Delayed Blood Sugar Determinations

—*Evaluation of a Blood Preservative*—

By WALTER M. BOWMAN, Sc.M., and PHILIP E. ENTERLINE, B.B.A.

EACH YEAR, millions of persons in the United States have blood tests for syphilis. If it were possible for an additional blood specimen to be drawn and shipped to a central laboratory at the same time, and there tested for glucose content, a diabetes case-finding program could be operated at little additional expense. Probably the most important technical problem would be preservation of the glucose content of the whole blood until the glucose determination could be made.

It is well known that the glucose content of whole blood disappears when the blood is stored for any period of time. Sunderman and others (*1*) stated "at room temperature glucose disappears from oxalated blood at an approximate rate of 5 percent per hour." A number of studies of various blood preservatives have been reported, often with contradictory results (*2*, *3*, *4*, *5*). Most of these studies indicated that sodium fluoride apparently has desirable properties as a preservative. In the prevention of glycolysis, Baldwin (*6*) showed that sodium fluoride inhibits enolase, the 11th enzyme in enzymic glycolysis, but does not inactivate the other enzymes. The most promising inhibiting agent for all enzymes seemed to be a combination of 1 mg. of thymol with 10 mg. of NaF per milliliter of whole blood, as reported by Sander (*2*). (Sodium fluoride in this amount is a satisfactory anticoagulant.)

The West Virginia State Health Department and the Public Health Service cooperatively tested this preservative to determine its usefulness in a diabetes case-finding program involving a delayed glucose determination. The results are presented in this paper.

At the outset, it was decided to use a method of blood glucose determination which was specific for true glucose. The method had to be highly reproducible and adaptable to testing a large volume of specimens. The Nelson method (*7*) seemed to meet these criteria because it is not as subject to interference from saccharoids as some of the older methods, and stability of color makes it possible to examine large numbers of specimens without danger of fading. Certain modifications of this method were made to increase its reproducibility and adaptability to large numbers.

In measuring changes in the glucose content of blood, the reproducibility of the test itself must be estimated. If reproducibility were poor, it would be difficult to decide whether deviations from some previously observed blood sugar level were due to inability to reproduce test results or due to actual changes in the blood

Mr. Bowman is chief of the microbiological and chemical section of the hygienic laboratory division, West Virginia State Department of Health. Mr. Enterline is assistant chief of the Program Analysis Section, Division of Chronic Disease and Tuberculosis, Public Health Service.

sugar level. Test reproducibility, as it applies to the experiments described here, in the main, is affected by two types of error: (*a*) the inherent error of the test itself, that is, the differences in glucose values which are observed in simultaneous replications on the same blood sample; and (*b*) the day-to-day error of the test, such as day-to-day changes in solutions used, calibration of equipment, temperature, and humidity, that affects test results. Error refers to the standard deviation of recorded results around the mean of these results (assumed to be the true glucose value). Estimates of error can therefore be interpreted as the range of deviations which would occur as the result of factors other than changes in glucose level in 68 out of 100 observations, while 2.58 times this error is the range which would occur in about 99 out of 100 observations. (The distribution of differences around assumed true glucose values appeared sufficiently normal to warrant this interpretation.)

Day-to-day error was estimated by performing the Nelson test on standard dextrose solutions on several days. The results obtained on five replications on standard solutions, representing five glucose levels tested on three different days, are shown in table 1. From the results of this experiment, both the inherent error and the day-to-day error of observed results on standard solutions could be estimated at each glucose level. Tests for statistical significance (F test applied to each glucose level, 2 and 12 degrees of freedom) show that the day-to-day error is probably negligible or nonexistent—there are no day-to-day changes in solutions used, calibration of equipment, and so on, that materially affect test results. In the experiments described, therefore, estimates of the inherent error of the Nelson test have been used to measure reproducibility. Thus, the standard deviations shown at the bottom of table 1 are measures of the reproducibility of the Nelson test when used on standard solutions.

