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USE OF GEL FILTRATION IN THE ASSAY OF URINARY ENZYMES

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SUMMARY

Gel filtration of urine provides a simple and rapid, yet reliable, method for the separation of lactate dehydrogenase, alkaline and acid phosphatase, leucine aminopeptidase, arylsulphatase, and β-glucuronidase from interfering substances. It removes inhibitors more completely than dialysis and separates all spurious lactate dehydrogenase and alkaline phosphatase activities from the protein enzymes.

INTRODUCTION

The determination of urinary enzymes is increasingly being used for detection, differential diagnosis and following the course of urinary tract diseases[1–6]. However, various physiologically excreted ions and substances of low molecular weight interfere with the assay of enzymatic activity in "native" urine. Specifically, inorganic phosphate[7] and a urinary pigment[8] competitively inhibit phosphatases, saccharo-1,4-lactone[9] and various organic acids[10,11] inhibit β-glucuronidase, and two peptides of low molecular weight[12,13] inhibit lactate dehydrogenase. The ionic strength of the specimen may also influence enzyme activity[14,15]. Dialysis of urine against water previous to enzyme assay has been proposed to remove these interferences[1–5,16–20]. The present experiments investigate: (I) the efficiency of the commonly recommended method of dialysis in removing substances that interfere with enzyme analysis from urine; and (II) the advantages of a simple gel filtration technique as an alternative to dialysis. The six enzymes studied were selected because they have found wide clinical interest. Assay methods are partly modified micro versions of standard techniques.

METHODS

Samples

Urines were kept without additive at 4° until analysed (at the most 4 h), samples with abnormal protein or glucose content were discarded. Samples (10 ml) were

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centrifuged for 5 min at 4,500 r.p.m. Only the top 5 ml were used for analysis. If removed carefully this fraction is free of cells.

Dialysis

About 25 cm of dialysis tubing (7 mm diameter, 0.02 mm thickness. Type 8/32, Nr. 44104, Visking Corp., Chicago, Ill.) was filled with 5 ml of urine and tied safely at both ends. Dialysis was performed at room temperature against running tap water (12–15°) for various lengths of time, routine use was for 2 h (arylsulphatase was routinely dialyzed for 18 h at 4° against six changes of tap water)[17]. The water uptake during dialysis was determined by weighing and corrected for when calculating enzyme activities.

Gel filtration

Glass columns with a small dead space (inner diameter 1 cm, height 40 cm) were filled to a height of 14.5 cm with hydrated Sephadex G-50 (Pharmacia, Uppsala, Sweden) giving a total gel bed of 11.6 cm^3. A rubber sponge of 1 cm thickness was placed on top of the gel bed to facilitate sample application and to prevent the column from running dry[21]. Separation was performed at room temperature. Physiological saline (0.154 *M* NaCl) was used as eluant. To analyze the separation patterns, eluates were collected in 1 ml fractions. For routine analysis the following elution procedure was adopted: the sample (3 ml) was washed into the column with 1 ml of eluant followed by another 1 ml of eluant. The liquid emerging from the column up to that time (5 ml) was discarded. 6 ml of saline were now placed on the column, and the corresponding eluates collected for enzyme analysis. The column was then filled and rinsed with saline to prepare it for re-use.

Assay methods

Lactate dehydrogenase (EC 1.1.1.27). This was determined according to DORFMAN, AMADOR AND WACKER[18] with the following modification: 0.2 ml sample, 0.4 ml buffer-substrate solution (6.2 g sodium pyrophosphate, 2.34 ml 90% lactic acid, and 1.1 g NAD in 200 ml, pH 8.8) were used. Kinetic measurement was at 334 nm and 25°. One unit is defined as an increase of 0.001 O.D./min.

Alkaline phosphatase (EC 3.1.3.1). This was determined according to AMADOR, ZIMMERMAN AND WACKER[16] with the following modification: 0.2 ml sample and 0.4 ml buffer-substrate solution (2-amino-2-methyl-1-propanol-HCl, 0.75 *M*, pH 10.3; disodium-*p*-nitrophenylphosphate, 4.5 m*M*) were used. Kinetic measurement was at 405 nm and 25°. One unit is defined as an increase of 0.001 O.D./min.

