TECHNICAL PAPERS

Direct-reading Photocolorimetry

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The use of photocolorimeters is becoming increasingly widespread and in many laboratories has completely supplanted the visual colorimeter. When large numbers of analyses are made, the calculation of results may become a time-consuming chore. It is possible to simplify such calculations by an expedient whereby the scale readings are made to express directly the quantities of the unknown substance analyzed. When applied to the analysis of blood sugar, the result in mg per 100 ml can be read directly from the scale of the Klett-Summerson photocolorimeter. The method is applicable also to other types of photocolorimeters.

The relationship between the density of the color in the solutions derived from the standard and unknown samples and their respective concentrations is illustrated in the equation

$$C_u = (R_u - R_b)\frac{C_s}{(R_s - R_b)}$$

in which C_u and C_s are the concentration of material analyzed in unknown and standard samples; R_u, R_s, and R_b are the readings in terms of optical density of unknown, standard, and blank samples.

If R_b reads zero or if the instrument is adjusted so that $R_b = 0$ then the foregoing expression becomes

$$C_u = R_u \frac{C_s}{R_s}$$

It will be seen that if $R_s = C_s$, then $C_u = R_u$ and no calculation is necessary. This relationship seldom is encountered; however, it can be established or closely approximated by selection of a filter or wavelength setting that will have the effect of expanding or contracting the light absorption for each unit of substance to make it divisible evenly into the units of the scale. An adjustment of shutter or galvanometer setting then can make R_s equal to C_s or to some simple multiple of C_s, nC_s. Then

$$C_u = (R_u - R_b + d)\frac{C_s}{(R_s - R_b + d)}$$

where d represents the effect of the shutter adjustment. If the relationship between light absorption of the solution and filter is such that the change from $R_b = 0$ to $R_s = nC_s$ is small then d will be small and for values of C_u near C_s, R_u will be approximately equal to C_u. The error at $C_u = 0$ is given by the deviation of R_b from zero.

To illustrate, the following procedure was used in adapting the Somogyi-Nelson blood sugar method to this system of measurement by means of a Klett-Summerson photocolorimeter. Three standard solutions containing glucose equivalent to blood sugar concentrations of 50, 100, and 200 mg per 100 ml respectively, and a blank tube containing reagents only were prepared together with the unknowns. A filter having maximum transmission at 540 mμ was tested first. With the cuvette containing the blank in position and the dial scale set at zero the shutter was adjusted to give a null reading on the galvanometer. Next the three standards were read and scale readings of 65, 120, and 245 were obtained. Since these values were not conveniently convertible to the corresponding blood sugar values, a filter having maximum transmission at 500 mμ was tested. The adjustment of the blank to zero was repeated, and the three standards now read 54, 103, and 205. These readings approximated the desired range and this filter consequently was selected. For measurement of blood sugar in the normal range, the cuvette containing the 100 mg standard was placed in the instrument. The dial was set to 100 and the galvanometer restored to the null point by means of the shutter adjustment. Readings of the unknowns were then taken directly from the dial. They agreed (within the limits of error of the readings) with the blood sugar values for the same specimens calculated by a conventional procedure.

When the method is applied to photocolorimeters scaled to read transmissions, a setting is selected that will give a simple fraction when converted to optical density. To illustrate, the method used in this laboratory for determination of cholesterol employs the Evelyn photocolorimeter. Two standards containing 0.4 mg and 0.8 mg of cholesterol together with the unknown samples are treated with acetic anhydride and sulfuric acid. With the reagents currently in use, and when conditions for development of color are kept uniform, galvanometer readings of the 0.8 mg standard closely and consistently approximate 32. With the colored solution derived from the 0.8 mg standard in place, the galvanometer is set at 31[3]. The optical density (L value) corresponding to this is 0.498. Dividing it into 0.8 gives a quotient of 1.6. While this quotient is not a whole number, selection of a serum sample of the proper size, in this instance 0.16 ml, simplified the final calculation. The result, expressed as mg of cholesterol in 100 ml of blood, is obtained by multiplying the L value by 1000. Similarly, the second standard, if needed, is set at 56[1] giving an L value of 0.250. Since the color developed is not entirely stable, each

[1] The technical assistance of Misses Jane Bourne and Rhoda Greenberg is acknowledged with thanks. The paper includes several helpful suggestions made by Dr. J. Harold Austin.

standard is replaced in the instrument at regular intervals and the setting readjusted to maintain the standards at the desired L values. Marked change in readings or lack of consistency in the color developed by the standards would preclude use of the simplified method, although the same objection would apply to results obtained by conventional procedures.

The accuracy obtained will depend on the extent to which the readings of the standards deviate from the desired scale values and the consequent extent of the shutter or galvanometer adjustment. It has been possible to select filters or alter sample sizes so that this deviation was kept to less than 5 mg per 100 ml for blood sugar, 1 mg per 100 ml for blood urea N, and 0.1 g per 100 ml for serum protein in a range of values ordinarily encountered. If the instrument adjustment required is appreciable, error will increase in proportion (1) as zero is approached, and (2) as the difference between concentration of the unknown and that of the standard increases.

