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THE DEVELOPMENT OF A CLINICAL REFERENCE METHOD FOR GLUCOSE IN SERUM†

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Abstract—The progress achieved as of July 1975 on the development of a clinical reference method for glucose is presented; this effort originated as a subcommittee function of the American Association of Clinical Chemists. The hexokinase glucose-6-phosphate dehydrogenase method for determining glucose was selected as the candidate clinical reference method. Its use in all laboratories performing the round-robin tests has provided high precision values. Its accuracy is being evaluated against the results of isotope dilution-mass spectrometry (ID–MS) as the definitive method for glucose. Work as yet incomplete shows some erratic differences between the ID–MS and the candidate clinical method. More is to be done with ID–MS to ensure the validity of its values before further efforts on the clinical reference method are undertaken.

1. INTRODUCTION

The development of a reference method for glucose in serum originated as an activity of the American Association of Clinical Chemists (AACC) when its Standards Committee, under Dr. George N. Bowers, Jr., asked the writer to organize a subcommittee to undertake that task, patterned on the prior development of the reference method for total calcium in serum.[1] For the glucose method subcommittee, a broad representation of the AACC membership was sought; however, members were chosen primatily because of their interest in participating in the work. The committee was finally composed of clinical chemists from industrial firms in the clinical chemistry field, from governmental laboratories that had specialized experience and resources, and from hospital laboratories. Service as a member of the subcommittee meant an agreement to participate in the round-robin (i.e. interlaboratory) testing of the selected candidate method and to help in all stages of progress with the subcommittee's task. The names of the original subcommittee members and of others added later are shown in Table 1. In starting the development of the glucose reference method, the subcommittee had to consider several problems. One was the goal for accuracy required on use of the reference method. With a recognized goal, once that accuracy was achieved by round-robin tests of the candidate reference method, it would follow that the subcommittee's experimental efforts in establishing the reference method work would be finished. The subcommittee needed also to select a definitive method‡ and the candidate clinical reference method. The answers to none of these requirements were immediately obvious.

Before proceeding further, the reader should recognize that this presentation is in the nature of a progress report of the subcommittee's work. Some of the results discussed here may be treated differently by the time the entire work is evaluated and considered complete.

2. INITIAL WORK

In selecting the candidate clinical reference method, the subcommittee was restricted to those (a) that could be performed manually, (b) for which quality reagents would be available and (c) in which performance specifications for analytical equipment could be described. The method needed to be sensitive, to exhibit high specificity, and to afford optimum precision and a high probability of accuracy. Whereas the clinical reference method would necessarily have to be a method that could be carried out at least in specialized, if not in many, clinical laboratories, the definitive method had no such restriction. The only consideration was the potential for accuracy that would be obtained by use of the definitive method, which meant that systematic and random errors in this method would need to be sufficiently low that their net effect would be insignificant with respect to the requirement for the accuracy of the results it would provide.

Table 1

Dr. R. J. L. Bondar	Worthington Biochemical Corp., NJ
Dr. C. Fasce	City of Kingston Laboratory, NY
Dr. N. Gochman	Veterans Administration Hospital, San Diego, CA
Dr. G. Kessler	Jewish Hospital of St. Louis, MO
Dr. R. B. McComb	Hartford Hospital, CT
Dr. J. W. Neese	Center for Disease Control, GA
Mr. W. T. Ryan	Beckman Instruments, CA
Dr. R. E. Vanderlinde	NY State Department of Health, NY
Dr. R. Schaffer (Chairman)	National Bureau of Standards, DC
Dr. C. A. Burtis	Oak Ridge National Laboratory, TN
Dr. P. Schroff	Warner Lambert Research Institute, NJ

The subcommittee was organized in the latter part of 1972. Its initial actions were to encourage the study of an isotope dilution–mass spectrometry(ID–MS) approach for obtaining definitive analyses for glucose at the U.S. National Bureau of Standards (NBS), Washington, D.C., and because the subcommittee recognized the need for additional information on the hexokinase glucose-6-phosphate dehydrogenase method before selecting a candidate clinical method from among the possible candidates, it encouraged study of that method in several subcommittee members' laboratories. The subcommittee specifically urged that a thorough study of the reaction parameters for the method and of interferences with the method, and a comparison of the method with other major methods for determining glucose in serum be carried out at the U.S. Center for Disease Control (CDC), Atlanta, Georgia. Although these studies were to fulfill the

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‡See previous paper by Cali (*Pure Appl. Chem.* **45**(2), 63–68 (1976) for definition of "definitive method."

information needs of the subcommittee, subcommittee members were asked to pursue these studies for publication as scientific contributions from their own laboratories. Two of these studies have now been published.[2,3] Two others have been presented[4,5] as papers but have not yet appeared as publications.

