1}. Caplow and Jencks (1962) report the rate of hydrolysis of *N*-benzoylimidazole at pH ≲ 1 as 3.67×10^{-2} sec^{-1} (25°, μ = 0.1). It seems likely then that the reaction of imidazole with **3** involves nucleophilic attack and displacement at the ester carbonyl group.

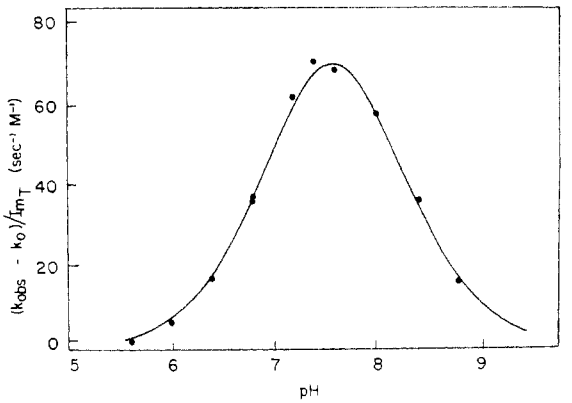


FIGURE 3: A plot of $(k_{obsd} - k_0)/[Im_T]$ *vs.* pH for reaction of **3** in imidazole buffers. The points are experimental and the curves generated by eq 4.

An entirely analogous reaction was observed between **4** and imidazole. Here the observed rate constants could be fitted to eq 4 using K_1 (from Table II) and K_2 as above and $k_N = 320$ $sec^{-1}\,M^{-1}$.

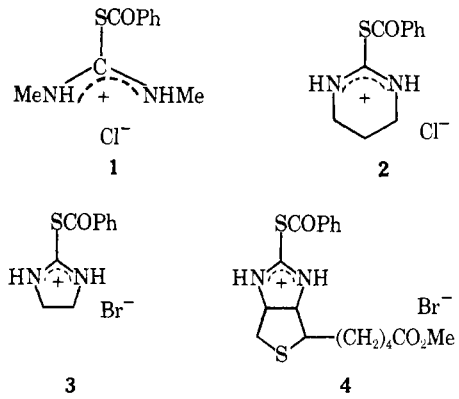
Other buffers used with **3** and the second-order rate constants k_N for reaction of the base species of the buffer with the protonated isothiourea are given in Table III.

The much larger rate constant of imidazole compared to that of phosphate indicates (as shown above for imidazole) that nucleophilic attack at the acyl group is involved (*e.g.*, Johnson, 1967). Nucleophilic attack by phosphate has also been confirmed. Hydroxamate analysis (Lipmann and Tuttle, 1945) after the reaction of **3** in 0.25 M phosphate buffer (pH 6.5) indicated 105% of the acyl moiety trapped (by comparison to a sample of **3** dissolved in 10^{-3} M HCl and assayed immediately). Benzoyl phosphate is known to hydrolyze very slowly at pH 6.5 (DiSabato and Jencks, 1961).

Nucleophilic attack by the carboxylates also is indicated by the much larger k_N value of formate with respect to an acetate of the same pK_a. In the case of acetate itself a second slower reaction was observed spectrophotometrically after the initial reaction of **3** was complete. The rate here ($\sim 10^{-3}$ sec^{-1}) is of the order expected for acetic benzoic anhydride (Bunton and Perry, 1960; Bunton *et al.*, 1963). This second reaction was not observed with the other carboxylates, presumably because of their relatively faster hydrolyzing mixed anhydrides.

Discussion

The reactions of *S*-benzoylisothiouronium salts **1–4** in aqueous solution have been studied kinetically and by product analysis. The pH–rate profiles for the disappearance of **1–4** in



aqueous solution in the absence of added buffer are given in Figure 1 and have been interpreted in terms of Scheme II.

At low pH the rates of disappearance of **1–4** are invariant with pH and the products are those of ester hydrolysis, benzoic acid and the respective thiourea. This reaction must involve attack of water on the protonated isothiourea. At high pH the products are again of hydrolysis and the rates of disappearance of isothiourea are first order in hydroxide ion. This reaction must arise from hydroxide ion attack on the ester carbonyl group of the neutral isothiourea. Analogous reactions and, in fact, very similar rate constants have been observed for the hydrolysis of the cyclic benzoylisourea studied by Hegarty and Bruice (1970b).

