SUMMARY

A procedure has been described for the spectrophotometric determination of the zinc content of various lots of insulin. This technique for quality control involves differential demasking as a means of precluding the effect of contaminating trace metals such as copper and iron. The process is simple, accurate, and capable of rapid analysis for large numbers of samples. It should be useful in the pharmaceutical industry for this important determination.

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Communications

Nonclassical Antimetabolites XVI. The Bridge Principle of Specificity with Active Site-Directed Irreversible Inhibitors, VII. Detection of Differences in Specificity of Enzymic Nucleophilic Sites by the Maleamyl Group

Sir:

The concept of (1) a new class of irreversible inhibitors that operate by active site-direction (exo-alkylation)[1] has been supported by strong experimental evidence (2); a properly designed inhibitor such as 4-(iodoacetamido)salicylic acid (I) can reversibly complex with the active site of an enzyme such as GDH,[2] then become irreversibly bound to the enzyme adjacent to the complexing site by an anchimerically assisted type of alkylation. Four other laboratories have subsequently and independently made related observations pertinent to this type of irreversible inhibition in the field of esterases (3–5) and in the field of immunochemistry (6). Our detailed version of the experimental evidence for active site-directed irreversible inhibition (7) led to the proposal of the bridge hypothesis of specificity:

Compared to a reversible inhibitor, the active site-directed type of irreversible inhibitor can have an extra dimension of specificity; this extra specificity is dependent upon the ability of the reversibly-bound inhibitor to bridge to and covalently link to a nucleophilic group on the enzyme surface and upon the nucleophilicity of the enzymic group being attacked.

Experimental evidence for the first corollary of the bridge hypothesis of specificity, namely, the difference in ability of certain reversibly bound inhibitors to bridge to and alkylate on enzymic nucleophilic site has been presented (8, 9). More recently experimental evidence for the second corollary, the difference in nucleophilicity of the enzymic groups being covalently linked, was demonstrated with compounds related to 5-(carbophenoxyamino)salicylic acid, whereby GDH was irreversibly inhibited, but LDH was not (10). In fact, the substrate-

TABLE I.—IRREVERSIBLE INHIBITION OF LDH BY 4-(MALEAMYL)SALICYLIC ACID (II)

Compound	I_{50}[c,d] GDH	I_{50}[c,d] LDH	Rate of Inactivation[f] GDH	Rate of Inactivation[f] LDH
I	3.4[e]	6.6[e]	1.0[g]	1.0[g]
II[a]	17[i]	7.3[i]	0[h]	0.9
III[b]	...	32[i]	...	0[h]

[a] Prepared in 96% yield from maleic anhydride and 4-aminosalicylic acid in boiling methyl ethyl ketone; light yellow crystals, m. p. 195–197° dec.; *Anal.*—Calcd. for $C_{11}H_9NO_6$: C, 52.6; H, 3.58; N, 5.57. Found: C, 52.7; H, 3.75; N, 5.68. [b] *Org. Syn.*, 41, 93(1961). [c] The I_{50} is defined as the concentration of inhibitor necessary to give 50% reversible inhibition in the presence of 1 millimolar of substrate. [d] 1 Millimolar α-oxoglutarate to L-glutamate or 1 millimolar pyruvate to L-lactate as previously described (9, 13). [e] From reference 9. [f] Rate of inactivation by 2 m*M* concentration of inhibitor was determined as previously described (7, 12). [g] Arbitrary value for comparison down the column; the absolute rates are different for the two enzymes (7). [h] Showed protection against thermal inactivation of the enzyme in some runs. [i] Estimated from the amount of inhibition obtained up to a 3 m*M* solution, the maximum concentration still permitting full light transmission. *i* Estimated from the amount of inhibition obtained up to a 16 m*M* solution, the maximum concentration permitting full light transmission.

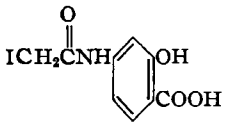
[1] The term "active site-directed irreversible inhibition" is preferable to the term "exo-alkylation" used in previous papers since, first, the term is more self-explanatory and, second, some of the irreversible inhibitors operate by acylation or Michael addition and not by alkylation.

[2] Abbreviations: LDH, lactic dehydrogenase from rabbit skeletal muscle; GDH, glutamic dehydrogenase from mammalian liver.

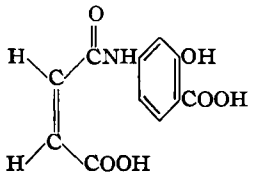
identical enzyme, LDH, from two different tissues could be irreversibly inhibited selectively by use of both corollaries (11, 12).

The fact that 4-(iodoacetamido)salicylic acid (I) could irreversibly inhibit both GDH and LDH was attributed to the low functional specificity of the iodoacetamido group. In contrast, 5-(carbophenoxyamino)salicylic acid irreversibly inhibited GDH, but not LDH; this selectivity was attributed to the specificity of the carbophenoxy group for reaction with a primary amino group which apparently was properly juxtapositioned in GDH to the carbophenoxy function for reaction, but was not in LDH. This paper describes a cross-over of functional specificity with 4-(maleamyl)salicylic acid (II).

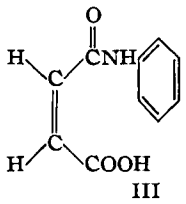
In Table I is listed a comparison[3] of both reversible and irreversible inhibition of GDH



I



II



III

and LDH by compounds I–III. Although 4-(maleamyl)salicylic acid (II) could reversibly bind to both enzymes, *only LDH was irreversibly* inhibited. Maleanilic acid (III) showed relatively weak reversible binding to LDH,[4] *but showed no irreversible inhibition.* The irreversible inhibition of LDH shown by II and not III is strong evidence that a properly oriented complex with the active site is an obligatory intermediate for irreversible inhibition; if II had inactivated LDH by random bimolecular reaction, then maleanilic acid (III) should have inactivated LDH even more rapidly than II, since II would protect the active site against bimolecular reaction (12). Bimolecular inactivation of enzymes by N-ethylmaleimide is a well known phenomenon, and maleanic acid also inactivates enzymes but at a much slower rate (14); since III does not inactivate LDH, the inactivation of LDH by II definitely operates by a different mechanism, presumably by active site-directed irreversible inhibition.

The α,β-unsaturated carbonyl system of II, maleimides, and maleamic acids react in general most rapidly with SH groups, much slower with amino groups, and extremely slowly with hydroxyl groups. Thus, it is probable that LDH has a properly juxtapositioned SH group within the LDH–II complex that allows for an anchimerically assisted Michael addition reaction, whereas GDH does not; in contrast, GDH is irreversibly inhibited by active site-directed acylation with the amine reagent 5-(carbophenoxyamino)salicylic acid, but LDH is not irreversibly inhibited by this reagent (10).

Further search for groups on a reversible inhibitor that can specifically bridge to and covalently link other enzymic functional groups is continuing; such group specific active site-directed irreversible inhibitors would be of use in both chemotherapy and protein structure studies.

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[4] III probably binds reversibly *via* the carboxylate in the fashion of benzoate and cinnamate (13), thus giving a conformationally different complex than I and II.