

Standardized Viral Hemagglutination and Hemagglutination-Inhibition Tests

I. Standardization of Erythrocyte Suspensions

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A spectrophotometric procedure for standardizing red blood cell suspensions for use in viral hemagglutination tests is described. The procedure is based on highly reproducible cyanmethemoglobin absorbance readings at 540 nm on any suitable spectrophotometer. Target values for milligrams of cyanmethemoglobin per 100 ml are given for the red cell suspensions of all mammalian and avian species employed in viral hemagglutination tests. By use of these values and a cyanmethemoglobin standard curve for a particular photometer, approximate 4% erythrocyte suspensions can be diluted to any lesser concentration. Coefficients of variation for the various diluted suspensions range from 4 to 7%.

Hemagglutination (HA) and hemagglutination-inhibition (HI) tests for the identification of viruses and serological diagnosis of virus infections have been subjected to endless procedural modifications since the inception of the HA test for influenza in 1941. Most of the variations in the performance of the hemagglutination tests are needless and confusing, and give rise to titers which depend as much on the test procedure as on the virus or serum being tested. In addition, small laboratories often do not have the manpower or budgetary allowance to conduct a variety of hemagglutination procedures and, as a result, do not use HA and HI tests to their full potential in the identification of viruses and specific antibodies.

Two considerations are paramount in establishing standardized HA-HI procedures. First, a study of diagnostic viral HA-HI tests needs to be performed in an effort to render them more sensitive and reliable. Second, a standardized protocol is needed to serve as a reference when comparing results in one laboratory with those in another.

Standardization of viral HA-HI tests necessarily involves all of the "arbitrary constants" that make up the tests: volume and concentration of red blood cells, volume of antigen, type of diluent, serum treatment, antigen-serum-cell volume ratio, and incubation time. Of these variables, the concentration of erythrocytes is the least controlled, despite its importance in the reproducibility of the test results. In many labora-

tories, suspensions of red blood corpuscles (RBC) are prepared by reading the packed cell volume in uncalibrated centrifuge tubes after centrifugation under variable conditions with no control on the sources of error.

The most accurate method currently available for preparing erythrocyte suspensions is by spectrophotometry. An approximate cell concentration is prepared and a sample is treated with a hemolytic reagent which liberates the hemoglobin. The absorbance or optical density (OD) of the solution is measured in a spectrophotometer and compared with the OD of a known cell suspension (called the "target OD") to obtain the dilution factor needed to adjust the suspension to the correct concentration. This target OD must be very accurately determined, since all future unknown suspensions are adjusted by the target OD values.

Clarke and Casals (3) discussed a photometric procedure for preparing cell suspensions for HA-HI tests, but their method was not applicable to all photometers or cell suspensions. Similar limited attempts to standardize sheep cell suspensions for the complement-fixation test have also been made (4, 5).

Since the use of red cell suspensions of accurate concentrations is an integral part of the standardized HA-HI tests, we describe in the present report the standardization of erythrocyte suspensions by spectrophotometry and the routine preparation of standardized cell suspensions. In a companion paper we discuss the other variables

of current hemagglutination procedures, outline the protocol for the standardized HA-HI tests, and present the statistical evaluation of these tests.

MATERIALS AND METHODS

Collection and washing of red blood cells. Bloods from Hampshire and Suffolk sheep, Hartley strain guinea pigs, type "O" humans, rhesus and vervet monkeys, Sprague-Dawley albino rats, white mice, Chinese geese, White Rock chickens, and White Leghorn 2-day baby chicks were used in these experiments. Blood was collected and stored in Alsever solution (consisting of 20.5 g of glucose, 8.0 g of trisodium citrate dihydrate, 0.55 g of citric acid monohydrate, 4.2 g of sodium chloride, and 1,000 ml of distilled water; pH 6.1; sterilized by membrane filtration) in the proportion of 1 ml of blood to 4 ml of anticoagulant. When needed, the red cells were washed and ultimately diluted in phosphate-buffered saline (PBS), pH 7.2, containing 0.010 M total phosphate and 0.146 M sodium chloride (consisting of 1.096 g of disodium phosphate anhydrous, 0.315 g of monosodium phosphate monohydrate, 8.5 g of sodium chloride, and 1,000 ml of distilled water).