At intermediate pH (around neutral) hydrolysis is a less important reaction, but other hydrolysis terms arising either from hydroxide ion attack on the protonated isothiourea or water attack on the neutral compound can be seen. Of these

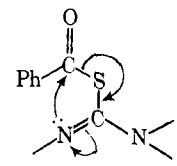
TABLE III: Second-Order Rate Constants for Reaction of **3** with Buffer Bases.

Buffer Base	pK_a	k_N ($sec^{-1}\,M^{-1}$)
Imidazole	7.20	140
Monohydrogen phosphate	6.50	0.50
Acetate	4.55	0.25
Ethoxyacetate	3.45	0.103
Chloroacetate	2.60	0.0388
Formate	3.55	1.94

kinetically indistinguishable possibilities the former, hydroxide ion attack on the protonated acylisothiourea is preferred because of the size of the rate constants involved. For instance, for **2** a first-order rate constant of 0.26 sec^{-1} (Table II) is required for water attack on the neutral compound. This can be compared to a constant of 2.05×10^{-4} sec^{-1} for water attack on the protonated compound. In view of the expected and observed preference of nucleophiles for the protonated species this interpretation seems unrealistic. On the other hand, interpretation in terms of hydroxide ion attack on the protonated species leads (by putting $k_2K_w/K_1 = 0.26$) to a second-order rate constant of 2.2×10^4 $sec^{-1}\,M^{-1}$ for this process. A comparison to rate constants for hydroxide ion attack on the neutral form (k_b, Table I) suggests that this value is quite reasonable.

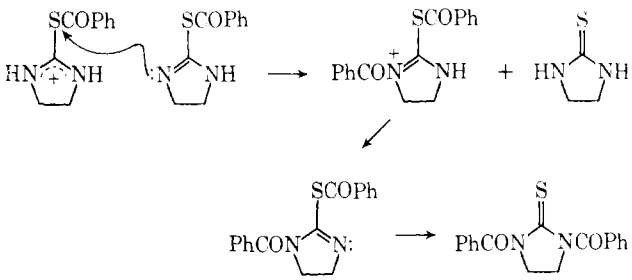
Buffer species also catalyze the disappearance of **1–4** from solution. This has been interpreted as from reaction (eq 4) between the protonated isothiourea and the buffer base species acting as a nucleophile in attack on the ester group of the substrate to form the benzoylated nucleophile and the thiourea. Second-order rate constants for this reaction with **3** are presented in Table III for imidazole, phosphate, and several carboxylates. These constants, and the water hydrolysis rate constants, k_1 (Table II) indicate the extremely high acyl-transfer potential of the protonated acylisothioureas which is, as indicated above, apparently very similar to that of acylisoureas. The rate constants of Tables II and III suggest they lie in reactivity between 2,4-dinitrophenylacetate and 1-acetoxy-4-methoxypyridinium ion (and much closer to the latter) in a series of esters of increasing reactivity (Jencks and Gilchrist, 1968). High acyl-transfer potential would, of course, be a useful property of a carboxyl-transferring biotin·CO_2 adduct; it is not a property of *N*-acylureas.

The most interesting reaction of **1–4** is, of course, the *S*- to *N*-acyl transfer yielding *N*-acylthioureas which is observed as the predominant reaction around neutral pH. The kinetics suggest that this is a unimolecular reaction of the neutral acylisothiourea molecule resulting from an intramolecular nucleophilic attack of the imino nitrogen on the ester carbonyl group as depicted below and as suggested by Curtin and Miller (1965, 1967) for the rearrangement of *O*-acylisoamides.



Analysis of the results in this fashion requires identification of the kinetically apparent pK_a (Table I) with the actual pK_a of the protonated acylisothiourea (Scheme II, Table II). Although no direct titration was possible because of the lability of the species involved at pH's about the pK_a and the apparently small spectral differences between the protonated and neutral forms these apparent values are not unreasonable for the actual pK_a's. Some evidence for this identity stems from the coincidence of the apparent pK_a's of **3** and **4** from the rates of acyl shift with those required by the rates of imidazole and phosphate nucleophilic displacement. The pK_a's of *O*-methylisourea and *S*-methylisothiourea are 9.80 and 9.83, respectively (Dippy *et al.*, 1959), and that of *S*-phenylisothiourea is 9.35 (T. C. Bruice and R. F. Pratt, unpublished data). As expected, replacement of SR with SCOR lowers the pK_a as in **1** and **2** (Table II). Incorporation of the isothiourea structure into a five membered ring also lowers the pK_a—the pK_a's of *O*-methylethyleneisourea and *S*-methylethyleneisothiourea are 9.14 and 9.32, respectively (Hegarty *et al.*, 1969). This is seen in the pK_a of **3** and of **4** where fusion of the second five-membered ring lowers it even further.