Acid phosphatase (EC 3.1.3.2). 0.1 ml sample, 0.25 ml acetate buffer (0.2 *M*, pH 5.4), 0.25 ml substrate (disodium-*p*-nitrophenylphosphate, 12.6 m*M*) were used. After 10 min at 25° incubation was terminated by adding 2.5 ml NaOH (1 *N*), and the optical density measured at 405 nm. Results were corrected for reagent and sample blanks. Activity is reported in U/l.

Leucine aminopeptidase (EC 3.4.1.1). Here the L-leucine-*p*-nitranilide cleaving enzyme was determined according to NAGEL, WILLIG AND SCHMIDT[22] with the following modification: 0.02 ml sample, 0.6 ml Tris-buffer (0.05 *M*, pH 7.5), and 0.02 ml substrate (L-leucine-*p*-nitranilide, 0.025 *M*) were taken. Kinetic measurement was at 405 nm and 25°. Activity is reported in U/l.

Arylsulphatase (EC 3.1.6.1). Only arylsulphatase A was assayed. This was determined according to BAUM, DODGSON AND SPENCER[17] with the following modification: 0.2 ml sample, 0.1 ml buffer (1.0 *M* acetate buffer containing 0.5 m*M* $Na_4P_2O_7$ and 10% w/v sodium chloride, pH 5.0), and 0.1 ml substrate (2-hydroxy-5-nitrophenyl sulphate, 0.02 *M*) were used. After 60 min at 37°, incubation was terminated by adding 0.6 ml NaOH (1 *N*), and the optical density was measured at 515 nm. Results were corrected for reagent and sample blanks. One unit is defined as 1 μg/h/ml liberated 4-nitrocatechol.

β-Glucuronidase (EC 3.2.1.31). This was determined according to SZASZ[23]: 0.05 ml sample, 0.4 ml acetate buffer (0.2 *M*, pH 4.0) nad 0.05 ml substrate *p*-nitrophenyl-β-D-glucuronide, 50 m*M*) being used. After 300 min at 37°, incubation was terminated by adding 0.2 ml NaOH (0.5 *N*), and the optical density was measured at 405 nm. Results were corrected for reagent and sample blanks. Activity is reported inU/l.

Inorganic phosphate was determined by the vanadium–molybdate method[24]. Optical densities at 280 nm and at 405 nm were measured on a PMQ II spectrophotometer (Zeiss, Oberkochen, Württemberg, West Germany). All determinations were performed in duplicate.

RESULTS

Dialysis of urine

Fig. 1 shows the activities of leucine aminopeptidase, lactate dehydrogenase, acid and alkaline phosphatase, β-glucuronidase, and arylsulphatase before and after dialysis. In all instances the dialyzed samples had accelerated substrate turnover. Fig. 2 shows the effect of varying lengths of time of dialysis. There was an almost exponential removal of all inorganic phosphate but substances absorbing light at 280 nm and at 405 nm (urochromes) were only partly removed. The apparent activity

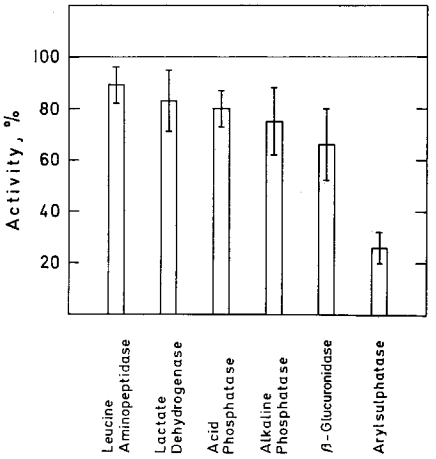

Fig. 1. Activities of six urinary enzymes, before and after dialysis. Activity before dialysis is expressed in per cent of the activity measured after dialysis. Ten urines were analyzed, mean values ± 1 standard deviation are plotted.