These studies were presented to the subcommittee before they were submitted for publication. From these studies, in particular the evaluation performed at the CDC, it was recommended to the subcommittee that the hexokinase glucose-6-phosphate dehydrogenase method be adopted as the candidate reference method for glucose in serum. The precision of the results obtained by the method, the ruggedness of the method, the relative freedom from interferences, and the comparisons of the results on patient samples with those by other methods were the major considerations that led the subcommittee to accept the recommendation and to undertake its evaluation as the clinical reference method.

The method[6] entails the conversion of glucose initially into glucose-6-phosphate using yeast hexokinase, adenosine triphosphate, and magnesium, and the subsequent oxidation of the glucose phosphate to 6-phosphogluconate with glucose-6-phosphate dehydrogenase and nicotinamide adenine dinucleotide. The reduced form of nicotinamide adenine dinucleotide produced by the second enzyme catalyzed reaction is then measured. The protocol which was developed at the CDC requires use of deproteinized serum and employs aqueous solutions of SRM glucose† as the standard. The enzymatic processes, carried out on these aqueous standard solutions, are tested to insure the linearity of the standardization. Although the candidate clinical reference method has not as yet been published in a clinical journal, it has in fact been printed in the Federal Register, Volume 39. No. 126, Part III, issued 28 June 1974 in Washington, D.C. as part of a Food and Drug Administration proposal for the "Establishment of a Product Class Standard for Detection or Measurement of Glucose." The protocol developed at the CDC for performing the candidate clinical reference method is detailed in that document. Only the scheme for pre-testing the necessary enzyme reagents and the specifications for the automatic pipettor-dilutor that may be used in one alternative of the protocol are not included. The round-robin tests that were carried out by the subcommittee followed that protocol.

Work on ID–MS as the definitive method for glucose was begun at NBS, but, at the time when the subcommittee was preparing plans for round-robin testing of the protocol for the clinical reference method, an estimation of the potential of this approach as the definitive method could not be made nor was the method ready for use. Hence, the first and second round-robin tests that were run could only examine the within-laboratory and the between-laboratory precision of the candidate clinical method.

A subcommittee consensus on the accuracy goal required of the clinical reference method was not reached during initial considerations of this subject. Given the unavailability of definitive measurements, the decision on the accuracy goal could be deferred until the time when the definitive method would be in use and afford the neans for estimating accuracy.

†D-Glucose, Standard Reference Material 917 supplied by the National Bureau of Standards, Washington, D.C.

3. CANDIDATE REFERENCE METHOD RESULTS

Round-robin testing of the candidate clinical method was begun without awaiting the definitive measurements on the specimens to be studied. The test specimens (some serum, some aqueous) were made available by the CDC. Also all of the reagents needed for the first round-robin were prepared at the CDC and sent to the individual subcommittee members for use in performing the analyses. Thus for the first round robin it was necessary only for participating laboratories to prepare their own standard solutions using the NBS–SRM glucose, carry out the testing according to the protocol, and report their results. The data collected from all of the laboratories on five tested specimens are presented in Table 2.

The first round-robin test utilized only manual pipetting of specimens and reagents, but for the second round-robin, the alternative protocol, which utilizes an automatic pipettor-dilutor, was also to be performed in subcommittee members' laboratories that were suitably equipped. The specifications for the accuracy and precision requirements of the pipettor-dilutor that might be used were prepared at the CDC. In the second round-robin, each laboratory was required to prepare its protein precipitation reagents, but the CDC continued to prepare and provide the enzyme reagents since at that time the specifications for pretesting of enzymes was not yet completed. The results of the second round-robin are summarized in Table 3.

