presented in Table II. Identity and purity checks by paper chromatography showed that (except as noted) no ergot alkaloids other than ergotamine or ergotaminine were detected in the products. In some older samples with low assay values, brown spots appeared at $R_f = 0$ on the paper chromatogram of the ergotaminine fraction. These probably represent ergotamine that has oxidized or otherwise deteriorated.

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Determination of Zinc in Insulin Preparations

By BENNIE ZAK and JERRY S. COHEN

A description of an accurate absorptiometric technique for the determination of zinc in insulin is presented. It involves differential demasking of zinc in the presence of contaminating interferences such as copper and iron, followed by a reaction with the color reagent, zincon. It appears to be a simple yet useful process for quality control.

An important analytical phase for the pharmaceutical industry involves quality control of products (1). The determination of zinc in insulin preparations for quality control purposes is an assay which presents some difficulty. Included among the procedural techniques for this element are processes employing spectrophotometry (2, 3), polarography (4, 5), and chelatometric titrations (6, 7). The first two types of procedure involve selective dithizone extractions followed by colorimetric measurement and retrograde extraction followed by polarographic measurement. The titrimetric procedure with a complexone titrant becomes more difficult and subjective as the concentration of zinc decreases. One milliliter of 0.001 *N* ethylenediamine tetraacetic acid corresponds to 65.4 mcg. of zinc (8); some insulin preparations, such as the unmodified variety, contain only 6 mcg. of zinc per ml. A visual titration involving a two-color indicator, such as Eriochrome black T, is subject to serious shortcomings when the titrant becomes very dilute, and the indicator color-change represents a slow transition from one form to the other (9).

The procedure for insulin zinc analyses to be discussed here involves an absorptiometric technique without extraction. It is capable of achieving an accurate determination for zinc yielding high absorbance data on samples which are never greater than 1 ml., even for those preparations containing the smallest amounts of zinc (10, 11). In the case of zinc-insulin solutions, the required amount of material is a micro quantity.

EXPERIMENTAL

Reagents.—Borate buffer, pH 9.0: dissolve 31 Gm. of boric acid in metal-free distilled water, add 53 ml. of 4 *N* NaOH, and dilute the solution to a liter with any necessary adjustment of pH. Zincon color reagent: dissolve 130 mg. of zincon in 2 ml. of the detergent, acationox, and dilute the solution to 100 ml. with metal-free distilled water. Stock standard zinc solution (100 mg./L.): dissolve 100 mg. of zinc in a minimum amount of HCl and dilute the solution to a liter with metal-free distilled water. This stock is diluted with water so that 0–18 mcg. per aliquot was used as a working standard.

Procedure.—An aliquot containing approximately 6–18 mcg. of zinc was pipeted into a centrifuge tube and diluted to 3.0 ml. with metal-free distilled water. This aliquot ranged from 0.05 ml. for an 80-unit globin-zinc-insulin preparation to 1.0 ml. for a 40-unit unmodified insulin preparation. To the diluted insulin solution, 1.5 ml. of 1 *N* HCl was added to liberate the zinc from its protein binding site. Ten per cent trichloracetic acid (1.5 ml.) was then mixed in to precipitate the proteins. Trichloracetic acid may be capable of performing both functions, but the stronger acid ensures that the zinc will become unbound. After a 5-minute stand, the mixture was centrifuged, and a 4.0-ml. aliquot was transferred to a Coleman 19 mm. O.D.

Table I.—Precision of Recovery of Absolute Quantities of Zinc

3.0 Mcg.	5.0 Mcg.	7.0 Mcg.	9.0 Mcg.	12.0 Mcg.
3.0	4.9	6.8	8.9	12.3
3.0	5.0	6.8	8.8	11.9
2.9	4.8	6.9	9.0	11.9
2.9	5.0	6.9	9.0	12.3
3.1	5.0	7.1	9.0	12.3
3.1	4.9	6.8	8.9	12.3
3.0	5.0	6.9	9.2	11.9
3.1	4.9	7.1	8.8	12.0
3.0	5.0	7.0	9.0	11.9
3.1	4.8	6.9	9.2	12.0

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cell. A quantity of 4.0 *N* NaOH was added to neutralize the acidity so the capacity of the buffer would not be exceeded (0.4 ml. was used in the described procedure). One milliliter of pH 9 buffer was added followed by 0.1 ml. of 3% NaCN solution to form complexes with the zinc and any contaminating copper and iron present in the sample (12) or in the reagents used. Zincon color reagent (0.6 ml.) was then added. Because the cyano complexes are too tightly bound to release the metals for reaction with zincon, each tube can be used as its own blank. The instrument was zeroed at 630 mμ with the tube, then 0.2 ml. of 60% chloral hydrate solution was quickly mixed into each tube of standard or sample to demask the zinc differentially. Within 1 minute after the final addition, the absorbance of each tube was obtained again at 630 mμ. Since the zinc cyanide complex breaks down first, contaminating trace metals do not interfere in the process.

