The *N*-Phosphobiotin Route: a Possible New Pathway for Biotin Coenzyme

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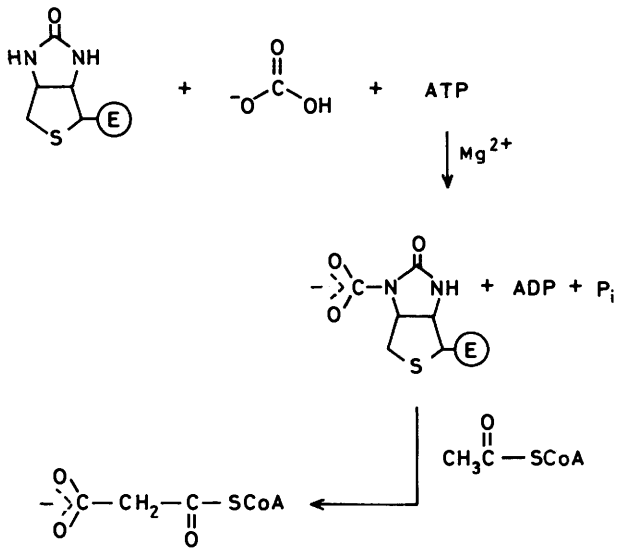
The formation of *N*-acylureas from *N*-phosphorylated ureas under very mild conditions is presented as a possible route to formation of carboxybiotin from biotin in biotin-mediated enzymatic carboxylations in which an *N*-phosphobiotin is the activated form. The detailed mechanistic pathway for the model reaction indicates that an intermediate acylphosphate is formed, the rate-determining step being nucleophilic attack of the ureido anion or its enol on this acylphosphate. In no case was an *O*-phosphorylated intermediate (of the *O*-phosphobiotin type) involved.

Despite progress in understanding biotin carboxylase reactions,[1] several points remain unclear in the detailed mechanism of coenzyme carboxylation (step 1, Scheme 1) and more precisely about the role of the adenosive triphosphate (ATP) molecule.



Scheme 1.

To account for the reactivity of the weak ureido nitrogen nucleophile on the poor carbonate electrophile, two activation modes have been postulated, one, the *O*-phosphobiotin[2] (Scheme 2), where a more nucleophilic nitrogen atom can react with the hydrogencarbonate by a concerted mechanism, the other with carboxyphosphate as a reactive species which transfers the carbonate group to the ureido nitrogen atom of the coenzyme.

However, the difference is not as clear cut as in Scheme 2 for the *O*-phosphobiotin route, because a carboxyphosphate is also formed in the concerted process.

Hydrolysis of a ureido-bearing phosphonate,[3] in which assistance by the oxygen atom of the ureido moiety is involved and rationalised in terms of formation of an *O*-phosphorylated intermediate, favours Scheme 1, while experimental evidence for the involvement of a carboxyphosphate intermediate in reactions of the carbamoyl phosphate synthetase[4] favours Scheme 2.

But for biotin-mediated reactions, no direct and definitive results have yet been obtained, neither in chemical nor biochemical studies, probably owing to the lack of suitable substrates in both circumstances.