In the third round-robin, the results of which are shown in Table 4, the participating laboratories prepared and tested their own enzyme reagents (from enzymes and coenzymes that each laboratory separately obtained from commercial suppliers). Again the protein precipitation reagents were the responsibility of the individual laboratory. Both the manual and automatic pipettor-dilutor versions of the protocol were carried out. It must be noted that not all the laboratory round-robin results were available when the data shown in Table 4 were compiled for this report.

Table 2

Specimen number	Observed mean (mg/dl)	Coefficient of variation between lab (1 detn/lab)
1	42·1	6·2
2	79·8	3·3
3	108·0	2·4
4	208·1	1·7
5	567·8	2·0

Table 3

		Manual pipetting		
		Coefficient of variation (%)		
Specimen number	Observed mean (mg/dl)	Within lab (1 detn)	Between lab (1 detn/lab)	Between lab (4 detn/lab)
1	40·4	1·5	5·7	5·6
2	75·4	1·1	2·7	2·5
3	134·3	1·9	2·1	1·3
4	205·9	1·4	1·7	1·1
5	450·0	0·6	1·3	1·2
		Semi-automated pipetting		
1	40·8	1·9	3·6	3·2
2	76·0	1·0	1·9	1·7
3	133·9	1·1	1·4	1·0
4	204·1	0·7	1·0	0·9
5	450·3	0·5	0·9	0·8

Table 4

Specimen number	Observed mean (mg/dl)	Coefficient of variation (%) Within lab (1 detn)	Between lab (1 detn/lab)	Between lab (4 detn/lab)
		Manual pipetting		
1	41·7	4·3	5·8	4·4
2	79·8	2·9	3·4	2·3
3	134·7	1·2	1·6	1·2
4	195·7	1·2	1·7	1·3
5	294·9	1·3	1·4	0·8
		Semi-automated pipetting		
1	42·0	3·3	3·3	1·6
2	79·1	1·1	1·1	0·6
3	134·2	1·1	1·3	0·9
4	193·8	0·6	0·9	0·8
5	292·6	0·8	1·0	0·7

The data from the three round-robins has shown the subcommittee that the precision obtained by use of the candidate protocol was acceptably high for the requirement of a reference method. Clearly the use of the automatic pipettor-dilutor afforded higher precision than manual pipetting.

4. DEFINITIVE METHOD RESULTS

The analyses for glucose by ID–MS utilizes a derivative of glucose, namely 1,2:5,6-di-*O*-isopropylidene-D-glucofuranose,[7] commonly known as diacetone glucose. The compound will be designated here as DAG. Its chemical structure is shown in Fig. 1. This derivative of glucose is prepared by condensing the sugar with acetone in the presence of a small amount of concentrated sulfuric acid and an excess of the dehydrating agents, calcium sulfate and copper sulfate. The DAG that forms is separated from other reaction material by sublimation and the sublimate is then further purified by preparative thin-layer chromatography. The crystalline DAG obtained is transferred to the mass spectrometer for analysis using a direct sample probe. Figures 2–4 illustrate the mass spectrum of DAG prepared from natural glucose, of

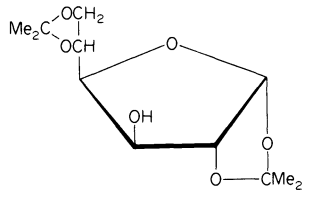

Fig. 1. Structure of 1,2:5,6-di-*O*-isopropylidene-D-glucofuranose.

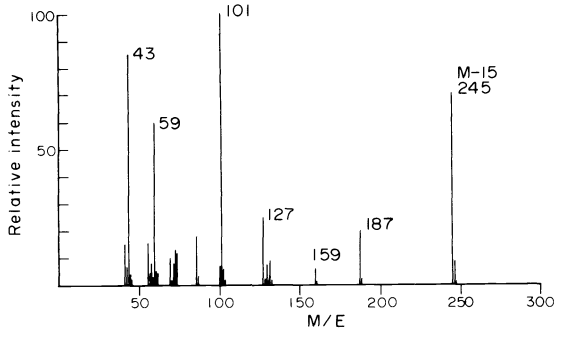

Fig. 2. Computer print-out of the mass spectrum of DAG.