Buffer species, *e.g.*, phosphate, carbonate, and Tris, had no effect on the rate of N → N transfer as would be expected for an intramolecular reaction of the type envisaged above. The bimolecular analog of the acyl shift reaction is probably exemplified by the production of 1,3-dibenzoylthiourea derivatives from **3** and **4** in concentrated solution. *S*-Alkylisothioureas are known to be powerful nucleophiles toward acyl



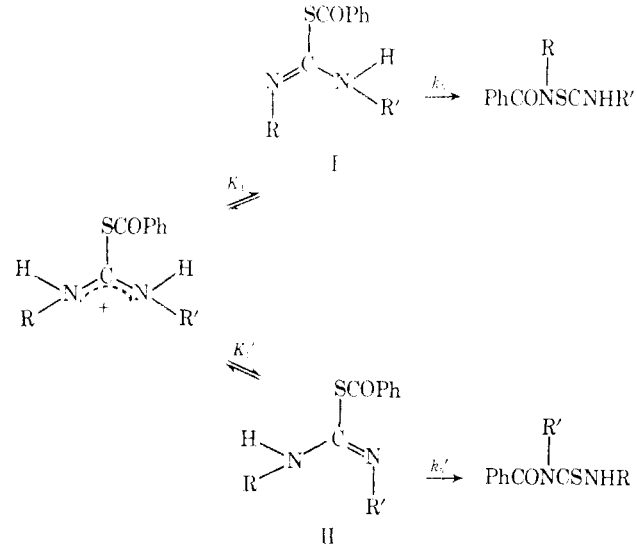
carbon (Hegarty *et al.*, 1969). Dibenzoylated products were not observed with **1** and **2** under the same conditions presumably because of the much faster intramolecular reactions in these cases.

Transfer of the benzoyl group from S to N is a rapid reaction for **1** and **2**, *e.g.*, half-life of the benzoyl group on S for **1** at pH 7 is *ca.* 1 sec. Comparison of rate constants, k_5, for the acyl-transfer reaction (Table II) shows that **2** is some 6 times less reactive than **1** whereas **3** and **4** are 4000 times less reactive. The greatly reduced susceptibility of **3** and **4** to acyl migration most likely reflects the steric strain encountered in the fusion of a presumably almost coplanar four-membered ring transition state (or intermediate) onto a five-membered ring. This effect is seen to a much lesser extent in **2** where a six-membered ring is involved.

In terms of the proposed biotin·CO_2 intermediate (Scheme I) and assuming that the relative rates of intramolecular acyl transfer for the *O*-acylisoureas analogous to **1**–**4** will be the same as that found for **1**–**4**, it is clear that the presence of a five-membered ring urea in biotin is a useful adaptation in that it leads to an acylated intermediate which is much less susceptible to conversion to the reaction terminating *N*-acyl isomer than an acyclic analog. The important assumption made above is not an unreasonable one; *e.g.*, Gregory and Bruice (1967) have shown that there is a direct linear relationship between the rate constants for nucleophilic displacement at the carbonyl group of esters and those of thiol esters where both have very good leaving groups such that nucleophilic attack is rate determining as would be expected for the acyl-transfer reaction currently under discussion.

There is no evidence from studies carried out on a variety of *S*-acylisothioureas that the S → N shift is reversible and in fact there is good evidence (T. C. Bruice and R. F. Pratt, unpublished data) that it is not, in aqueous solution at least. The isomerization of 3′- to 1′-benzoylthiobiotin methyl ester, described in the Results section and discussed further below, could proceed *via* a reverse N → S intramolecular acyl migration but an intermolecular transfer mechanism, suggested by the appearance of 1′,3′-dibenzoylthiobiotin methyl ester as one of the rearrangement products, seems more likely.