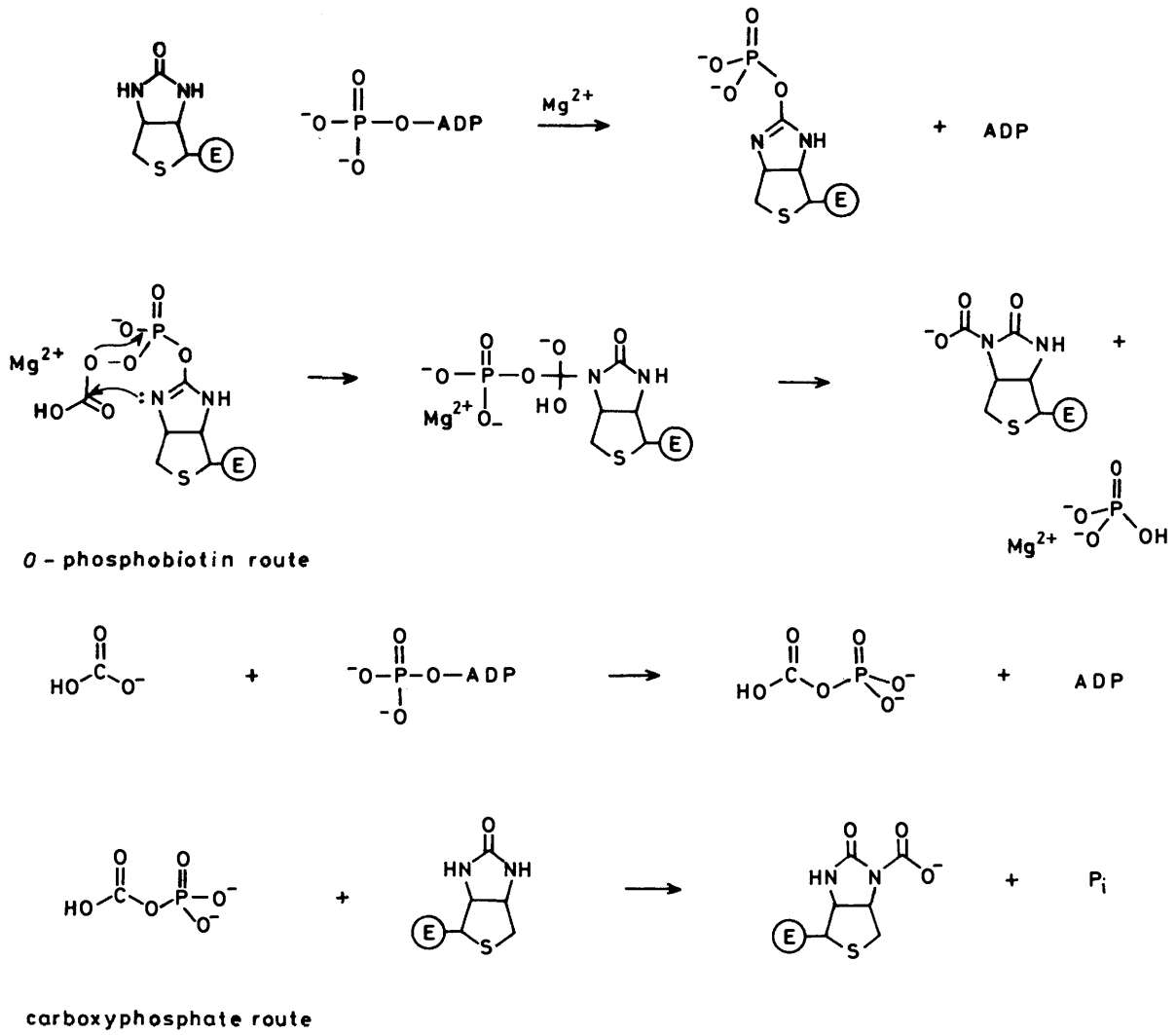
Results

The work presented here, employing model reactions, provides evidence for an alternative pathway, a reaction proceeding by a carboxyphosphate formed *in situ* from an *N*-phosphorylated species. This we call the *N*-phosphobiotin route. The substrates are indicated in Scheme 3.

Reactions were performed by adding stoicheiometric amounts of 1:1 carboxylic acid–triethylamine mixture to the phosphorylated derivative in acetonitrile at room temperature. The anion $RCOO^-$ is considered to represent the $HCOO^-$ reactant of the enzymatic reaction. Progress was followed by ^{31}P n.m.r., the *N*-acylated product being characterised after isolation.

With substrate (**1**), which equilibrates in solution with (**1′**),[5] no acylation was observed. The preferred reaction is fast base-catalysed elimination of the thiophosphoryl group in (**1′**). In a detailed study of the rearrangement (**1**) $\rightleftharpoons$ (**1′**), as a model for phosphoryl group transfer, we noted that this elimination, which occurs even in neutral conditions, is very fast when base is present.[6] With substrate (**2**), no reaction occurred, even after several hours at reflux in acetonitrile. By contrast, substrates (**3**) and (**4**) gave *N*-acylated products quantitatively and under very mild conditions. Compound (**3**) led to (**5**), whereas (**4**) gave a mixture of mono and/or diacylated products (**6**) and (**6′**) depending on the reaction conditions (Scheme 3). In both cases activation of the substrate by phosphorylation is required since no reaction occurs with the unphosphorylated substrate, even under more drastic conditions. This activation takes place *via* the *N*-phosphorylated form and not by an *O*-phospho structure as with the *O*-phosphobiotin in Scheme 2. This *O*-phosphorylated form might result from equilibrium with the *N*-phosphorylated form, as observed for the (**1**) $\rightleftharpoons$ (**1′**)[5] (Scheme 3).