Although incidental to the experiments described here, table 1 illustrates an important point in the establishment of the calibration curve. Even with quantitatively known dextrose solutions, a single observation will frequently deviate from the expected value. As can be seen by the magnitude of the standard deviations shown at the bottom of the table,

Table 1. Observed replicate values for 3 different days on dextrose solutions of known glucose content

Observed values	Replication No.	Known glucose value (mg. percent)				
		50	100	150	200	250
1st day	1	51. 2	100. 6	149. 4	195. 5	255. 9
	2	50. 0	99. 4	151. 2	204. 1	250. 0
	3	48. 8	100. 0	150. 6	204. 1	254. 7
	4	50. 0	100. 0	153. 5	200. 0	248. 8
	5	49. 4	100. 0	150. 0	197. 1	246. 5
2d day	1	49. 4	101. 2	151. 8	198. 8	250. 0
	2	48. 2	100. 6	147. 6	197. 1	248. 8
	3	48. 8	98. 2	144. 7	201. 2	248. 2
	4	48. 8	102. 9	152. 9	198. 2	250. 6
	5	51. 2	100. 6	150. 6	201. 8	248. 2
3d day	1	48. 8	101. 2	147. 1	195. 9	257. 6
	2	48. 2	100. 0	152. 4	201. 2	247. 1
	3	51. 2	100. 0	151. 8	201. 2	245. 9
	4	48. 8	98. 8	150. 0	198. 8	252. 9
	5	51. 2	97. 6	151. 8	197. 1	254. 1
Mean value		49. 6	100. 1	150. 4	199. 5	250. 6
Range of value		48. 2	97. 6	144. 7	195. 5	245. 9
		51. 2	102. 9	153. 5	204. 1	257. 6
Standard deviation (inherent error)		1. 13	1. 28	2. 38	2. 74	3. 60

the range of these deviations tend to increase with the height of the glucose level, so that multiple observations are particularly important at the higher levels.

In determining blood sugar levels in the experiments reported below, quantitative dextrose solution controls for a glucose control of 100 mg. percent were always incorporated with each "run" of blood specimens. When the reading on the control in a given run deviated from the expected value by more than 3 mg. percent, procedures were checked and specimens were retested on the assumption that a procedural mistake had occurred. (This tended to minimize day-to-day error, if it existed, in the experiments described.)

Experiment With Replications

The following experiment was designed to evaluate the effectiveness of 10 mg. NaF and 1 mg. thymol per milliliter of blood in preserving the glucose content, as measured by the Nelson method.

Twelve tubes of blood were collected in B-D Vacutainers from each of six donors. Six of each donor's tubes contained 50 mg. of NaF and 5 mg. of thymol; the remaining six contained only 10 mg. of potassium oxalate as an anticoagulant. The fluoride and thymol were thoroughly pulverized and were added to the tubes with a microspatula calibrated to hold 55 mg. of powder. The tubes were evacuated for a volume of 5 ml. immediately before use to insure sufficient vacuum at the time of collection. Sterility was not maintained, but gross contamination was avoided by wiping the outside of the tubes with 70 percent ethanol before they were opened for the addition of the powder.

Before the tourniquet was applied, the donor's arm was sponged with iodine-ethanol, and the needle with holder attached was introduced into the vein with the Vacutainer in place. The tourniquet was removed and the tubes containing NaF-thymol and those containing oxalate were filled alternately. The tubes were inverted and shaken 6 to 8 times, as they were filled, to mix the blood with the preservative or the anticoagulant.

The tubes of blood from each donor were divided into 3 groups, each comprising 2 oxalated tubes and 2 tubes containing NaF-thymol. One group was examined exactly 1 hour after collection. The second group was mailed to a town 135 miles away; then remailed to the laboratory and tested 96 hours after collection. The third group was kept, without agitation, in the dark at a constant room temperature, and tested 96 hours after collection (except blood from donor No. 4 which was tested at 144 hours). Within each group, 3 independent blood sugar determinations were made from each tube. Since 2 tubes were treated and preserved identically, 6 replications for each observation were effected. The mean results of the 6 replications on blood from each donor are shown in table 2 along with their standard deviation from this mean.

As would be expected on the basis of Sunderman's report (*1*), the glucose content of unpreserved specimens dropped considerably; the amount of glucose remaining at the end of 96 hours varied with the initial level. It is evident that glycolysis in unpreserved specimens started immediately if we assume that the true glucose content at the time the blood was drawn was about that observed at 1 hour in the preserved specimens. (This assumption is supported by the fact that, as will be shown later, blood sugar levels in preserved specimens remained relatively constant with the passage of time.) As shown in table 2, the mean results obtained on the six replications tested at 1 hour were lower on all unpreserved specimens than on preserved specimens. They were, in fact, about 5 percent lower on the average, as suggested by Sunderman. In general, the differences between results at 1 hour on preserved and unpreserved specimens are highly significant statistically, indicating a real difference in sugar content. (F test with 1 and 10 degrees of freedom, applied to 1-hour observations on each donor.)