Preparation of 4.0% RBC suspensions by centrifugation. The red cells were washed three times in an International PR-II centrifuge at $2,264 \times g$ (3,000 rev/min) for 30 min at 4 C (8). Kolmer 10-ml centrifuge tubes, which had previously been calibrated at the 0.40- and 10.0-ml marks, were used for this purpose. Following each centrifugation, the buffy coat (white cell layer) and excess red cells were carefully aspirated so that the final packing would read exactly 0.40 ml. The packed RBC volume was diluted to 10.0 ml with PBS to obtain a 4.0% RBC suspension accurate to the first decimal place.

Preparation of approximate 4% RBC suspensions. Approximate cell suspensions were prepared in the "usual" manner. The cells were washed three times in 12-ml conical graduated centrifuge tubes in a clinical centrifuge at approximately $1,500 \times g$ for 5 min at room temperature. The buffy coat was removed by aspiration after each wash. The packed cell volume was read after the final wash and then diluted to an approximate 4% concentration with PBS. The use of centrifuge tubes which had been checked for accuracy at the 0.2-, 0.4-, and 0.6-ml marks lessened the error in reading the packed cell volume but did not substantially reduce the overall variation in this procedure.

Cyanmethemoglobin assay. The hemoglobin content of each RBC suspension was determined spectrophotometrically by the Hycel cyanmethemoglobin procedure. (Hycel Inc., Houston, Tex.; see Hycel literature for further explanations of reagent, standard curve, and assay.) In this method, erythrocytes are lysed in a hypotonic solution, and the liberated hemoglobin is then oxidized to cyanmethemoglobin which has a strong absorption band at 540 nm.

The hemolytic reagent was prepared by dissolving one vial of Hycel powder reagent in 2 liters of distilled water. Use of the reagent at one-half strength helped to overcome the "resistant-cell" phenomenon.

The reagent was stored in a brown, screw-capped polyethylene bottle at room temperature.

The cyanmethemoglobin standard curve was prepared from various amounts of the Hycel standard with 80 mg of cyanmethemoglobin per 100 ml and Hycel cyanmethemoglobin reagent to give concentrations of 80, 60, 40, 20, and 0 mg of cyanmethemoglobin per 100 ml (80 mg of cyanmethemoglobin per 100 ml equals 20 g of hemoglobin per 100 ml). The absorbance readings at 540 nm were used to calculate a factor by which the OD of unknown samples was converted to milligrams of cyanmethemoglobin per 100 ml. Calibration of the cyanmethemoglobin method and calculation of the factor are described in the Hycel literature and elsewhere (2). A convenient way to calculate the factor is to divide the sum of the concentrations of the standards (80, 60, 40, 20; and where the 0 standard is the reagent blank) by the sum of the OD readings (at 540 nm) of the standards. This factor may be used without change if the same instrument is employed.

The cyanmethemoglobin assay of RBC suspensions was performed by adding 1 ml of the well-mixed suspension to a 25-ml volumetric flask which was then filled to the mark with one-half strength cyanmethemoglobin reagent. The flask was inverted 10 times to thoroughly mix the contents and then allowed to stand at room temperature for 15 to 45 min—a length of time sufficient for cell lysis to occur. If the cell stroma were not completely dissolved, the solution was clarified by centrifugation at $1,650 \times g$ before it was transferred to cuvettes.