This point was demonstrated by following the progress of the reaction of substrates (**3**) and (**4**) by ^{31}P n.m.r. at low temperature (−12 °C). The results are shown in the Figure. Spectrum (a) shows results obtained with (**3**) after 10 min; three peaks were observed corresponding respectively to the diphenylphosphate formed, the remaining starting material, and a third phosphorylated species which is an intermediate as indicated by its complete disappearance with time [spectrum (b)]. Reaction with (**4**), more complicated owing to successive reactions of the two phosphoryl groups, gave spectrum (c) indicating the formation of an intermediate having the same chemical shift as for the reaction with (**3**) [species (**7**)]. The other peaks correspond respectively to the starting material, the phosphate formed, and a monoacylated, monophosphorylated product. The intermediate (**7**) is rapidly formed at low temperature and its formation parallels that of the phosphate as indicated by relative intensities of the corresponding peaks 2 and 3 at low temperature [spectra (d) and (e)]. This means that no diacylated product is formed at low temperature. By contrast, at room temperature [spectra (f) and (g)], the intensity of peak 3



Scheme 2.

decreases compared with that of the phosphate and the starting material completely disappears [spectrum (g)]. A possible explanation of this fact is that diacylation occurs only after migration of the first acyl group from the sulphur atom to the nitrogen in the *S*-acylated *N*-monophosphorylated intermediate (**7**) (Scheme 4), implying acylation first on the sulphur atom.

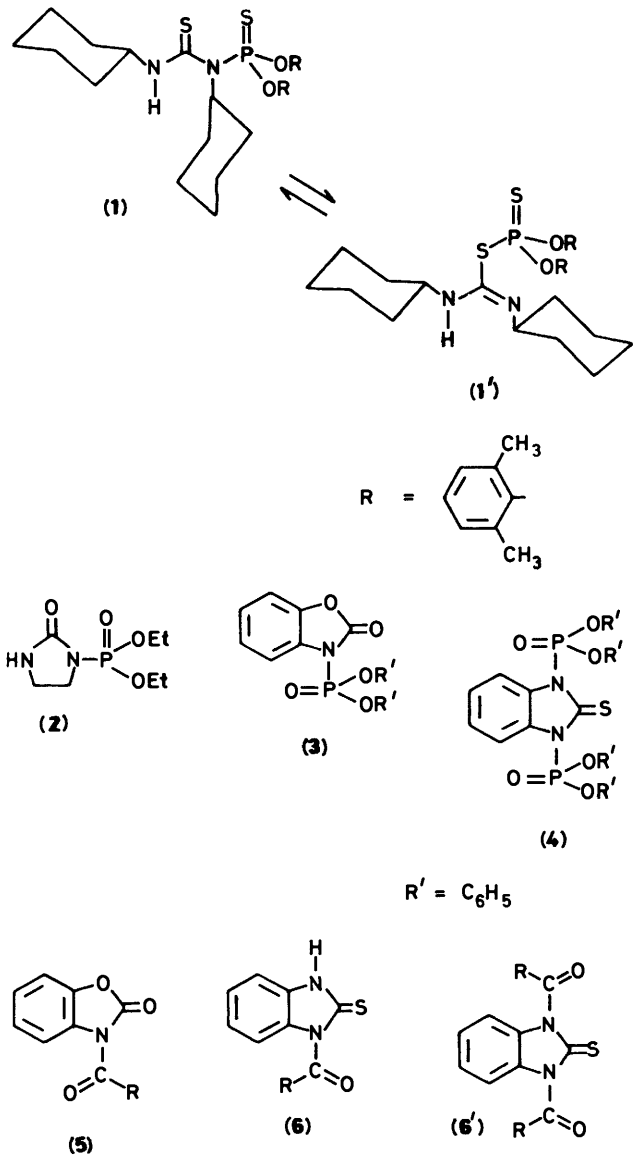
This acyl migration, investigated with other cyclic thioureas, has been shown to proceed intramolecularly with a rate constant of *ca.* 10^{-3} s^{-1} at 30 °C;[7] it therefore should be rather slow at −12 °C.

Experiments with both substrates (**3**) and (**4**) demonstrate that the common intermediate in the Figure cannot correspond to an *O*-phospho or *S*-phosphorylated species of (**3**) and (**4**), respectively, since their ^{31}P chemical shifts would be very different, δ *ca.* −10 p.p.m. for an *O*-phosphourea[8] and δ *ca.* 40 p.p.m. for an *S*-phosphourea.[9] Owing to the fact that corresponding *S*-phospho intermediates are rather stable their presence would have been easily shown by ^{31}P n.m.r.; *O*-phospho intermediates are less stable[10] but can also be characterised at low temperature.[8,10] The intermediate with δ_p −19.3 p.p.m., which accumulated up to 13% with substrate (**3**), is formed directly from the *N*-phosphorylated starting material. Therefore, path b of Scheme 5 has to be ruled out.

The Nature of the Intermediate.—Path a of Scheme 5 indicates that one of two intermediates should be considered, either a pentavalent species formed by reaction of the carboxylate with the *N*-phosphorylated form which would lead after a concerted reaction and O/S ⟶ N acyl migration[7,11] to the *N*-acylated product (path a_1), or an acylphosphate formed from the pentavalent intermediate which would be the reactive species (path a_2).