This experiment provided some information on whether changes occurred in the glucose content of blood preserved with NaF-thymol when tested at 1 hour, when mailed and tested at 96 hours, or when tested after being stored at room temperature for 96 hours. If the preservative were completely effective, one would expect the mean values for preserved blood from each

Table 2. Means and standard deviations of 6 replicate glucose values on preserved and unpreserved specimens from 6 donors showing results before and after mailing and storage

Donor No.	Preserved specimen (sodium fluoride-thymol)						Unpreserved specimen (potassium oxalate)					
	1 hour		Room temperature 96 hours		In mail 96 hours		1 hour		Room temperature 96 hours		In mail 96 hours	
	Mean	S. D.	Mean	S. D.	Mean	S. D.	Mean	S. D.	Mean	S. D.	Mean	S. D.
1	100. 7	0. 52	100. 3	1. 03	101. 5	1. 38	96. 5	1. 38	<5	------	<5	------
2	77. 8	1. 72	77. 3	2. 07	72. 5	2. 59	73. 3	1. 21	<5	------	<5	------
3	73. 5	1. 05	75. 7	2. 16	74. 7	0. 52	63. 7	1. 86	<5	------	<5	------
4	154. 3	2. 34	[1] 172. 8	2. 79	[1] 170. 0	4. 77	145. 5	3. 89	[1]<20	------	[1]<10	------
5	336. 5	3. 33	329. 0	3. 29	332. 5	4. 28	326. 2	1. 33	173. 8	4. 07	224. 8	2. 79
6	220. 5	4. 18	201. 5	3. 21	202. 7	2. 73	213. 3	4. 45	52. 5	3. 02	137. 5	2. 51

[1] 144 hours after withdrawal.
S. D.—Standard deviation.

donor (table 2) to be not significantly different from each other. There were numerical differences. Application of a test for statistical significance to observations on preserved blood from each donor indicates that a change probably occurred in preserved blood from 3 of the 6 donors. (F test with 2 and 15 degrees of freedom.) Two of these changes were decreases and 1 was an increase.

It is apparent that many of the factors which affect the glucose content of blood were uncontrolled in this experiment. These could not be identified. No doubt some of these factors are inherent in the donor's blood, as suggested by Bose and De (*8*), while others are probably due to differences in handling specimens. Stored and mailed portions of preserved specimens generally differed considerably less from one another than either differed from the initial value, indicating that time had more effect than did shipment versus storage.

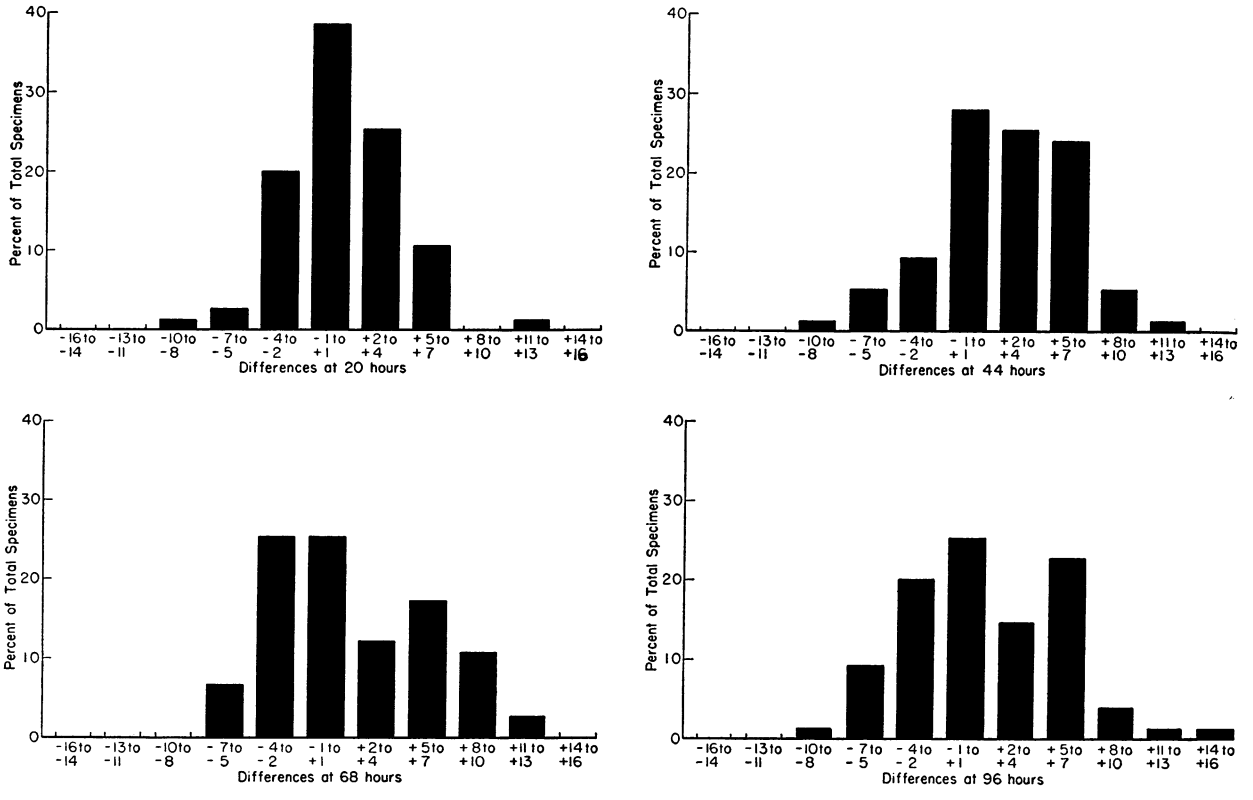
The experiment summarized in table 2 provides estimates of the inherent error in the Nelson test which, in the absence of day-to-day error, are measures of its reproducibility when used on blood—measures which were used in the test for statistical significance. Data shown in table 1 provide measures of the reproducibility of the Nelson test when used on standard solutions. These are shown as standard deviations on both tables and demonstrate a direct relationship between reproducibility and glucose level. In general, reproducibility appeared to be slightly poorer on blood than on standard solutions. This was to be expected since more steps are involved in making glucose determinations on blood than on standard solutions. Roughly, it would appear from table 2 that the Nelson test can measure the glucose content of blood within 1.5 percent of the true glucose level 68 times out of 100, while 99 times out of 100 (2.58 S. D.) it can measure within 4 percent. This estimate of reproducibility was of considerable importance in deciding whether changes in glucose content of blood actually occurred and was used in evaluating the results of further experiments described below.

