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Technical methods

Improved method for the automatic determination of serum inorganic phosphate

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The methods currently available for the automated determination of phosphate suffer from technical disadvantages. The Fiske and Subbarow (1925) method, which is the basis of the Technicon N method, is relatively insensitive and requires the use of a concentrated mineral acid and a heating bath. None of the three methods recently published by Baginski, Epstein, and Zak (1964) avoid the possibility of contamination of one specimen by another. All three methods were designed for a rate of analysis of 40 specimens per hour. Two require incubation of reagents at 95°C. while the third involves the use of a 7-foot time-delay coil. Delsal and Manhouri (1958) described a method for the determination of inorganic phosphate which they claimed was 3·5 times more sensitive than the Fiske and Subbarow procedure. This method has now been adapted to the AutoAnalyzer (Technicon Instruments Company, Ltd.) and can be used to make 60 determinations per hour, with very little contamination of one specimen by another.

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PRINCIPLE

Inorganic phosphate is separated from protein by dialysis and allowed to react with ammonium molybdate at *p*H 4·0. The colourless phosphomolybdic acid which is formed is reduced by metol (p-methylaminophenol sulphate) to a blue complex which is measured colorimetrically.

REAGENTS

SAMPLE DILUENT NaCl, 9·0 g., per litre distilled water with 10 drops 10% Brij (Honeywell & Stein, Ltd.) added.

RECIPIENT SOLUTION Acetate buffer prepared by dissolving 2·5 g. $CuSO_4 \cdot 5H_2O$ in about 800 ml. distilled water after which 23 g. hydrated sodium acetate is added, followed by 116 ml. glacial acetic acid and the solution diluted to 1 litre with water. Fifteen drops of Brij are added.

AMMONIUM MOLYBDATE Ammonium molybdate, 5 g., dissolved in 100 ml. distilled water (no wetting agent).

METOL (P-METHYLAMINOPHENOL SULPHATE) Metol, 2 g., is dissolved in 320 ml. water with 10 g. hydrated sodium sulphite ($Na_2SO_3 \cdot 7H_2O$) and the solution made up to 400 ml. with distilled water and filtered. Eight drops of Brij are added. This solution is stable for several weeks if stored at 4°C. but in order to ensure good peaks on the recorder chart it should be brought to room temperature before use.

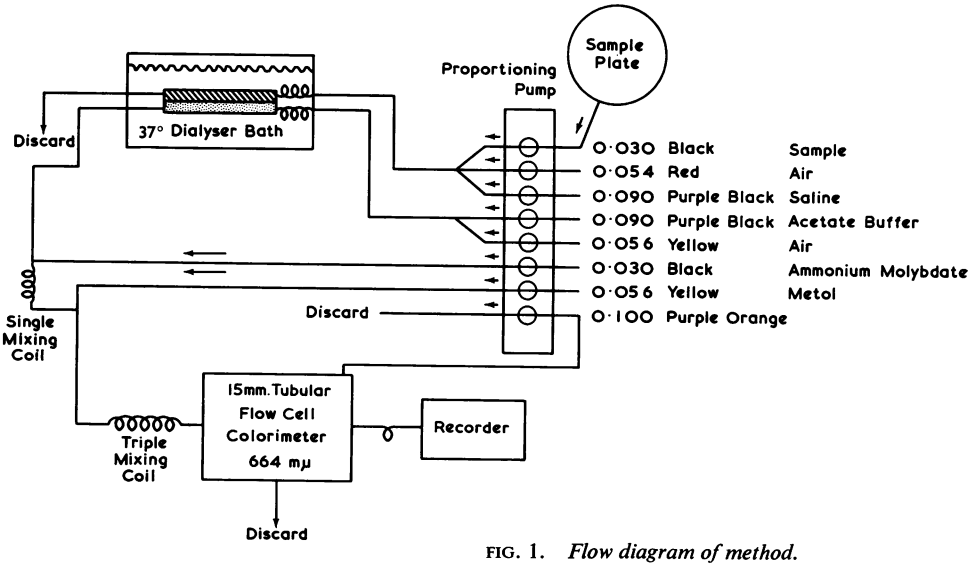

FIG. 1. *Flow diagram of method.*

STANDARD SOLUTIONS With each batch of determinations a set of standards containing from 1 to 5 mEq. inorganic PO_4 per litre (1·7-8·6 mg./100 ml.) and 10 mEq. per litre (17·2 mg./100 ml.) are run in ascending order. The flow diagram is shown in Fig. 1 and a typical chart in Figure 2.