The following experiments indicated that path a_2 is preferred. A 1:1 mixture of diphenyl chlorophosphate and benzoic acid–triethylamine at −12 °C in acetonitrile gave a unique phosphorylated product, the chemical shift of which was δ −19.3 p.p.m. A mass spectrum of a sample of this solution indicated a peak at *m/e* 354 for $(C_6H_5O)_2P(O)OC(O)C_6H_5$, with the corresponding fragments 326 and $[(C_6H_5O)_3P{=}O]$; 105 ($C_6H_5C{=}O$) (*I* 100%). Addition to this solution of the starting material (benzoxazolin-2-one) at room temperature led to the *N*-benzoyl product in quantitative yield.

Experiments with other substituted carboxylate anions indicated that this intermediate plays a key role. Under the conditions for the benzoate anion, reactions followed by ^{31}P n.m.r. showed disappearance of starting material (**3**) (Table). With *p*-methoxybenzoic acid anion, an intermediate was



Scheme 3.

Table. Half-lives of reaction between substituted benzoic anion (0.3M) with compound (**3**) in acetonitrile at −12 °C

X in p-$XC_6H_4CO_2$	$t_{\frac{1}{2}}$/min
NO_2	25
H	18
CH_3O	11

observed [δ_p −19.4 p.p.m., corresponding to p-$CH_3OC_6H_4$-$C(O)OP(O)(C_6H_5O)_2$] whereas no intermediate was observed for the p-nitro analogue. From these two facts, it follows that the reaction is very sensitive to electronic effects, since a change from methoxy or hydrogen to nitro at the *para* position of the aromatic ring slows down the reaction rate, implying that the rate-determining step is nucleophilic attack of benzoate anion on the phosphorus atom of the starting material. This first step is followed by attack on the carbon of the acyl group of the mixed anhydride intermediate which is much more electrophilic than the phosphorus atom (Scheme 5, steps a and a_1). This high