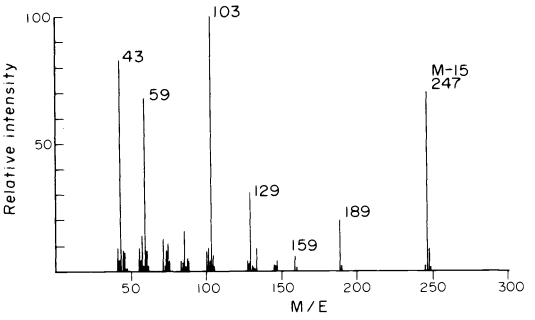

Fig. 3. Computer print-out of mass spectrum of DAGD2.

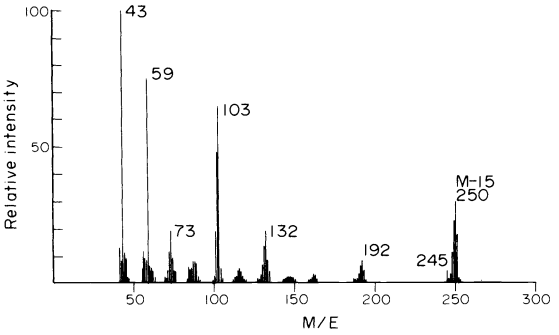

Fig. 4. Computer print-out of DAG13C.

DAGD2 from dideuteroglucose and of DAG13C from C-13-labelled glucose, respectively. DAG has a molecular weight of 260 but the ion corresponding to this parent molecule is not found as a significantly large peak on electron impact–mass spectroscopy (EI–MS). Instead, the highest molecular weight principle ion is found at a mass to charge ratio (m/e) of 245 and it results from the loss of a CH_3 group from the isopropylidene group attached at C5 and C6 of the glucose.[8] DAG was selected as the glucose derivative for this study because of its ease of preparation, volatility, and this simple, well-characterized fragmentation on EI–MS.

Analysis for the isotopic forms of glucose is based on the relative peak heights of this principle ion, i.e. for DAG the peak at m/e 245, for DAGD2 that at m/e 247, and for DAG13C the peak at m/e 250. As may be visualized, with mixtures of DAG and DAGD2 the ratios of the 245 and 247 peaks observed by EI–MS would reflect the relative proportions of the unlabelled and labelled molecules that are present. Likewise mixtures composed of the DAG and DAG13C would provide relative peak heights at m/e 245 and 250 that reflect the relative proportions of the natural and isotope-labelled molecules.

As shown in Fig. 5 for mixtures of DAG and DAGD2, the ratios of the peaks at 245 and 247 linearly reflect the ratios of the natural and labelled forms of glucose in the mixtures which are plotted (although the DAG isotopic molecules are measured). It should be noted that the corrected peak ratios at 245 and 247 are used. Correction of the peak ratios observed is necessary because, as may be seen from the mass spectrum of DAG (Fig. 2), a small peak at m/e 247 is present due to the natural occurrence of stable isotopes of carbon, hydrogen and oxygen in the natural sugar, and in the spectrum of DAGD2 (Fig. 3), there is a small peak present at m/e 245 from unlabelled molecules of DAG. The mixtures, for which the ID–MS data are plotted in Fig. 5, were prepared in 2 ways:

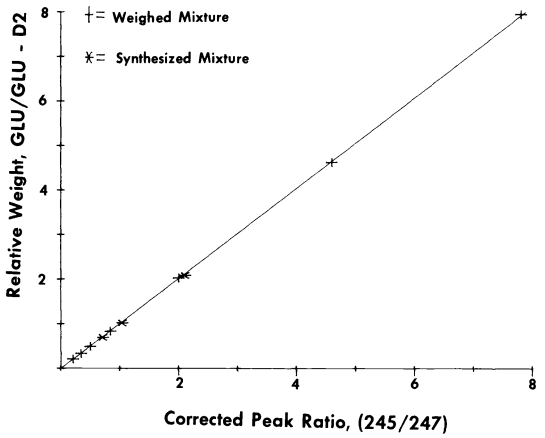

Fig. 5. The linearity of the relationship between the relative weights of natural glucose and dideuteroglucose and the corrected peak height ratios as measured on the diacetone derivatives of the sugars.