DISCUSSION AND RESULTS

The self-blanking described under procedure was quite convenient and yielded accurate results. Slight variations in different blanks were not significant because the absorbance difference before and after demasking could be obtained precisely. Background color characteristics of samples did not appear to affect the answers and this was decidedly advantageous. Copper and iron are ubiquitous trace contaminants of reagents. It was felt therefore that masking them in a system wherein demasking occurred much later than for the desired zinc would ensure accurate determinations.

A precision and accuracy study was carried out using replication on several absolute concentrations of zinc. The findings are shown in Table I. The values obtained were quantitatively in accord with those values known to be present in every instance. Conclusions concerning the precision as well as the accuracy of the determinations were therefore obvious in this experiment.

Twenty lots of different types of insulin unknowns were analyzed for their zinc content by the described procedure. The preparations varied from approximately 6 mcg. of zinc per ml. to 240 mcg. per ml. The results of this blind spectrophotometric study were compared to an ethylenediamine tetraacetate titration technique carried out in another laboratory where neither laboratory knew the results obtained by the other in advance. The comparative values are shown in Table II. The values obtained for the spectrophotometric procedure compare well enough to the EDTA titration since visual titrations of low concentration constituents with two-color indicators such as the Eriochrome black T used here are difficult (13) even if screening is employed as a visual aid (14).

Different quantities of zinc were added to aliquots of each of several lots of insulin. Zinc determinations were carried out on the insulins where a prior analysis of the materials established the base values. Because the zinc contents for different types of insulin are of such divergent composition (6–240 mcg./ml.), it was necessary to vary the size of the starting sample to conform to the best spectrophotometric characteristics of the system. The values found for base plus addition were quantitative and these results make up Table III.

TABLE II.—BLIND COMPARISON OF TWO PROCEDURES FOR ZINC BY TWO LABORATORIES

Lot	Lab A[a]	Lab B[b]	Lot	Lab A[a]	Lab B[b]
1	6	6	11	10	9
2	13	12	12	11	9
3	6	6	13	22	18
4	7	6	14	23	18
5	8	6	15	21	18
6	14	12	16	126	120
7	15	12	17	247	240
8	11	9	18	122	120
9	22	18	19	247	240
10	11	9	20	247	240

[a] Described procedure. [b] Chelatometric titration, courtesy of E. R. Squibb and Sons, Technical Services Department.

TABLE III.—RECOVERY OF VARIOUS ADDITIONS OF ZINC TO INSULIN

Type of Insulin	Sample Size, ml.	Present, mcg./ml.	Added	Total Present	Total Found
Unmodified	0.50	13.5	3.0	9.8	9.4
Unmodified	0.50	13.5	3.0	9.8	9.4
Unmodified	0.50	13.5	6.0	12.8	12.8
Unmodified	0.50	13.5	6.0	12.8	12.5
Unmodified	0.50	13.5	9.0	15.8	16.0
Unmodified	0.50	13.5	9.0	15.8	15.4
NPH	0.10	23.3	3.0	5.3	5.1
NPH	0.10	23.3	6.0	8.3	8.1
NPH	0.10	23.3	9.0	11.3	10.8
NPH	0.25	23.3	3.0	8.8	8.4
NPH	0.25	23.3	6.0	11.8	11.6
NPH	0.25	23.3	9.0	14.8	13.8
Globin	0.05	122	3.0	9.1	9.4
Globuin	0.05	122	3.0	9.1	9.7
Globuin	0.05	122	6.0	12.1	12.4
Globin	0.05	122	6.0	12.1	12.2
Globin	0.05	122	9.0	15.1	15.4
Globin	0.05	122	9.0	15.1	15.4

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-

[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.

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

	I_{50}[c,d]		Rate of Inactivation[f]	
Compound	GDH	LDH	GDH	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[j]	...	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. [j] Estimated from the amount of inhibition obtained up to a 16 m*M* solution, the maximum concentration permitting full light transmission.