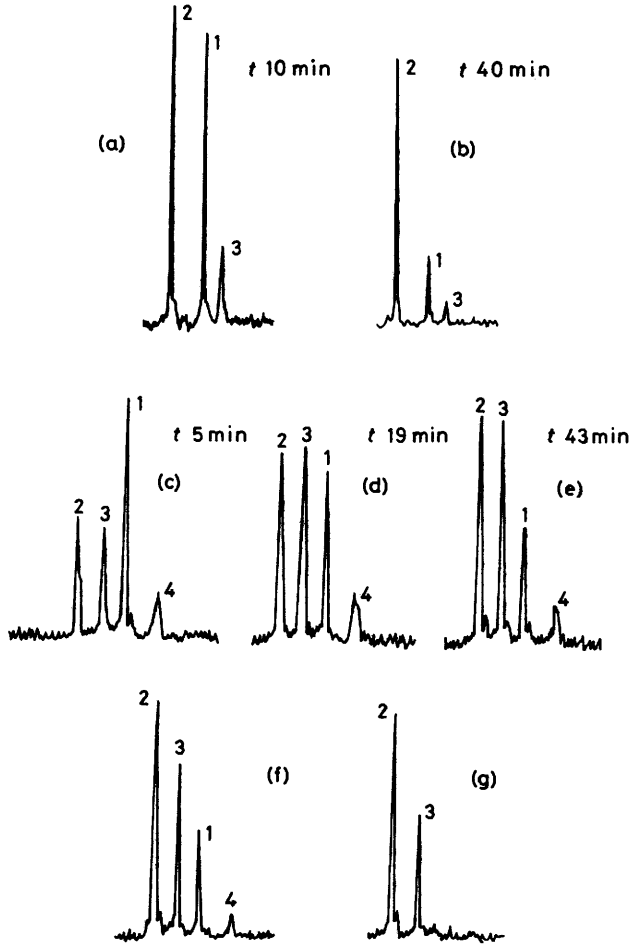
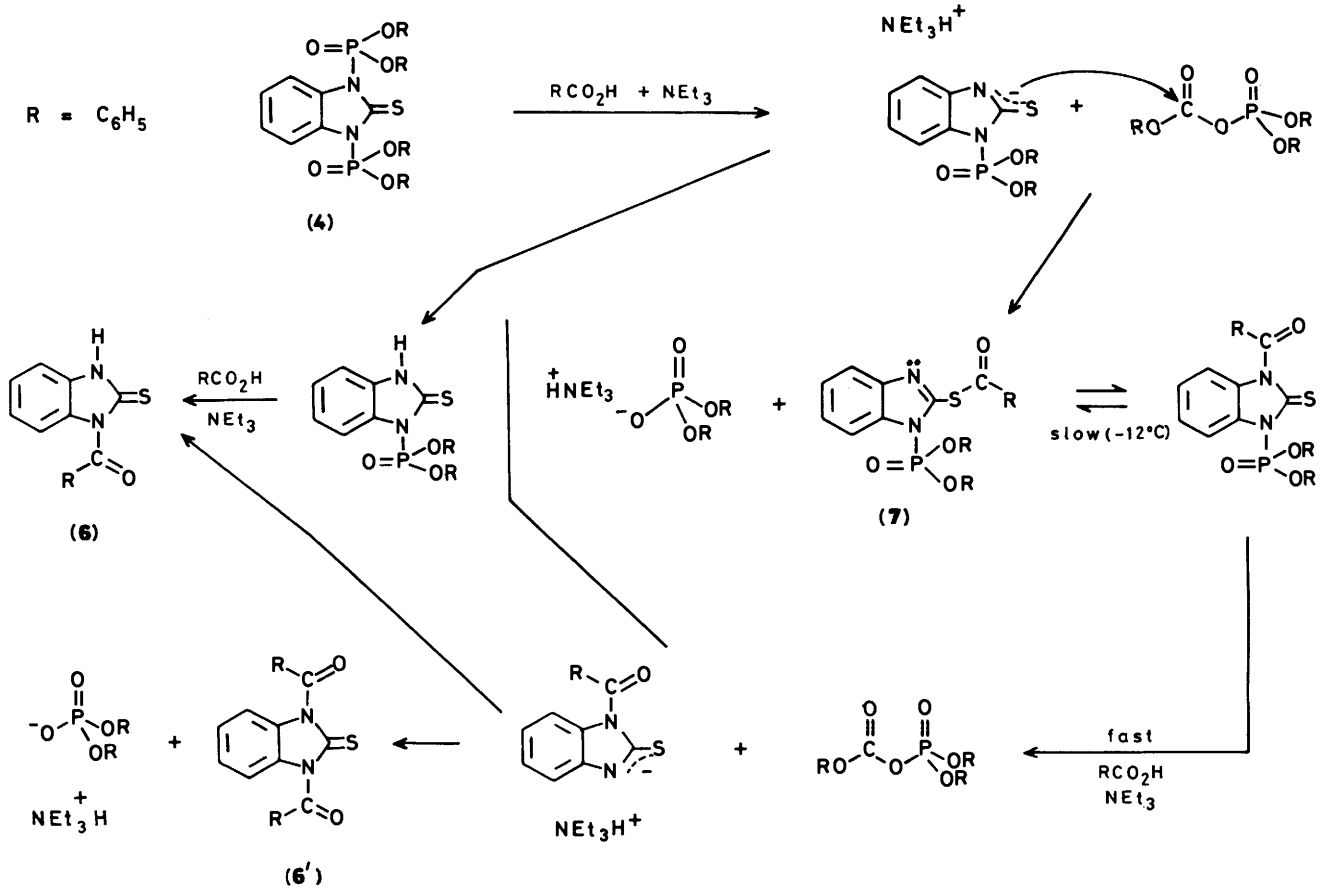


Figure 1. ^{31}P N.m.r. spectra of reaction of compounds (**3**) and (**4**) with benzoic acid in the presence of triethylamine at −12 °C at room temperature. (a), (b): 1, (**3**) (δ_p −17 p.p.m.); 2, $(RO)_2P(O)O^-$ Et_3NH^+ (δ_p −12 p.p.m.); 3, intermediate (δ_p −19.3 p.p.m.). (c)—(g): 1, (**4**) (δ_p −16.2 p.p.m.); 2, $(RO)_2P(O)O^-$ Et_3NH^+ (δ_p −12 p.p.m.); 3, monophosphorylated species (δ_p −14.2 p.p.m.); 4, intermediate (δ_p −19.3 p.p.m.).

reactivity have been noted for other acyl phosphates.[12] The actual nucleophile in step a_3 could be either the anion or the corresponding enol since an acid is present (Et_3NH^+) and also because anions become strong bases in acetonitrile.[13] The anion should react faster than the enol.