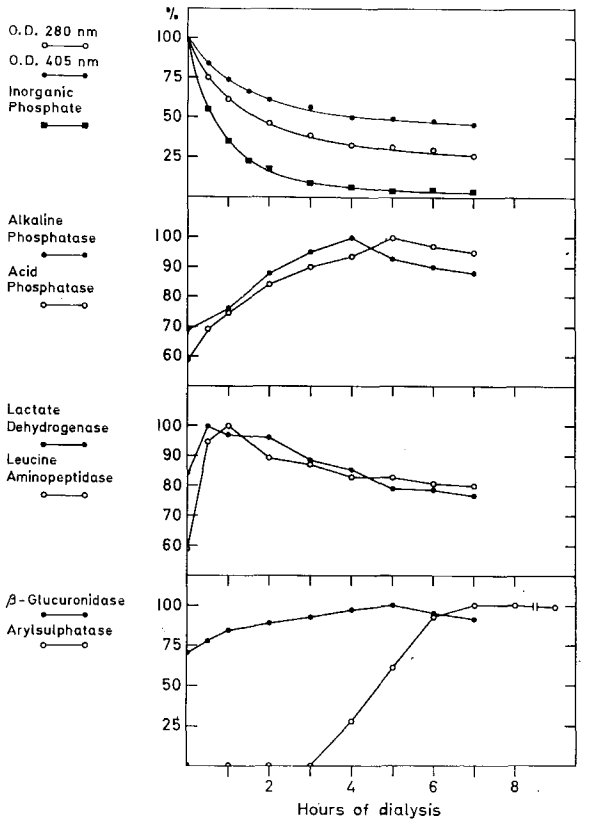

Fig. 2. Effect of varying the length of time of dialysis upon the optical densities at 280 nm and 405 nm, the concentration of inorganic phosphate, and the apparent activities of six enzymes in urine. Results are reported in percent of the highest measured value.

of the six assayed enzymes initially increased. Lactate dehydrogenase and leucine aminopeptidase reached peak activity during the first hour, alkaline phosphatase, acid phosphatase and β-glucuronidase from the fourth to the fifth hour, and arylsulphatase at the seventh hour. Subsequently the activities of all enzymes, except arylsulphatase, decreased.

Gel filtration of urine

The first 5 ml of the eluates were free of enzymes activity (see Fig. 3). The activity of all the assayed enzymes reached a peak in the next 6 ml with a maximum in the eighth ml. Lactate dehydrogenase and alkaline phosphatase showed a second peak of apparent activity with a maximum at the 16th and 17th ml, where substances absorbing light at 280 nm and inorganic phosphate also emerged. Heating to 95° abolished all lactate dehydrogenase and alkaline phosphatase activities found in the first peak, while the same treatment eliminated only a verying fraction of the apparent activities found in the second peak. Fig. 4 compares the separation of the two activity