Stored Specimens

In the experiment just described, it was possible that bloods from each donor, preserved and handled under identical conditions, would show the same behavior pattern with respect to changes in glucose content. This was not the case. In blood from 1 donor the glucose content increased, in 2 it decreased, and in 3 it did not change significantly. In effect then, this experiment gave only 6 observations on what turned out to be an important source of variation and, while showing that the combination of NaF and thymol is generally effective as a preservative, the experiment provided inadequate information on the frequency with which deviation in individual specimens would probably occur when used in large-scale projects.

To learn more about the behavior of indi-

Figure 1. Glucose differences in milligrams per 100 ml. of blood between initial and subsequent Nelson readings, 75 preserved specimens stored at room temperature



vidual specimens and to obtain more information regarding the manner in which preserved bloods tend to behave with the passage of time, specimens from 97 donors were collected in tubes containing the preservative and were examined at various intervals up to 120 hours. All specimens were drawn as in the preceding experiment. Examinations were made on 75 of these specimens 1 to 4 hours after collection, then at 20, 44, 68, and 96 hours; on 22 specimens examinations were made 1 to 4 hours after collection, then at 20, 44, and 120 hours. Between examinations, the specimens were kept in the dark at a constant room temperature and were not agitated except for mixing to permit glucose determination.

On the basis of the reproducibility of the Nelson test, estimated above to give results within 4 percent of the true value 99 times in 100, differences of 6 percent or more probably represent real changes in the glucose content of the specimens (when a ratio of the difference between specimens to the standard error of the difference in excess of 2.58 is considered significant). At the last test, the observed glucose content on 43 of the 97 specimens deviated from the initial content by as much as this amount. It is probable, therefore, that the glucose content actually changed in nearly half of these specimens. Of these 43 changes, all but 6 were increases.

The results on the specimens stored for 96 hours were considerably better than those stored for 120 hours. Of the 75 specimens stored for 96 hours, only 29 changed significantly; of the 22 specimens stored for 120 hours, 14 changed significantly (all increases).

Figure 1 shows the glucose differences observed between the first test and the 20-, 44-, 68-, and 96-hour tests on the 75 specimens on which the last test was made at 96 hours.