Application of this method of calculation does not necessarily require that standards be included with each run. In clinical laboratory procedure, however, this is desirable regardless of the system of calculation employed, particularly when equilibrium reactions like those involved in blood sugar determinations are utilized.

The method described deviates partially from the accepted principles of photometry and any new applications should be thoroughly tested. Furthermore, it deliberately introduces an approximation and thus should be used with discrimination. However, it is suitable for many purposes and has proven to be of great convenience where large numbers of analyses are required in a minimal period as in the clinical laboratory. Here it has expedited analyses and decreased the number of errors arising in calculation of results.

The simplified procedure has been applied to determinations of blood sugar, urea nitrogen, serum total protein, and free and esterified cholesterol. The results in each instance have agreed closely with those obtained by the standard method of readings. Differences have been small and without statistical significance.

The Separation of Purine and Pyrimidine Bases and of Nucleotides by Ion Exchange

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As a prelude to tracer studies on the enzymatic formation and degradation of nucleic acids, we have undertaken to develop ion exchange techniques for the separation and analysis of the various bases, nucleosides, and nucleotides at the milligram level. Although this technique is finding increasing application in biochemical studies for separation of these compounds (*8, 9*) and others (*1, 2, 6, 7*), we were led independently to its use through our earlier experience with radioisotope separation by ion exchange chromatography (*4, 11*). The present note describes briefly methods we have developed for the separation of each of the purine and pyrimidine bases and of the ribose nucleotides.

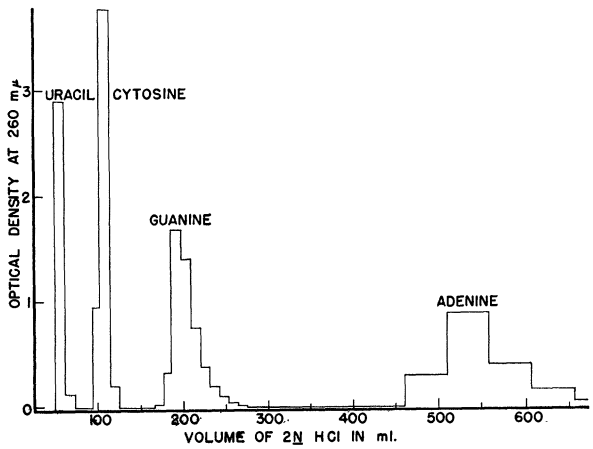

FIG. 1. Separation of purine and pyrimidine bases by cation exchange. *Exchanger*: 8.1 cm × 0.74 cm² Dowex-50, ca. 300 mesh, H⁺ form. *Eluting solution*: 2*N*-HCl at 0.6 ml/min. *Test material*: 0.5 mg uracil, 1.0 mg each of adenine, guanine, and cytosine, in 7.5 ml 2*N*-HCl. *Recoveries*: 98.5–101.5% (based on optical density at 260 mμ).

Of the five naturally occurring purine and pyrimidine bases, three (cytosine, guanine and adenine) exist as cations in solution of pH < 4 and hence are able to combine with cation exchangers. Since their affinities for the cation exchanger differ, they may be eluted successively with such simple reagents as HCl or NaCl; this is demonstrated in Fig. 1. This procedure does not separate

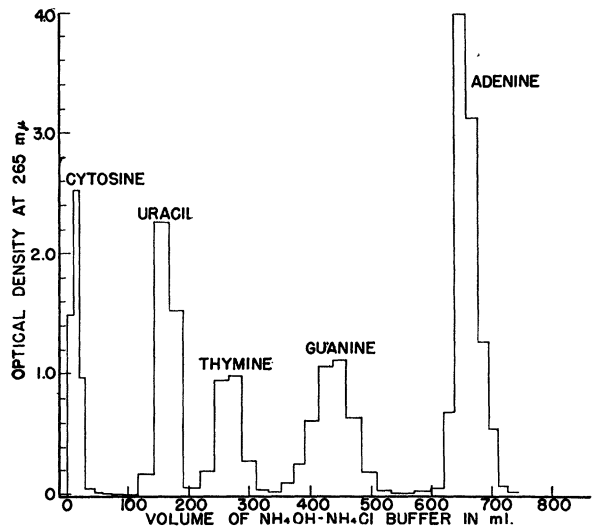

FIG. 2. Separation of purine and pyrimidine bases by anion exchange. *Exchanger*: 8.5 cm × 0.74 cm² Dowex-Al, ca. 300 mesh, Cl^- form. *Eluting solution*: 0.2*M*-NH_4OH-NH_4Cl buffer, pH 10.6, 0.025 *M*-Cl^- (at 575 ml, pH changed to 10.0, Cl^- to 0.1 *M*), at 0.25 ml/min. *Test material*: 1 mg cytosine, 2 mg each of other bases, in 10 ml eluting solution. *Recoveries*: 97.5–99% (based on optical density at 265 mμ).