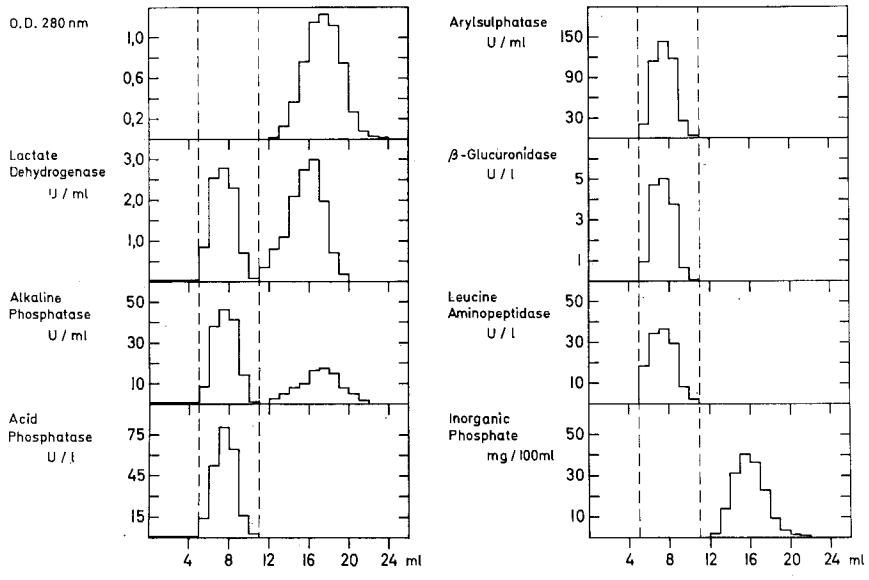

Fig. 3. Gel filtration of urine on Sephadex G-50, fine. Optical density at 280 nm (eluates diluted 1:20), apparent activities of six enzymes and concentration of inorganic phosphate in the eluates are shown. The dashed lines indicate the eluate fraction used in the routine procedure.

peaks on coarse and fine Sephadex G-50. In both instances the first peak emerged earlier on the coarse gel but only the fine gel separated the two peaks completely. This gel was used in all further experiments.

To test the accuracy of gel filtration urine samples had the interfering substances removed by a first gel filtration, then the samples were filtered a second time. 96% to 109% of the lactate dehydrogenase activity found after the first column passage

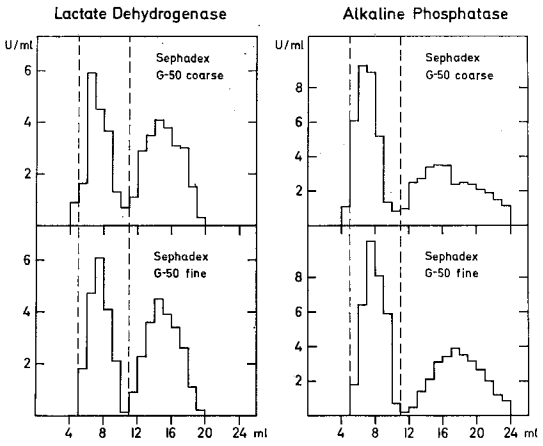

Fig. 4. Gel filtration of urine on Sephadex G-50, coarse (top), and Sephadex G-50, fine (bottom). The apparent activities of lactate dehydrogenase and alkaline phosphatase in the eluates are shown. The dashed lines indicate the eluate fraction used in the routine procedure.

TABLE I

ACCURACY OF GEL FILTRATION

Enzymatic lactate dehydrogenase activity was separated from interferences by gel filtration, subsequently the enzyme fraction was chromatographed a second time.

Urine	*Activity after first gel filtration U/ml*	*Activity after second gel filtration U/ml*	*Recovery %*
1	10.3	11.2	108.7
2	9.8	10.4	104.1
3	8.4	8.2	97.6
4	8.4	8.6	102.4
5	6.1	6.0	98.4
6	5.8	5.6	96.6
7	2.4	2.6	108.3
8	2.0	2.0	100.0
Mean	6.7	6.8	102.0

were thus recovered (Table I). Precision was tested by four replicate filtrations of the same urine on three different columns. The coefficient of variation of these twelve determinations (which includes the error inherent in the enzyme assay) was 2.4% (Table II).

Comparison of dialysis and gel filtration

Table III lists the enzyme activities of 25 urines after gel filtration and after dialysis. In all cases the activities of acid phosphatase, leucine aminopeptidase and β-glucuronidase were higher after gel filtration. The activity of arylsulphatase after 18 h dialysis and after gel filtration was not significantly different. (χ^2-test for positive and negative signs: $0.05 < P < 0.10$). The apparent activities of lactate dehydrogenase and alkaline phosphatase on the other hand were lower after gel filtration than after dialysis. For both activities substrate turnover, however, was linear after gel filtration, while substrate turnover after dialysis usually decreased with time. To investigate this difference a urine specimen was dialyzed for varying

TABLE II

PRECISION OF GEL FILTRATION

The arylsulphatase activity was assayed in twelve specimens of the same urine after gel filtration on three different columns. All results are reported in U/ml.