Cyanmethemoglobin readings were taken on a Bausch & Lomb Spectronic-20 colorimeter, two Coleman Junior spectrophotometers (model 6A), and a Beckman DU spectrophotometer. All were operated at a wavelength of 540 nm. Cuvette sizes were 13×100 mm for the Spectronic-20 colorimeter, 12×75 mm for the Coleman Junior spectrophotometers, and $10 \text{ mm}^2 \times 45$ mm for the Beckman DU spectrophotometer. All samples were read against a reagent blank, and a standard curve was included with each series of test samples. The average of five OD readings for each sample was converted to grams of hemoglobin or milligrams of cyanmethemoglobin per 100 ml by multiplying the OD by the factor calculated from the appropriate standard curve: grams of hemoglobin or milligrams of cyanmethemoglobin per 100 ml of suspension $x = (\text{OD of suspension } x) \times (\text{factor})$.

Cell counts. Cell counts were taken on the 4.0% suspensions with a Coulter model B counter and a Particle Data Corporation Celloscope. Both were operated at the appropriate lower threshold and amplification settings required to rule out background noise. The upper threshold was disengaged. Cell suspensions were diluted 1:30,000 in Gower's solution which had been passed through a 0.22- μm filter (Millipore Corp., Bedford, Mass.) to minimize the background count.

RESULTS

Development of the cyanmethemoglobin procedure. Several preliminary tests were conducted to

determine which RBC concentration and volume of hemolytic reagent would be most practical for a routine procedure. The logical choice for the reagent volume was the 25-ml volumetric flask, since it is readily available and is a convenient size. The use of 4% cell suspensions, to be diluted 1:25 in the reagent, was dictated by two factors: (i) this hemoglobin concentration gave readings in the middle of the most sensitive portion of the absorbance scale (0.2 to 0.4 OD), and (ii) a 1:25 dilution of a 4% suspension approximates the clinical procedure recommended by the reagent manufacturer, namely, 0.02 ml of whole blood into 5.0 ml of reagent, which is a 1:251 dilution of blood (32 to 45% hematocrit). On the basis of these considerations, we established that the method of choice was to prepare 4% RBC suspensions and measure the hemoglobin content by the absorbance of a 1:25 dilution of this suspension in cyanmethemoglobin reagent. Using this procedure, we found that mammalian cells which had been stored for 2 weeks or less lysed within 30 min at room temperature and were completely soluble in the hemolytic reagent. After lysis and thorough mixing, the solution was transferred to cuvettes for spectrophotometric readings.

Avian cells were more difficult to handle. Complete lysis was achieved only when the cells were fresh (0 to 2 days) and when they had been properly collected in the anticoagulant. For complete lysis, 3- to 5-day-old cells usually required the addition of a few milligrams of saponin, heating at 60 C for 2 hr, or multiple freeze-thawings. Cells older than 6 days could be completely lysed only by sonic oscillation. In addition to the hemolysis problem, some fragments of avian cells were not completely soluble in the hemolytic reagent. The cell stroma tended to form long, viscous aggregates which were free from hemoglobin but which would interfere with photometric read-

ings if not removed. Therefore, avian red cell solutions were clarified by centrifugation at $1,650 \times g$ before measurements were made in the spectrophotometers.

Hemoglobin and cell count values of 4.0% suspensions. The standardization of RBC suspensions by spectrophotometry is based entirely upon the establishment of accurate target values for milligrams of cyanmethemoglobin per 100 ml. Such values were determined by using the cyanmethemoglobin assay procedure to measure the absorbance of a large number of accurately prepared 4.0% cell suspensions. The OD values were converted to milligrams of cyanmethemoglobin per 100 ml through standard curves. This procedure established accurate target values for 4.0% suspensions; from these, the target values were calculated for the 0.4 and 0.5% suspensions that were to be used in the standardized HA-HI tests.

The absorbance and grams of hemoglobin per 100 ml for centrifugally prepared 4.0% suspensions of eight species of erythrocytes were measured on four spectrophotometers as given under Materials and Methods. The results are presented in Table 1. Although the OD values were quite far apart from one instrument to another, owing to different slopes of the standard curves, the grams of hemoglobin per 100 ml were within a very close range. This indicates that any of these instruments is usable for standardizing red cell suspensions. Agreement on grams of hemoglobin per 100 ml between spectrophotometers was 98%, and the standard deviation in hemoglobin values for each species was 4 to 7%. This error range includes normal variation between animals as well as experimental error. The Coleman Junior spectrophotometer was the most difficult instrument to read and consequently gave the highest error between readings on identical samples.