Discussion

Our chemical results are related to other reports in the literature relevant to the same mechanism. Kunieda *et al.*[14] obtained carboxyactivating agents for peptide synthesis by the reaction of a protected amino-acid on a oxazol-2-one bearing a phosphoryl group on nitrogen. Wakselman[15] described the reaction of the carboxy anion of an amino acid with a phosphoramidate. This leads through an assumed pentavalent intermediate to the formation of a peptide bond, after intramolecular acyl transfer reaction from phosphorus to the nitrogen atom of the phosphoramidate. The synthesis of *N*-acylphthalimides from *N*-phosphorylphthalimides[16] may follow the same pathway as the one that we propose. Interesting uses of mixed anhydrides of amino acids and phosphoric acid derivatives in peptide synthesis have been investigated by Ramage.[17]



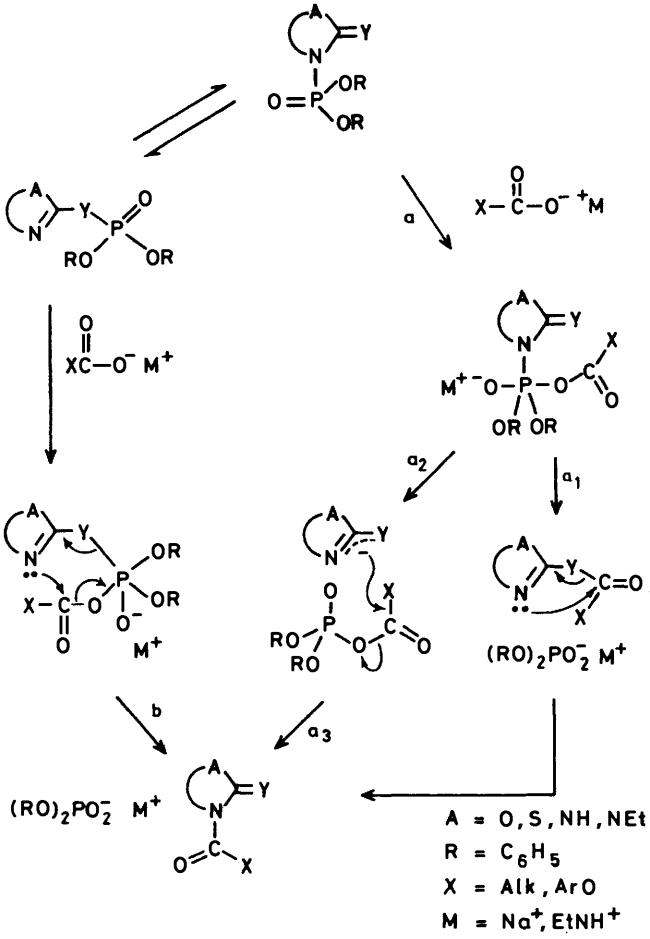
Scheme 4.

The extension to biotin of the results decribed here for model reactions (*N*-acylation under very mild conditions of *N*-phosphorylated derivatives) would imply the formation of 1′-*N*-carboxybiotin, from 1′-*N*-phosphobiotin, either by a stepwise mechanism (a_2 and a_3 in Scheme 5) as for the model reactions, or by concerted one (step *a*, Scheme 6). An important difference between the reaction with substrates (**3**) and (**4**) on the one hand, and the enzymatic reaction on the other, relies on the different leaving-group abilities for a stepwise mechanism between the benzoxazolin-2-one anion and the anion of biotin respectively. Experiments with (**2**), where no acylation is observed, show that leaving-group departure is critical for acylation to occur in the model reaction. Therefore, the equivalent enzymatic carboxylation would require either protonation of the biotin oxygen by an acid of the enzyme in the case of a stepwise process, or a concerted reaction. This question has been recently discussed by Knowles[18] on the basis of results obtained in the enzymatic carboxylation of pyruvate by pyruvate carboxylase, a biotin-mediated reaction, in the presence of chiral ATP at the γ position. The observed inversion at phosphorus is rationalised in terms of either a carboxyphosphate intermediate formed by direct reaction of ATP with the carbonate, or by a concerted mechanism of the *O*-phosphobiotin type (Scheme 2), but not by a stepwise mechanism as suggested by model reactions. If these conclusions can be extended to all biotin carboxylases, they would imply a concerted process for the *N*-phosphobiotin route, with carboxylation at oxygen followed an O ⟶ N carboxyl group migration to produce 1′-*N*-carboxybiotin (Scheme 6). According to Baldwin's rules[19] these rearrangements are unfavourable but not forbidden; for instance in the case of the S ⟶ N acyl migrations mentioned above,[7,11] such reactions are first order and therefore intramolecular, and proceed at an appreciable rate, even though they are 4 000 times slower than those for the equivalent isothioureas. Another example where these rules are not followed has recently been given.[20] In our case it is not a true 4-*endo-trig* rearrangement (C=O *exo*); the strain in the four-membered-ring intermediate is reduced because of the sulphur atom. It follows that route *a* of Scheme 6 represents a possible pathway for formation of carboxybiotin compatible with both the *N*-acylation mechanism described in this work, and also with the inversion stereochemistry at phosphorus observed by Knowles.