While the nominal values for the internal diameters of the manifold tubing are shown in Fig. 1 the tracing shown in Fig. 2 was obtained when the actual volumes shown in Table I were aspirated. During this run the speed of the pump was 1 revolution per 15 seconds, room temperature was 21 °C., and the dialyser bath temperature was 36·5 °C.

Determinations are made at a rate of 60 per hour and filters with a wavelength of 664 mμ are used in conjunction with a tubular flowcell of a nominal 15 mm. light path. The sample is added to the diluent through an 'HI' cactus with a fine polyethylene insert to insure uniform distribution of the sample into the diluent. One hundred per cent transmission is set while the manifold is pumping reagents. To reduce the contamination from one specimen to the next the sample line may be flushed with water between samples. The Technicon sampler (model 2) performs this automatically.

TABLE I

COMPARISON OF ACTUAL AND THEORETICAL VOLUMES OF SOLUTIONS ASPIRATED

Solution	*Actual Volume (ml./min.)*	*Theoretical Volume (ml./min.)*
Sample	0·32	0·32
Saline diluent	2·80	2·90
Acetate buffer	2·75	2·90
Ammonium molybdate	0·34	0·32
Metol	1·17	1·20
Discard	3·39	3·40

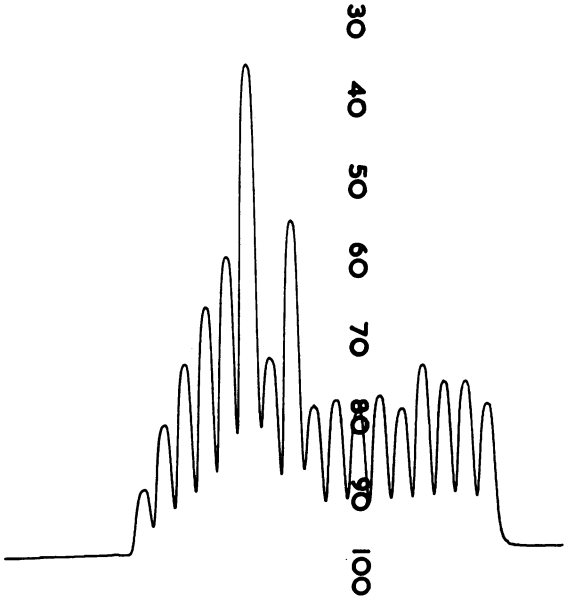

FIG. 2. *Reproduction of recorder tracing without water wash between samples with the conditions mentioned in the text. Six standards are followed by 11 serum samples.*

RESULTS

REPRODUCIBILITY A serum pool was analysed in random positions during routine analyses on 63 separate occasions without any automatic water-wash between samples. The recorded answers varied from 1·9 to 2·3 mEq./l. with a mean of 2·09 and a standard deviation of 0·07 mEq./l.

COMPARISONS WITH AUTOMATED FISKE AND SUBBAROW METHOD One hundred and twenty sera were analysed by both methods. Specimens were analysed in random order without aspirating water through the sample line between

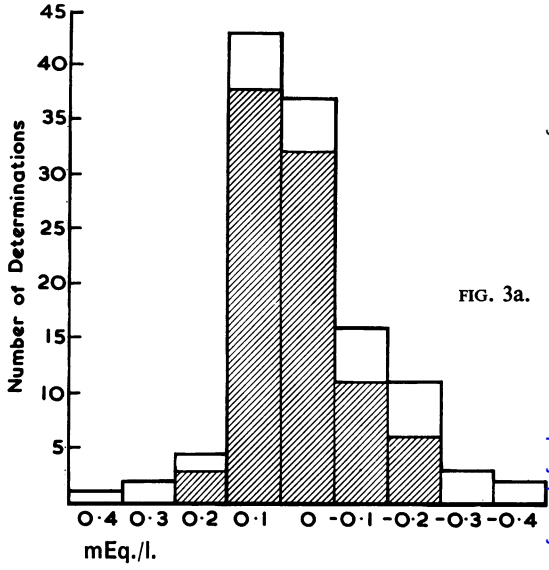

FIG. 3a.

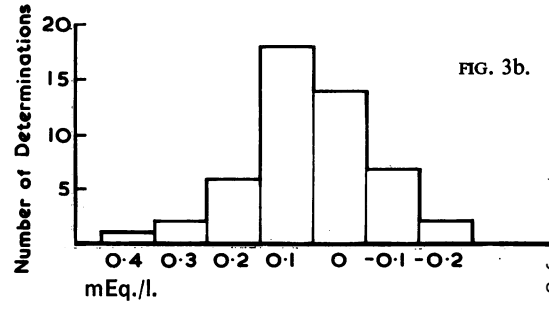

FIG. 3b.