The hemoglobin values are based on a standard

TABLE 1. Summary of hemoglobin determinations on different spectrophotometers

Source of cells	Spectronic-20				Beckman DU				Coleman Junior "6A"				
	No. of tests	Avg OD	Avg g of hgb/100 ml ^a		No. of animals used	Total no. of tests	Avg OD	Avg g of hgb/100 ml ^a		No. of tests	Avg OD	Avg g of hgb/100 ml ^a	
			Mean	SD				Mean	SD			Mean	SD
Sheep	11	.382	12.76	0.33	8	13	.340	12.66	0.55	6	.297	12.51	0.82
Guinea pig	7	.379	12.70	0.86	9	13	.343	12.74	0.98	6	.307	12.92	1.47
Human	4	.381	12.62	0.40	5	8	.338	12.56	0.95	6	.290	12.22	1.28
Monkey	7	.353	11.56	0.88	5	8	.319	11.93	0.92	6	.272	11.47	1.16
Rat	8	.371	12.13	0.52	4	8	.330	12.44	0.64	3	.288	12.21	0.84
Goose	13	.358	11.86	0.37	6	16	.320	12.04	0.62	9	.295	12.24	0.38
Baby chick	6	.323	10.52	0.26	12	12	.295	10.98	0.57	12	.262	10.98	0.44
Chicken	11	.307	10.07	0.46	9	11	.270	10.12	0.41	6	.247	10.01	0.19

^a The values for grams of hemoglobin (hgb) per 100 ml are based on a standard curve of 0 to 20 g of hgb per 100 ml (0 to 80 mg of cyanmethemoglobin per 100 ml).

TABLE 2. Comparison of hemoglobin and cell count data with published values

Source of cells	Hemoglobin (g/100 ml), Beckman DU values			Hemoglobin (g/100 ml), literature values ^a adjusted to 40% hematocrit		Per cent agreement of means	Cell count/ml on 4.0% suspensions	Cell count/ml, avg literature values ^a adjusted to 4.0% hematocrit	Per cent agreement of means
	Mean	SD	Range of one SD	Mean	Reported range				
Sheep.....	12.66	0.55	12.11-13.21	13.7	12.5-14.9	93	11.6×10^8	13.0×10^8	89
Guinea pig....	12.74	0.98	11.76-13.72	13.7	10.5-15.8	93	5.0×10^8	5.3×10^8	94
Human.....	12.56	0.95	11.61-13.51	13.4	11.9-15.8	94	6.2×10^8	4.7×10^8	76
Monkey.....	11.93	0.92	11.01-12.85	12.0	9.5-15.2	99	4.9×10^8	5.0×10^8	98
Rat.....	12.44	0.64	11.80-13.08	12.9	10.4-15.2	97	7.1×10^8	7.7×10^8	92
Goose.....	12.04	0.62	11.42-12.66	11.4	10.6-12.1	95	3.6×10^8	2.6×10^8	72
Baby chick....	10.98	0.57	10.41-11.55	11.5	Not reported	96	10.1×10^8	6.9×10^8	68
Chicken.....	10.12	0.41	9.71-10.53	11.1	8.2-14.5	91	4.7×10^8	3.6×10^8	77

^a See references 1, 6, 7, and 8.

TABLE 3. Combined target values for 4.0% suspensions^a

4.0% RBC	No. of tests	Target OD (DU only)		All spectrophotometers			
				Target hemoglobin (g/100 ml)		Target cyanmethemoglobin (mg/100 ml)	
		Mean	SD	Mean	SD	Mean	SD
Mammalian.....	50	.336	.023	12.52	0.84	50.05	3.37
Goose ^b	16	.320	.018	12.04	0.62	48.14	2.49
Baby chick.....	12	.295	.014	10.98	0.57	43.93	2.26
Chicken.....	11	.270	.010	10.12	0.41	40.47	1.63

^a The 4.0% suspensions were diluted 1:25 in Hycl cyanmethemoglobin reagent (Table 1).