For further comparison between **4** and the biotin·CO_2 intermediate it is interesting to examine the acyl-transfer products of **4** in more detail. For an asymmetric compound such as **4** it is necessary to expand Scheme II somewhat as shown below (for the acyl transfer only). In this case k_s =



$(k_5K_1 + k_5'K_1')/(K_1 + K_1')$ and $K_a = K_1 + K_1'$, where k_s is the portion of k_a (eq 1) giving *N*-acylthioureas and K_a is the apparent dissociation constant from the kinetics (eq 1, Scheme II).

From the product analysis $k_5K_1/k_5'K_1' = 0.26$, where k_5K_1 is the rate of production of 1′-benzoylthiobiotin methyl ester and $k_5'K_1'$ that of the 3′ isomer. This result is unexpected since migration of the *S*-benzoyl group to the less hindered 1′ position would seem more favorable. Certainly the 1′ isomer should be the more thermodynamically stable of the two. Benzoylation of thiobiotin methyl ester with benzoyl chloride in refluxing benzene gave apparently only the 1′ isomer. When the 3′ isomer was heated above 140° for 1 hr, partial conversion into the 1′ isomer was observed. Further heating appeared to lead to an equilibrium situation with the 1′ isomer predominant (~77%). The products observed from **4** where this steric factor favoring the 1′ isomer is more than offset requires that either or both of the following be true: (a) $K_1 < K_1'$, *i.e.*, II is more stable than I and (b) $k_5 < k_5'$. Both conditions could be satisfied by the proposal of a favorable interaction (*e.g.*, hydrophobic) between the benzoyl group and the aliphatic side chain of biotin in the reactant II, thus (a), or in the transition state leading to 3′-benzoylthiobiotin methyl ester and

hence (b), but not in the product since the 3′ isomer is apparently less stable thermodynamically. The (apparently unfavorable) proximity of the benzoyl group to the side chain in the 3′ product is seen in the greater than tenfold slower alkaline hydrolysis rate of the 3′ *vs.* the 1′ isomer. A study involving other acyl groups would be useful.

In contrast to the mixed products from rearrangement of **4**, Knappe *et al.* (1962, 1963) obtained only 1′-carbomethoxybiotin from methylation of the β-methylcrotonyl-CoA carboxylase·biotin·CO_2 complex with diazomethane. Also, 1′-carboxybiotin is the product of carboxylation of free biotin by β-methylcrotonyl-CoA carboxylase (Lynen *et al.*, 1961), by acetyl-CoA carboxylase (Stoll *et al.*, 1968), and by the biotin carboxylase fraction of acetyl-CoA carboxylase (Alberts *et al.*, 1969). Given that carboxylation occurs initially at oxygen and is followed by rearrangement as shown in Scheme I and that the change from a urea to a thiourea derivative does not affect product distributions, this difference must result from the absence of the side-chain interaction discussed above with a much less hydrophobic carbomethoxy or carboxy migrating group so that here the thermodynamically favored product is also favored kinetically. The results obtained from the action of heat on 3′-benzoylthiobiotin methyl ester and those of Knappe *et al.* (1961) where *ca.* 7% of the 3′-carbomethoxybiotin methyl ester was obtained from reaction of biotin methyl ester and methyl chloroformate suggest that relative thermodynamic stabilities may not be sufficient to explain the complete preference for the 1′ position shown by the biotin intermediate. In terms of energy differences, however, the discrepancy is not great and could readily be accommodated by ground-state (K_1) or kinetic (k_5) factors.

This work then does lend support to the suggestion that a fast *O*- to *N*-acyl migration could explain the isolation by Lynen, Knappe, and coworkers of 1′-carbomethoxybiotin from an enzyme·O·CO_2·biotin complex. These intramolecular acyl-transfer reactions have been shown to proceed *via* the neutral isourea species and occur readily at pH's around 7. Five-membered cyclic acylisothioureas (and presumably acylisoureas) are characterized by a slow intramolecular acyl-transfer rate compared to six-membered cyclic and acyclic analogs. Since intermolecular acyl-transfer rates are not similarly depressed, the presence of a five-membered cyclic structure would be a desirable feature of a urea acting as a catalyst of intermolecular acyl transfer *via* an *O*-acyl intermediate, *e.g.*, biotin, possibly. Facile intermolecular acyl-transfer reactions are characteristic of the protonated isourea species.

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