FIG. 3. *Comparison of automated Fiske and Subbarow methods* (FIG. 3a) *and* (FIG. 3b) *manual Delsal and Manhouri methods. Where the other methods yield a higher value than the present method the difference is recorded as a positive value. If the present method gives a higher value the discrepancy is indicated by a negative sign. The shaded area in Fig. 3a compares sera with phosphate values within the normal range.*

TABLE II

EFFECT OF AUTOMATIC WATER WASH ON APPARENT VALUES OF INORGANIC PHOSPHATE

Concentration of Preceding Sample (mEq./l.)	*Actual Concentration of Sample (mEq./l.) without Water Wash*						*Actual Concentration of Sample (mEq./l.) with Water Wash*					
	10·0	5·0	4·0	3·0	2·0	1·0	10·0	5·0	4·0	3·0	2·0	1·0
10·0	10·1	5·2	4·25	3·25	2·3	Swamped	9·95	5·05	4·05	3·05	2·1	Swamped
5·0	—	—	4·05	3·1	2·1	1·1	—	—	4·05	3·05	2·05	1·0
4·0	—	—	—	3·05	2·05	1·0	—	—	—	3·0	2·05	1·0
3·0	—	—	—	—	2·05	0·95	—	—	—	—	2·05	0·95
2·0	—	—			—	0·95	—	—	—	—	—	1·0

samples. The results are shown in Figure 3a. The values ranged from 0·8 to 8·7 mEq./l. The greatest discrepancies were found in those sera with most elevated values. If only the 90 sera with PO_4 values within the normal range of 1·6 to 2·4 mEq./l. (Wootton, 1964) were considered there was no variation of greater than 0·2 mEq./l. as shown in Figure 3a.

COMPARISONS WITH MANUAL DELSAL AND MANHOURI METHOD (WOOTTON, 1964) Fifty comparisons were made on sera with phosphate values ranging from 1·0 to 7·5 mEq./l. The results are shown in Figure 3b.

CONTAMINATION OF ONE SPECIMEN BY ANOTHER Because of the poor wash obtainable in the AutoAnalyzer phosphate methods there is a greater possibility of contamination of specimens than in most automated procedures. Similar contamination during the analysis of urea has been reported by Thiers and Oglesby (1964). With the present phosphate method it was found that contamination was reduced by aspirating water through the sample line in the interval between sampling from the cups in the sampler plate as shown in Table II. It is probable that the contamination occurring with the other available automated phosphate methods would be similarly reduced by flushing the sample line with water but this was not investigated.

DISCUSSION

The phosphate method described combines simplicity with accuracy. The reagents are easily prepared without careful weighing or the use of concentrated mineral acids or poisonous substances. Gomori (1942) has already stressed the ease of preparation and stability of metol as the reducing agent in comparison with the aminonaptholsulphonic acid of the Fiske and Subbarow (1925) procedure.

Most of the experimental work on the automated Delsal and Manhouri procedure was performed without automatic water-wash between samples. The wash characterestics are still better than those of the methods of Baginski *et al.* (1964), but may be further improved by means of an automatic water wash between samples. The phosphate method described can be used to measure urinary phosphate by making a 1 in 5 or 1 in 10 dilution of the urine with distilled water. Serum samples with phosphate values of greater than 10 mEq./l. may be diluted 1 in 2 with normal saline.

It is possible to dispense with a long-time delay coil in the automated Delsal and Manhouri procedure because the reduction of the phosphomolybdic acid by metol is complete within three minutes. Delsal and Manhouri (1958) stated that their procedure might yield lower results than other methods for the determination of serum inorganic phosphate because of the possibility of hydrolysis of labile phosphate esters when heat and strong acids are used as in the Fiske and Subbarow procedure. This might partly account for the average lower results yielded by the automated Delsal and Manhouri procedure shown in Figure 3a. In our experience the presence of a heating bath is one of the most common sources of trouble in automated procedures due to the difficulty of cleaning the inside of the coil. It has been found that the automated Delsal and Manhouri procedure has given rise to fewer problems than the Fiske and Subbarow procedure during routine use, which may be attributable to the absence of a heating bath.

SUMMARY

A new method for the automatic determination of serum inorganic phosphate is described. The method combines accuracy with simplicity. The advantages of this method are discussed.

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