^b Recent limited studies with feral pigeon blood have shown that the target absorbance and hemoglobin values for pigeon red cells are nearly identical with those for goose RBC.

curve of 0 to 20 grams of hemoglobin per 100 ml as outlined by Hycl. The Hycl standard curve is designed for a dilution of 0.02 ml of whole blood in 5.0 ml of reagent; this 1:251 dilution of blood is equivalent to a 1:627.5 dilution of packed RBC if an average hematocrit of 40% is used. This closely approximates the 1:625 cell dilution in the present work ($4.0\% \times 1/25$).

The results in Table 2 afford a comparison of the hemoglobin values obtained in these experiments with those reported in the literature. The very close agreement observed supports the accuracy of the 4.0% suspensions. The importance of the accuracy of these suspensions cannot be overlooked because the target values for milligrams of cyanmethemoglobin per 100 ml which will eventually be calculated (Table 3) can be no more accurate than the cell suspensions used to obtain them.

The average cell counts on the 4.0% suspensions for each species tested are listed in Table 2. Most of these counts are reproducible and agree with those given in the literature cited. For instance, 4.0% sheep cell suspensions were found to contain an average of 11.6×10^8 cells/ml, com-

pared with the literature values of 13.0×10^8 (1, 7) and 9.6×10^8 (4, 5). It should be noted here that comparison of our cell count data with previously reported counts is difficult because of the considerable variation in the literature values.

Calculation of target OD values. Since the mean grams of hemoglobin per 100 ml for the five mammalian species were not significantly different (F ratio = 1.342 with 4 and 49 degrees of freedom), they can be combined into a single figure for calculation of the target OD values (Table 3). The mammalian species were combined to give an average cyanmethemoglobin value of 50.05 mg/100 ml with a standard deviation of 3.37 mg/100 ml [a coefficient of variation (CV) of 6.7%]. Only the Beckman DU numbers from Table 1 were used, because this instrument is calibrated at the factory to minimize variation between machines. The OD at 540 nm of mammalian cells measured with the Beckman instrument averaged 0.336 with a standard deviation of 0.023 (CV = 6.8%). Variation among different Spectronic-20 instruments or among different Coleman Junior instruments is too great to permit

the establishment of an absolute absorbance for these spectrophotometers.

Even though the mean hemoglobin value for goose red cells falls within the mammalian group, this value is not included because goose red cells present the same difficulties of incomplete lysis and stroma insolubility as the other avian red cells. The cyanmethemoglobin value of goose cells is 48.14 mg/100 ml with a CV of 5.2%. Because the arithmetic means for chicken and 2-day-old baby chick erythrocytes were significantly different (F ratio = 16.860 with 1 and 22 degrees of freedom), they were not combined. The value for baby chick cells is 43.93 mg of cyanmethemoglobin per 100 ml (CV = 5.2%) and for chicken cells is 40.47 mg of cyanmethemoglobin per 100 ml (CV = 4.0%). The proposed target values for the RBC suspensions in viral hemagglutination tests are presented in Table 4. These values were calculated by simple proportionality from the accurate cyanmethemoglobin figures in Table 3.

Since it is more accurate to base hemoglobin concentration on a known weight of cyanmethemoglobin per unit volume rather than on grams of hemoglobin per 100 ml of blood, the target values of Table 4 are calculated in the form of milligrams of cyanmethemoglobin per 100 ml of solution. (A value for grams of hemoglobin per 100 ml of blood is very dependent upon hematocrit and several other hematologic variables and is thereby highly subject to error.) The figures in Tables 1 and 2 were expressed as grams of hemoglobin per 100 ml so that a direct comparison with the literature could be used to substantiate the accuracy of the 4.0% suspensions. Once this was accomplished, the emphasis was shifted to milligrams of cyanmethemoglobin per 100 ml. The multiplication factor to convert grams of hemoglobin per 100 ml to milligrams of cyanmethemoglobin per 100 ml is 4.00.