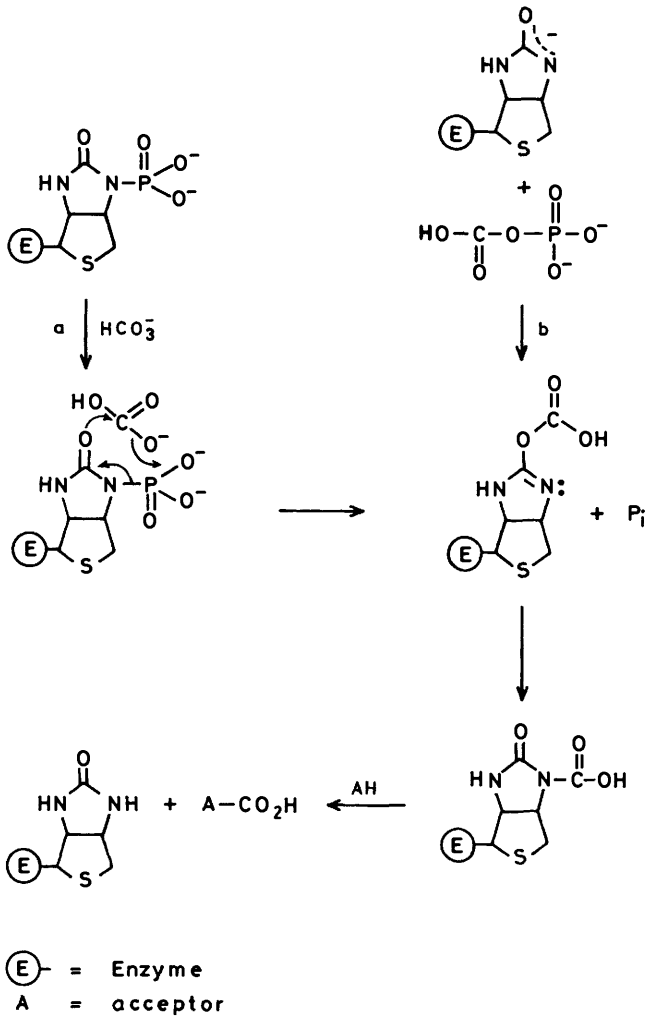
Our last point is concerned with the requirement of the additional step, initial formation of *N*-phosphobiotin compared with the direct formation of carboxyphosphate from ATP and carbonate. A tentative proposal is suggested: carboxyphosphate, being a short-lived species,[21] cannot accumulate in living cells; by contrast, *N*-phosphobiotin, like other *N*-phosphoureas, is a stable enough compound, and therefore is able to intervene in the regulation of carboxylation reactions.

Experimental

I.r. spectra were recorded on a Beckman IR 20 A instrument, ^1H and ^{31}P n.m.r. at 90 MHz on a Fourier transform Bruker instrument (for phosphorus, pulse 3.5 μs, time delay 15 s, acquisition time 0.33 s; chemical shifts are relative to 85% H_3PO_4, with positive values to low field of the reference). Spectra were decoupled with hydrogen and locked on deu-



Scheme 5.



Scheme 6.

terium. Mass spectra were obtained with a Varian-Mat 311 A instrument. Imidazolin-2-one, benzoxazole, 2-mercaptobenzimidazole, triethyl phosphite, and diphenyl chlorophosphate were from Aldrich. The synthesis of (**1**) was described earlier.[10]

Synthesis of 1-*Diethylphosphoroimidazolidone* (**2**).—This was formed in 72% yield by reaction of triethyl phosphite in acetonitrile at 0 °C with *N*-chloroimidazolidone.[22] The latter was obtained by adding chlorine to an aqueous solution of imidazolidone containing 1 equiv. zinc oxide. Bubbling was stopped when the solution became limpid. The chlorourea was extracted with dichloromethane and the solution dried ($MgSO_4$). Elimination of the solvent gave a powder which must be used immediately (explosive hazards on storage even at 0 °C). The product (**2**) was obtained by reaction of triethyl phosphite for 4 h. The oil obtained after evaporation of acetonitrile was purified by precipitation three times from dichloromethane–light petroleum (1:1), δ_p −3.55 p.p.m.; *m/z* 222 (M^+), 180, 179 (100%), 166, 164, 137, 124, and 85.