Column	*A U/ml*	*B U/ml*	*C U/ml*
Run 1	39.2	38.8	37.6
2	36.9	35.8	37.2
3	37.1	38.2	37.3
4	37.9	38.0	37.3
Mean for column	37.8	37.7	37.4
Grand mean		37.6	
Standard deviation		0.9	

TABLE III

COMPARISON OF THE APPARENT ACTIVITY OF SIX ENZYMES AFTER GEL FILTRATION (G) AND AFTER DIALYSIS (D)

The difference between the two results is expressed in per cent of the value found after gel filtration.

Urine	*Lactate dehydrogenase*			*Alkaline phosphatase*			*Acid phosphatase*		
	G U/ml	*D* U/ml	*Diff.* %	*G* U/ml	*D* U/ml	*Diff.* %	*G* U/l	*D* U/l	*Diff.* %
1	2.8	5.4	+93	3.8	6.8	+79	22	13	−41
2	3.6	7.6	+111	16.5	20.0	+22	450	341	−24
3	1.1	4.8	+336	4.1	7.6	+85	227	209	−8
4	4.1	6.9	+68	9.2	8.5	−8	43	20	−53
5	11.1	9.9	−11	50.2	54.5	+9	301	272	−10
6	3.4	5.3	+56	9.0	7.0	−22	242	187	−23
7	4.9	5.1	+4	9.6	10.9	+14	37	34	−8
8	5.3	6.2	+17	18.0	22.1	+23	691	274	−60
9	4.0	5.6	+40	7.3	10.3	+41	237	174	−27
10	4.0	3.6	−10	4.9	7.3	+49	25	14	−44
11	5.3	6.1	+15	23.7	28.4	+20	629	319	−49
12	3.5	4.1	+17	6.7	7.9	+18	245	203	−17
13	3.5	3.8	+8	5.2	7.3	+40	30	24	−20
14	8.1	8.0	−1	29.3	34.7	+18	1018	850	−17
15	5.1	6.8	+33	9.7	12.5	+29	260	256	−2
16	2.6	4.6	+77	6.0	8.4	+40	20	16	−20
17	4.6	5.3	+15	11.7	16.5	+41	94	71	−24
18	21.2	21.6	+2	18.8	38.1	+103	204	187	−8
19	163.6	169.8	+4	16.9	13.6	−20	238	211	−11
20	2.9	4.5	+55	2.6	5.3	+104	23	18	−22
21	2.2	4.7	+114	15.3	25.7	+68	633	617	−3
22	4.4	6.2	+41	23.7	35.4	+49	63	50	−21
23	3.5	4.8	+37	6.9	9.4	+36	292	271	−7
24	1.5	4.2	+180	6.5	9.7	+49	29	22	−24
25	1.5	4.6	+207	6.5	9.0	+38	41	38	−7
Mean	11.1	12.8	+60	12.9	16.7	+37	244	188	−22

lengths of time before gel filtration (Fig. 5). As dialysis progressed the first peak retained its heat-labile lactate dehydrogenase and alkaline phosphatase activities unchanged while the apparent activities in the second peak decreased. After two hours dialysis some of the apparent activity in the second peak, however, still contributed to the total activity. Proceeding in reverse order, eluates containing the second peak were collected from gel filtration and dialyzed. Under these circumstances, dialysis removed all apparent lactate dehydrogenase and alkaline phosphatase activity within half an hour.

DISCUSSION

Dialysis increases the measurable urinary activity of all the six investigated enzymes by removing inhibitory substances. Different enzyme activities, however, reached their peak at different times during dialysis and subsequently decreased (except arylsulphatase); therefore, the same dialysis procedure cannot be applied to prepare a specimen for the assay of multiple enzymes. With gel filtration on Sephadex G-50,