Tests on the reliability of the target OD values.

Tests on the reliability of the derived target values for milligrams of cyanmethemoglobin per 100 ml were conducted along three lines. In the first trial, the packed cell volume was checked after standardization and dilution. Sixteen sheep cell suspensions were prepared to approximately 4% by centrifugation, measured spectrophotometrically, and then diluted to 2.8% by the formula:

$$\frac{\text{test OD}}{\text{target OD}} \times (\text{volume of test susp.}) \\ = \text{final volume of 2.8\% suspension}$$

The target OD in this experiment was 0.267 on the Spectronic-20 colorimeter in our laboratory. A 10-ml amount of the final 2.8% suspension was pipetted into a Kolmer 10-ml centrifuge tube and centrifuged for 30 min at $2,264 \times g$. In 14 of the 16 tests, the packed cell volume read exactly 0.28 ml, as would be expected if the target OD were correct. The two errant values were 0.26 and 0.29 ml. In a similar trial, 19 of 20 standardized 1.0% rhesus monkey RBC suspensions read 0.10 ml after centrifugation, and one was estimated at 0.105 ml. The target OD on the Spectronic-20 colorimeter was 0.0954.

The second check on the target values for milligrams of cyanmethemoglobin per 100 ml was carried out by using the standardized cell procedure over a suitable period of time. Several serology laboratories provided this practical test by routinely making 0.4% mammalian and 0.5% chicken cell suspensions according to the standardized cell protocol. Target OD values on the Spectronic-20 colorimeter were 0.0382 for the 0.4% guinea pig, rhesus, rat, vervet, and human cells, and 0.0390 for the 0.5% chicken cells. Several hundred cell suspensions were prepared by the standardized cell procedure over a 2-year

TABLE 4. Proposed target values for RBC suspensions in viral HA-HI tests

Desired suspension	Target values ^a		Suggested use
	OD ₅₄₀ , DU only	Cyanmethemoglobin (mg/100 ml), all spectrophotometers	
Mammalian, 0.40%	.0336	5.005	Adenovirus HA-HI (monkey, rat); measles HA-HI (monkey); reovirus HA-HI (human); enterovirus HA-HI (human); myxovirus HA-HI (human, guinea pig); murine viruses HA-HI (human, guinea pig, sheep, mouse)
Goose, 0.25%	.0200	3.009	Arbovirus HA-HI
Baby chick, 0.25%	.0184	2.746	Rubella HA-HI
Chicken, 0.50%	.0338	5.059	Poxvirus HA-HI; myxovirus HA-HI

^a Calculated from the average values of 1:25 dilutions of 4.0% RBC suspensions (Table 3).

period, and all were used satisfactorily in HA-HI tests.

A third test on the accuracy of the derived target values for milligrams of cyanmethemoglobin per 100 ml was conducted by reversing the usual order of preparing standardized RBC suspensions. Since the target values are based on 4.0% RBC suspensions accurately prepared by centrifugation under specific conditions in calibrated Kolmer tubes, it was important to deter-

mine whether the reverse of this procedure would yield spectrophotometrically correct readings. Suspensions of 4.0% RBC were prepared in Kolmer centrifuge tubes as described in Materials and Methods. Other suspensions were prepared by carefully diluting the 4.0% suspensions in PBS. Cell counts and hemoglobin measurements were then made on each original and diluted suspension. The results are given in Table 5. It should be noted that the range of suspensions was limited to 1.0 to 4.0% so that accurate measurements could be obtained on the spectrophotometer without further treatment. Different lots of cells, representing different animals, appeared to have no effect in this experiment. The agreement of the measured values for milligrams of cyanmethemoglobin per 100 ml with the calculated values obtained from Table 3 supports the principle of preparing RBC suspensions of various concentrations by diluting suspensions of known hemoglobin concentration.