Synthesis of 3-*Diphenylphosphorobenzisoxazolin*-2-*one* (**3**) *and* 1,3-*Bisdiphenylphosphorobenzimidazole*-2-*thione* (**4**).—These were obtained by reaction, at room temperature in dichloromethane, of diphenyl chlorophosphate with the corresponding carbamate, or thiourea, in the presence of 1 equiv. triethylamine. The progress of the reaction was followed by ^{31}P n.m.r. After completion, the chlorohydrate was eliminated by filtration, the solvent was evaporated, and the residue was crystallised from ether–pentane (3:7) for (**3**), ether–dichloromethane (1:4) for (**4**). Compound (**3**) was formed in 98% yield, m.p. 62 °C; *m/z* 367 (M^+), 323, 135 (100%), 106, 91, and 79; δ_p (0.3M; CH_2Cl_2) −17.3 p.p.m.; $\nu_{max.}$ (2% KBr) 1 810 and 1 800 cm^{-1} (C=O) (Found: C, 61.2; H, 3.8; N, 3.8; P, 8.3. Calc. for $C_{19}H_{14}NOS$: C, 62.1; H, 3.8; N, 3.8; P, 8.45%). Compound (**4**) was formed in 90% yield, m.p. 131—132 °C; δ_p (0.3M; CH_2Cl_2) −16.1 p.p.m. (Found: C, 60.0; H, 4.0; N, 4.8; S, 5.4; P, 9.9. Calc. for $C_{31}H_{24}N_2O_6P_2S$: C, 60.6; H, 3.9; N, 4.6; S, 5.2; P, 10.1%).

Acylated Products (**5**), (**6**), *and* (**6′**) (R = Ph).—To a solution of 1 equiv. substrate (**3**) and (**4**) and 2 equiv. triethylamine in acetonitrile, was added at room temperature 1 equiv. benzoic acid. The reaction was completed after a few minutes, and the product was obtained by evaporation of solvent and filtration on a silica gel column (Kieselgel 60; Merck) using dichloromethane as eluant. Compound (**5**; R = Ph) was formed in 85% yield, m.p. 179 °C (lit.,[23] 170 °C); *m/z* 239 (M^+), 135, 105 (100%), and 77; $\nu_{max.}$ (2% KBr) 1 805—1 820 and 1 695 cm^{-1} (Found: C, 70.2; H, 3.8; N, 5.55. Calc. for $C_{14}H_9NO_3$: C, 70.3; H, 3.8; N, 5.9%). Compound (**6**; R = Ph) was formed in 51% yield, m.p. 203 °C (lit.,[21] 187—190 °C); *m/z* 358 (M^+) 254, 231, 222, 105 (100%), and 77; $\nu_{max.}$ (2% KBr) 1 705 (C=O) and 730 (C=S) cm^{-1} (Found: C, 69.4; H, 4.0; N, 7.8; S, 8.7. Calc. for $C_{21}H_{14}N_2O_2S$: C, 70.4; H, 3.9; N, 7.8; S, 8.9%). Compound (**6′**; R = Ph) was formed in 30% yield, m.p. 220 °C (lit.,[24] 192—

195 °C); *m/z* 254 (M^+), 222, 150, 118 (100%), 105, and 91; $\nu_{max.}$ (2% KBr) 3 100—3 120 (NH) and 1 710 (C=O) cm^{-1} (Found: C, 64.8; H, 4.0; N, 10.8; S, 12.6. Calc. for $C_{14}H_{10}N_2OS$: C, 66.1; H, 3.8; N, 11.0; S, 12.6%).

Low-temperature N.m.r. Experiments.—Samples were prepared as follows. To a solution containing (**3**) or (**4**) (0.6 mmol) and triethylamine (respectively, 0.6 or 1.2 mmol) in dry acetonitrile (1 ml) was added at −20 °C a second solution of benzoic acid (0.6 or 1.2 mmol) in acetonitrile (1 ml). The progress of reactions was followed by ^{31}P n.m.r. at −12 °C. Results are in the Figure, the formation of (**7**) confirmed by characterisation of product (**6′**) obtained after hydrolysis of the low-temperature solution, extraction with dichloromethane, and comparison of t.l.c. results with an authentic sample of (**6′**) (silica gel plate, eluant 1:1 dichloromethane–pentane, R_F 0.68). N.m.r. experiments with *p*-nitro- and *p*-methoxy-benzoic acids were made under the same conditions.

Intermediate Diphenyl Benzoyloxyphosphate.—This was prepared by adding at 20 °C a solution of diphenyl chlorophosphate (0.6 mmol) in acetonitrile (1 ml) to a solution containing benzoic acid (0.6 mmol) and triethylamine 10.6 mmol) in acetonitrile (1 ml). ^{31}P N.m.r. showed a unique peak at δ −19.3 p.p.m. Addition to this solution of a mixture of benzoxazolone (0.6 mmol) and triethylamine (0.6 mmol) in acetonitrile led to the immediate disappearance of the peak at δ −19.3 p.p.m. and the appearance of a peak at δ −12 p.p.m. corresponding to the diphenylphosphate anion. The acylated product was identified by comparing its m.p. and i.r. spectrum with those of an authentic sample.

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