different proportions of separately synthesized preparations of DAG and DAGD2 were weighed, combined in solution, and then allowed to crystallize by evaporating the solvent. The ID–MS data from such mixtures are represented in the graph by crosses. Alternatively, mixtures were prepared by weighing different proportions of the natural glucose (SRM glucose) and (highly purified) dideuteroglucose, combining them in solution, and then converting the combination into DAG–DAGD2 mixtures; these are shown on the graph as stars. A similar plot of mixtures containing the natural glucose and C-13-labelled glucose is given in Fig. 6. The measurements as represented in Figs. 5 and 6 reveal linearity over a wide range of proportions of the natural with either of the labelled glucoses. However, if the data obtained with the natural and dideuteroglucose mixtures is examined at closer range as illustrated in Fig. 7, the 2 kinds of mixtures are seen to be measurably different, suggesting the occurrence of an isotope effect and the potential for error in the use of dideuteroglucose for accurate ID–MS analysis. A similar differentiation of the 2 kinds of mixtures is not found in the case of mixtures of natural glucose with C-13-labelled glucose when they are examined similarly. As shown in Fig. 8, no differentiation is apparent from the plot of all the presently available data

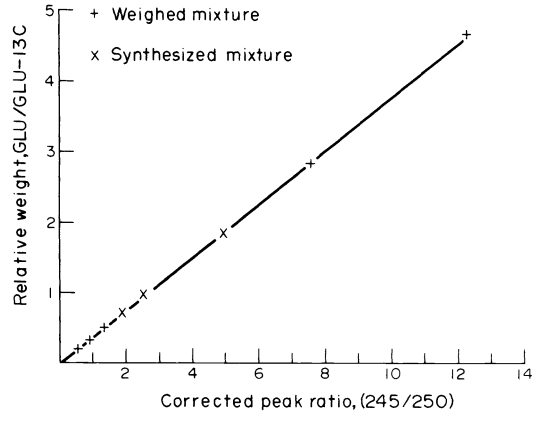

Fig. 6. The linearity of the relationship between the relative weights of natural glucose and uniformly labelled C-13-glucose and the corrected peak height ratios as measured on the diacetone derivatives of the sugars.

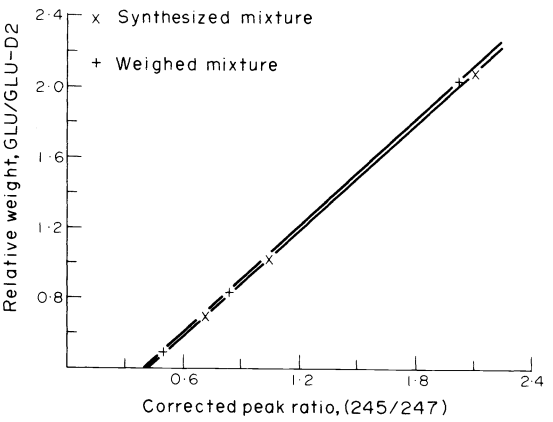

Fig. 7. The measurable differences in the corrected peak height ratios for known mixtures of the natural glucose and dideuteroglucose prepared by different means.

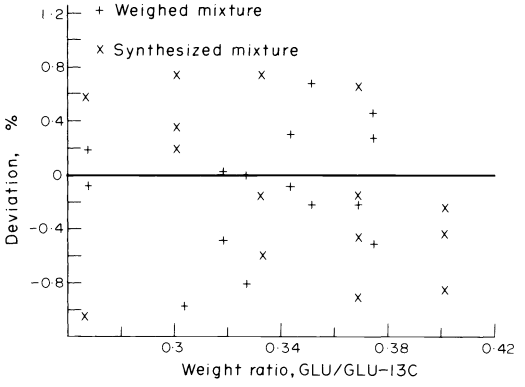

Fig. 8. Data showing the % error in determinations of the weight ratios of glucose and C-13-glucose in known mixtures prepared by 2 different methods.

obtained with a narrow range of proportions of the natural and C-13-labelled glucose (as DAG). The error of individual measurements as plotted in Fig. 8 is within 1% of the expected values (which are known from the method of preparation of the mixtures). The expectation of equivalent accuracy when ID–MS is applied to serum samples is not entirely proved by these measurements. Additional work is needed.