DISCUSSION

The usual procedure for preparing red blood cell suspensions is by reading the packed RBC volume in a conical centrifuge tube after the third wash and then diluting to the desired concentration. Often no attempts are made to use calibrated and accurate centrifuge tubes or to pack the cells under known, controlled conditions of centrifugal force, time, and temperature. Commonly used 12- and 15-ml conical centrifuge tubes often have an error of 10 to 25% or greater in their graduated markings, and conditions of centrifugation can contribute another 20% variation in reading the packed cell volume. An additional source of error is the investigator's failure to remove the buffy coat of white blood cells during the washing process, which results in the white cell layer being inadvertently included in the packed cell volume. These errors, especially if compounded, result in erythrocyte suspensions of unknown concentration. The RBC concentration in such preparations usually varies considerably from day to day and from one laboratory to another.

Variations in the cell suspensions affect the end points in HA and HI tests either by making the end point difficult to see (if the suspension is "light") or by lowering the end point with an excess of nonagglutinated cells (if the suspension is "heavy"). Consistency and accuracy in performing this basic part of the HA and HI tests is therefore essential for reproducibility within the same laboratory and for accuracy when results are compared between laboratories.

Accurate suspensions prepared by centrifugation under known and controlled conditions were

TABLE 5. Hemoglobin and cell count data on diluted suspensions

Cell suspension	Lot no.	No. cells/ml	Cyanmethemoglobin (mg/100 ml)		
			Measured	Calculated ^a	
Sheep 4.0%	1	12.5×10^8	48.0	50.0	
	1	12.0×10^8	54.4		
	2	10.8×10^8	49.2		
	2	9.8×10^8	48.5		
	3	11.0×10^8	49.4		
	3	9.7×10^8	49.5		
2.0%	3	4.0×10^8	26.0	25.0	
	3	4.0×10^8	25.0		
Guinea pig 4.0%	1	6.5×10^8	47.1	50.0	
	2	4.0×10^8	48.5		
	3	4.5×10^8	45.5		
	2.0%	1	2.5×10^8	23.9	25.0
		2	2.5×10^8	24.1	
		3	2.2×10^8	22.8	
1.0%	1	1.2×10^8	12.0	12.5	
	2	1.7×10^8	12.3		
	3	1.0×10^8	11.8		
Human 4.0%	1	6.0×10^8	50.7	50.0	
	2	6.0×10^8	52.1		
	3	7.0×10^8	49.9		
	2.0%	1	3.5×10^8	26.4	25.0
		2	3.5×10^8	26.7	
		3	3.0×10^8	25.8	
1.0%	1	2.0×10^8	13.4	12.5	
	2	2.0×10^8	13.4		
	3	1.6×10^8	12.8		
Monkey 4.0%	1	4.2×10^8	48.8	50.0	
	2	5.0×10^8	46.6		
	3	5.5×10^8	45.5		
	2.0%	1	2.3×10^8	23.8	25.0
		2	2.2×10^8	24.1	
		3	2.5×10^8	23.9	
	1.0%	1	1.2×10^8	11.8	12.5
		2	1.0×10^8	12.1	
		3	1.7×10^8	11.9	

^a Calculated from the target values for milligrams of cyanmethemoglobin per 100 ml in Table 3.

TABLE 6. Factors for converting OD_{640} to grams of hemoglobin and milligrams of cyanmethemoglobin per 100 ml

Instrument no.	Spectrophotometer make-model	No of standard curves made	Grams of hemoglobin per 100 ml/OD (avg factor and range)	Milligrams of cyanmethemoglobin per 100 ml/OD (avg factor and range)
1	Spectronic-20	13	33.05 (32.52-33.64)	132.19 (130.11-134.56)
2	Beckman DU	13	37.40 (36.95-37.82)	149.57 (147.82-151.29)
3	Coleman Junior "6A"	4	39.80 (39.28-40.92)	159.20 (157.12-163.68)
4	Coleman Junior "6A"	6	41.91 (40.47-43.63)	167.65 (161.89-174.54)

employed to establish target OD values, so that future unknown cell suspensions could be standardized by spectrophotometric comparison to this accurate OD. There is little difference in time or convenience between preparing accurate suspensions by centrifugation or by photometry; however, spectrophotometry is clearly the method of choice because it measures a more constant factor than does centrifugation. Centrifugation measures the packed cell volume, which is dependent upon the physical characteristics of size, shape, and mean corpuscular volume of the erythrocytes. These physical measurements vary considerably among the different mammalian species studied in this report. For example, the mean corpuscular volume alone ranges from 30 to 101 μm^3 (1).