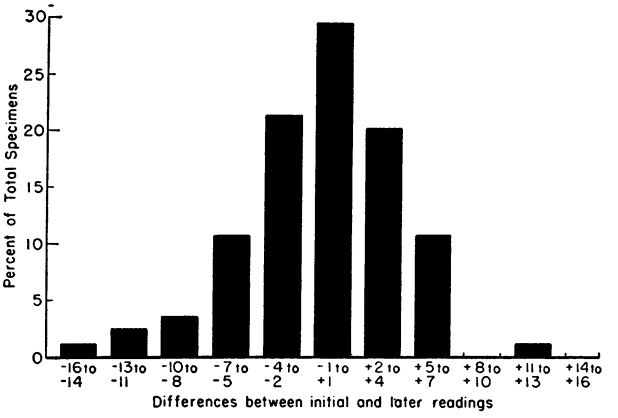
Shipped Specimens

An experiment was conducted to approximate the conditions under which the preservative might be used in a diabetes case-finding program and to obtain further information on the

effect of shipping. During a 30-day period in the late spring of 1952, 85 blood specimens were collected in tubes containing the preservative. A portion of each specimen was examined shortly after collection at the West Virginia Hygienic Laboratory. The remaining portion was mailed to a point 135 miles away and remailed to the West Virginia Hygienic Laboratory. This subjected the specimens to more varied handling than would normally occur in transmitting specimens from a collection point to a central laboratory. From the time the specimens were collected until they were examined the second time, they had been agitated a minimum of 18 times and had traveled by truck 9 times and by train twice. The time between the testing of the first portion and the testing of the second portion varied from 48 to 120 hours.

Five of the 85 specimens apparently changed by as much as 10 percent and 21 by as much as 6 percent. Again, lack of test reproducibility probably would not account for those deviations in excess of 6 percent, and it is likely that the glucose content actually changed in about a quarter of these specimens. Generally, this group of specimens behaved better than the 97 stored specimens, partly due, no doubt, to the shorter period between the first and last test. The results of testing the 120-hour specimens were relatively poor. Of 30 specimens tested at 120 hours, the glucose content of 10, or a third, had changed, whereas, of the 55 tested at or before 96 hours, the glucose content of 11, or a fifth, had changed. The distribution of differences between the first and last test on these 85 specimens is shown in figure 2.

Figure 2. Glucose differences in milligrams per 100 ml. of blood between initial and 48–120-hour Nelson readings, 85 preserved shipped specimens.



Discussion

Experiments involving 188 different blood specimens and 849 independent blood sugar determinations indicate that changes in the glucose content of whole blood preserved with NaF-thymol occurred when these specimens were stored at a constant room temperature or shipped and then were tested as long as 120 hours after collection. For the 135 stored and shipped specimens tested within 96 hours, the preservative appeared to be quite satisfactory. Of these, it is estimated that the glucose content actually changed in less than a third (in 42). These changes were small (only 6 were 10 mg. percent or greater) and were about equally divided between increases and decreases in glucose content. On the other hand, the glucose content changed in nearly half (25) of the 53 specimens stored for 120 hours or more; 22 of these changes were increases.

It was concluded that, beyond 96 hours, NaF-thymol loses some of its effectiveness and may actually cause increases in the glucose content of some specimens. However, for blood sugar determinations delayed for 96 hours or less, the preservative appears adequate, and the changes it permits are not the type which would cause any appreciable diagnostic error. Subsequent use of NaF-thymol in a statewide diabetes detection program in public health clinics, involving the collection of specimens in parallel with those drawn for syphilis and testing them for glucose content in the State hygienic laboratory at Charleston, W. Va., has shown the preservative to be quite satisfactory.

Summary and Conclusions

1. A series of experiments is presented to determine the value of the combination of 10 mg. of sodium fluoride and 1 mg. of thymol per milliliter of whole blood in maintaining the glucose content of blood, under conditions which would be characteristic of a diabetes case-finding program, conducted in conjunction with blood-collection programs for venereal disease.

2. As a basis for evaluating observed changes in the glucose content of blood when tested at varying intervals after being drawn, an estimate of the reproducibility of the Nelson test for glucose determinations was made. This estimate indicates that results obtained by the Nelson test will be within 4 percent of the actual glucose value 99 times in 100 and that differences between observed values in excess of 6 percent probably represent real differences in the glucose content of the specimen.

3. In three experiments involving a total of 188 different blood specimens and 849 independent glucose determinations, statistically significant increases and decreases were observed in the glucose content of stored and shipped specimens containing the preservative.

4. For periods up to 96 hours, it was estimated that less than a third of the blood specimens actually changed in glucose content. These changes were small. They were about equally divided between increases and decreases in the glucose content of specimens and would probably be of little or no clinical significance in diabetes case-finding programs.

5. The preservative was not considered satisfactory for periods beyond 96 hours.

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A detailed version of this paper, containing a more complete description of procedures and results, may be obtained by writing to the Hygenic Laboratory Division, West Virginia Department of Health, Charleston, W. Va.

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The Communicable Disease Center of the Public Health Service is offering a refresher course in communicable disease control to public health nurses and instructors in communicable disease nursing, April 26 through May 14, in Atlanta, Ga.

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