Arylsulphatase			*β-Glucuronidase*			*Leucine amino-peptidase*		
G U/ml	*D* U/ml	*Diff.* %	*G* U/l	*D* U/l	*Diff.* %	*G* U/l	*D* U/l	*Diff.* %
4.4	4.6	+4	0.20	0.12	−40	4.4	2.9	−34
29.0	28.2	−3	1.29	0.92	−29	9.9	5.6	−43
14.1	14.9	+6	0.43	0.26	−40	6.6	4.6	−30
9.4	5.0	−47	0.46	0.30	−35	4.6	4.0	−13
46.7	40.2	−14	2.08	1.21	−42	15.1	13.2	−13
16.4	6.6	−60	0.62	0.51	−18	6.0	4.7	−22
9.7	9.6	−1	0.46	0.29	−37	4.3	3.6	−16
19.8	17.9	−10	0.61	0.33	−46	7.9	7.0	−11
27.3	26.3	−4	0.55	0.45	−18	6.6	5.4	−18
3.3	3.7	+12	0.27	0.12	−56	4.1	2.8	−32
23.3	23.2	0	1.33	0.78	−41	7.9	7.0	−11
15.1	15.2	+1	0.54	0.43	−20	5.1	4.3	−16
15.9	16.6	+4	0.44	0.32	−27	7.1	4.1	−42
16.3	13.4	−18	1.12	0.62	−45	15.8	9.6	−39
7.6	7.5	−1	0.80	0.66	−17	10.1	6.6	−35
7.6	7.5	−1	0.56	0.45	−20	6.0	3.6	−40
10.3	7.0	−32	0.81	0.58	−28	7.5	5.5	−27
5.8	5.4	−7	2.42	1.66	−31	8.6	6.8	−21
8.9	8.7	−2	3.47	2.59	−25	10.1	7.6	−25
6.2	6.2	0	0.45	0.26	−42	6.0	2.3	−62
3.8	4.0	+5	1.31	0.79	−40	7.5	6.1	−19
1.6	1.2	−25	1.51	0.85	−44	6.4	4.8	−25
20.0	19.7	−1	1.54	1.05	−32	10.4	8.5	−18
5.1	5.2	+2	1.04	0.77	−26	6.2	3.6	−42
11.8	10.8	−8	0.66	0.48	−27	6.2	3.4	−45
13.6	12.3	−9	1.00	0.67	−33	7.6	5.5	−28

enzymes with a molecular weight over 30,000 emerge at the elution front and are also separated from smaller interfering substances. Even relatively large inhibitors and urochromes are thus completely removed from the urinary enzymes.

Although the eluant flow is more rapid on columns of coarse gel beads, the fine gel is preferable, since separation is sharper and elution from small columns remains sufficiently swift. Use of a sponge on top of the gel bed, or of a narrow constriction of the glass column immediately above the gel bed[25] prevents it from running dry. This makes constant column supervision unnecessary, and allows bulk elution for use in a clinical laboratory. Physiological saline is used as eluant, since proteins and other substances may adsorb to the gel when water is used. In the determination of multiple enzymes assay conditions differ, and buffers are, therefore, not suited as eluants. Sample volume and column dimensions are adjusted to minimize the dilution during column passage: 3 ml urine are eluated in 6 ml, a 1:2 dilution.

The method of gel filtration described is both accurate and precise. Higher urinary activities of acid phosphatase, β-glucuronidase and leucine aminopeptidase are found than after dialysis because gel filtration removes their inhibitors more