5. COMPARISON OF ID–MS AND THIRD ROUND-ROBIN MEASUREMENTS

ID–MS measurements were made on the serum samples that were studied in the third round-robin. All of the ID–MS data available to the time of the present report is shown in Table 5. Each of the 5 serum samples (labelled as pools A–E) were independently analyzed 3 times; known quantities of the C-13 glucose was added to three, separate, weighed specimens from each pool, and each of these combinations was separately treated to provide DAG for analysis by ID–MS. Note that the data presented in Table 5 are reported as milligrams (of glucose) per gram (of serum).

The averages shown in Table 5 are presented in Table 6 as milligrams (of glucose) per deciliter (of serum) for direct comparison with the overall average results obtained on these specimens by the clinical reference method. A statistical comparison is not given for lack of all of the data. However, a graphic display of this

Table 5

Sample-detn	Pools A	B	C	D	E
1-1	0·4194	0·8156	1·318	1·920	2·913
1-2	0·4114	0·8206	1·316	1·941	2·922
1-3			1·328		
2-1	0·4156	0·8063	1·328	1·927	2·904
2-2	0·4198	0·8094	1·326	1·917	2·917
2-3	0·4126	0·8090	1·337		
3-1	0·4085	0·8219	1·330	1·910	2·934
3-2	0·4093	0·8197	1·333	1·929	2·920
3-3			1·355		
Av.	0·4138	0·8146	1·330	1·924	2·918
s	0·0046	0·0064	0·011	0·011	0·010
C.V. (%)	1·1	0·8	0·8	0·6	0·3

Table 6

ID–MS method	HK/G6PD method Manual	Semi-automated
42·4	41·7	42·0
82·7	79·8	79·1
135·1	134·7	134·2
197·8	195·7	193·8
300·2	294·9	292·6

comparison is presented in Fig. 9 which shows the relationship of the 5-day average values from the individual laboratories as obtained by the manual method and by the automatic pipettor-dilutor method with the average result obtained by ID–MS. The individual laboratory averages are shown as the per cent difference from the ID–MS average. Examination of Fig. 9 shows the high precision of the analyses by the candidate clinical reference method as performed on samples having glucose concentrations at 80 mg/dl or greater. The differences between these values and those by ID–MS should not at present be considered as established. Clearly there is an erratic difference at the 80 mg/dl level that suggests the possibility of entirely different samples having been measured by the two methods. Whether this is the case or not, this difference must be and will be explored and clarified. Again, the growing difference apparent at higher glucose concentrations should not be regarded as proved. These are only the first intercomparisons of the definitive and candidate clinical methods. It should be helpful to know in examining these data, that at a recent meeting of the subcommittee the goal being

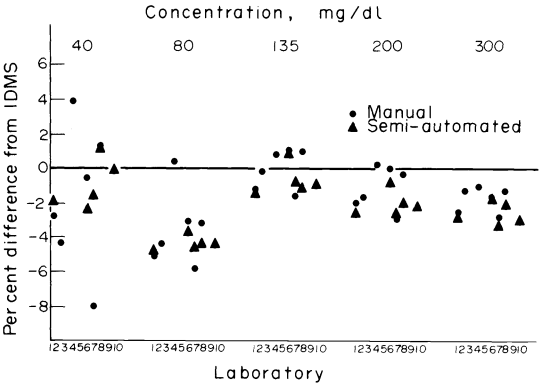

Fig. 9. Graph of individual laboratory 5-day averages plotted as per cent difference from ID–MS average values of 5 concentrations of glucose in serum.

discussed for the accuracy of the clinical reference method was the larger of either ±3% or ± 3 mg/dl of the definitively determined concentration.

6. FUTURE WORK

It should be clear from this presentation, that the subcommittee has not completed its experimental work. It recognizes that additional study of the definitive method is needed, i.e. blanks, recoveries, interferences, additional intercomparisons. The need for additional round-robins is not presently obvious, but will be considered when the source and the extent of the differences (if any) between the definitive and canditate clinical reference methods are better understood.

Acknowledgements—The author considers it special privilege to work with his colleagues on the AACC subcommittee and to discuss the work of the subcommittee. A large number of chemists in the laboratories of subcommittee members have contributed significantly to this study. Their help is gratefully recognized.

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