On the other hand, spectrophotometry measures hemoglobin concentration, which is a more constant factor within members of one species and among species (1, 8). The mean corpuscular hemoglobin concentration varies from 30 to 34.5 g per 100 ml of erythrocytes for all the mammalian species in this report. Hence, the overall greater accuracy in measuring hemoglobin concentration and the considerably smaller variation between animals indicate that the spectrophotometric standardization of red cell suspensions would yield more consistent results than any centrifugation procedure.

The choice of spectrophotometer or diffraction-grating colorimeter for use in standardizing red cell suspensions is not critical if a properly calibrated instrument is used. Glass-filter photometers and photoelectric colorimeters which employ glass filters are not recommended but may be used, although sometimes with a sacrifice in precision.

Different spectrophotometers will give different slopes of the standard curve, and it is essential to perform a standard curve on each instrument to calculate the correct factor. This factor will remain constant for the particular instrument provided the machine is not moved or repaired. The average and range of factors for the standard curves made during this study are given in Table 6. These factors are listed to illustrate the difference

between instruments of the same and different brands and will not necessarily apply to other spectrophotometers of the same type.

The values for milligrams of cyanmethemoglobin given in Tables 3 and 4 embody the most recent molecular weight for hemoglobin (64,458), established by the Congress of the International Society for Hematology in 1964. All current lots of cyanmethemoglobin standard should also be employing this new molecular weight.

Outline of the procedure for standardizing RBC suspensions by spectrophotometry. The initial step in this procedure is the calculation of the target OD values on the photometer which will routinely be used for cell standardization. Prepare a standard curve of 0 to 80 mg of cyanmethemoglobin per 100 ml, and compute the "factor" (see Materials and Methods). Using this factor, calculate the target OD for each cell suspension to be used:

target OD

$$= \frac{\text{target mg of cyanmethemoglobin per 100 ml (Table 4, column 3)}}{\text{factor (mg of cyanmethemoglobin per 100 ml/OD)}}$$

Once calculated and recorded, the target OD for each of the four possible RBC suspensions (Table 4) will be available for all subsequent cell standardizations on the spectrophotometer involved.

Thereafter, daily use of the spectrophotometer for standardization of red cells is a simple procedure and may be performed as follows. Obtain fresh RBC by mixing one volume of blood with four volumes of Alsever solution. Wash the red cells three times in PBS and carefully remove the "buffy coat" after each wash. Read the packed cell volume after the last wash and dilute to an approximate 4% suspension with PBS. Mix well and transfer 1.0 ml of the suspension into a 25-ml volumetric flask with a long-tipped Mohr measuring pipette or one of similar accuracy. Fill to the mark with cyanmethemoglobin reagent and mix thoroughly to obtain a uniform hemoglobin solution. Allow the solution to stand for 15 to 45 min at room temperature. If avian cell solu-

tions appear turbid, add a few milligrams of saponin and mix well. All avian solutions must be briefly centrifuged after lysis to remove the undissolved cell stroma.

Check the calibration of the spectrophotometer by reading the absorbance of the 1:2 standard (40 mg of cyanmethemoglobin per 100 ml) and checking this reading with that on the previous standard curves. Transfer some of the cell solution to a cuvette and read the OD at 540 nm against a reagent blank. Calculate the dilution necessary to obtain the desired suspension:

$$\frac{(\text{OD of test suspension}) \times (\text{volume of test suspension})}{\text{target OD}} = \text{final volume of desired suspension}$$

This procedure for preparing accurate RBC suspensions by spectrophotometry is suggested or the standardized HA-HI tests and for all

serological tests in which erythrocyte suspensions are used.

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