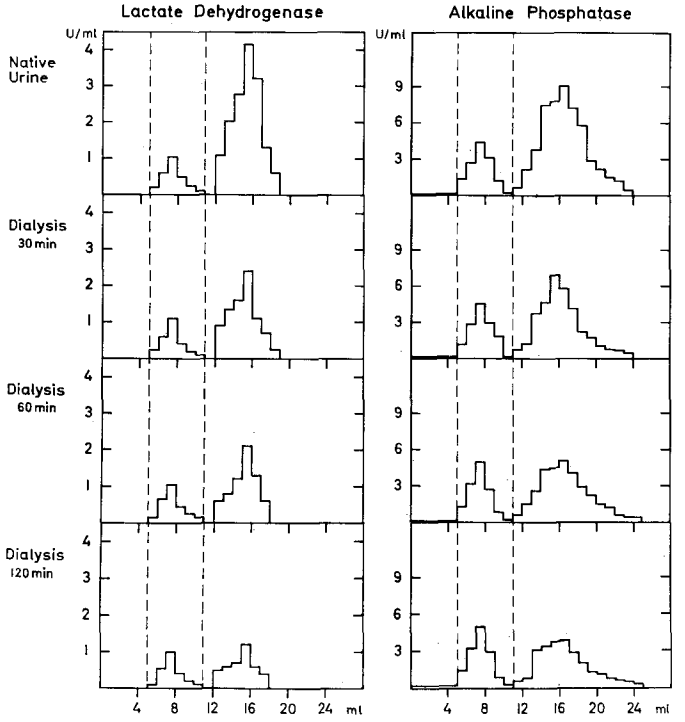

Fig. 5. Gel filtration of urine before and after dialysis of varying lengths of time. The apparent activities of lactic dehydrogenase and alkaline phosphatase in the eluates are shown.

completely. On the other hand the urinary activities of lactate dehydrogenase and alkaline phosphatase are higher after dialysis, as dialysis does not remove all the heat stable interfering substances causing spurious activity, which gel filtration separates from true enzyme activity. Since the sources and amounts of interfering substances in "native" urine are varied and unpredictable, changing from day to day even for the same individual[26], it is thus important that they be eliminated entirely.

REFERENCES

1 P. J. Butterworth, D. W. Moss, E. Pitkanen and A. Pringle, *Clin. Chim. Acta*, 11 (1965) 212.
2 O. Daniel, P. R. N. Kind and E. J. King, *Brit. Med. J.* 1 (1954) 19.
3 U. C. Dubach and R. Rediger, *Urol. Intern.* ,17 (1964) 65.
4 G. Golisch, *Klin. Wochschr.*, 38 (1960) 968.
5 W. Jösch and U. C. Dubach, *Z. Klin. Chem.*, 5 (1967) 59.
6 F. J. W. Lewis and C. H. J. Plaice, *Brit. J. Cancer*, 14 (1960) 106.
7 S. D. Hilliard, J. F. O'Donnell and S. Schenker, *Clin. Chem.*, 11 (1965) 570.
8 C. L. Walters, *Enzymologia*, 20 (1958) 33.
9 C. A. Marsh, *Biochem. J.*, 86 (1963) 77.
10 M. A. M. Abu-Fadl, *Biochem. J.*, 65 (1957) 16P.
11 S. Kushinsky and V. L. Chen, *Emzymol. Biol. Clin.*, 8 (1967) 266.
12 W. E. C. Wacker and G. A. Schoenenberger, *Biochem. Biophys. Res. Commun.*, 22 (1966) 291.
13 G. A. Schoenenberger and W. E. C. Wacker, *Biochemistry*, 5 (1966) 1375.

14 O. HYGSTEDT AND O. R. JAGENBURG, *Scand. J. Clin. Lab. Invest.*, 17 (1965) 565.
15 S. KUSHINSKY, V. L. CHEN AND J. OTTERNESS, *Nature*, 214 (1967) 142.
16 E. AMADOR, T. S. ZIMMERMANN AND W. E. C. WACKER, *J. Am. Med. Assoc.*, 185 (1963) 953.
17 H. BAUM, K. S. DODGSON AND B. SPENCER, *Clin. Chim. Acta*, 4 (1959) 453.
18 L. F. DORFMAN, E. AMADOR AND W. E. C. WACKER, *J. Am. Med. Assoc.*, 184 (1963) 1.
19 J. D. SCHMIDT, *Invest. Urol.*, 3 (1966) 395.
20 J. D. SCHMIDT, *Invest. Urol.*, 3 (1966) 405.
21 G. P. HICKS AND S. J. UPDIKE, *Anal. Biochem.*, 10 (1965) 290.
22 W. NAGEL, F. WILLIG AND F. W. SCHMIDT, *Klin. Wochschr.*, 42 (1964) 447.
23 G. SZASZ, *Clin. Chim. Acta*, 15 (1967) 275.
24 P. FLEURY AND M. LECLERC, *Bull. Soc. Chim. Biol.*, 25 (1943) 201.
25 R. L. PATRICK AND R. E. THIERS, *Clin. Chem.*, 9 (1964) 283.
26 W. PAUL, A. SCHAPIRO AND H. GONICK, *Enzymol. Biol. Clin.*, 8 (1967) 47.