Urinary isoamylases in the diagnosis of chronic pancreatitis

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EDITORIAL COMMENT The proportion of pancreatic to salivary isoenzymes of alpha amylase has been measured in 24-hour samples of urine and the absolute value of the isoenzymes calculated from urinary amylase values. In seven of eight patients exocrine deficiency of the pancreas was accompanied by an altered ratio of pancreatic to salivary isoamylases.

The quantitation of isoenzymes in body fluids has obvious applications in the diagnosis of disease. α -Amylase (E.C. 3.2.1.1.), of molecular weight 50,000, also occurs in multiple molecular forms. This heterogeneity was initially discovered in animals and later extended to man. The evidence has been principally electrophoretic (Nørby, 1964; Muus and Vnenchak, 1964; Berk, Searcey, Hayashi, and Ujihira, 1965; Kamaryt and Laxová, 1965; Aw, 1966). Extracts of salivary glands and pancreas, saliva, serum, and urine were shown to contain cathodally running bands of amylolytic activity differing only slightly in mobility. Recently the isoenzymes of amylase (isoamylases) from human pancreas, saliva, and milk have been partially purified with the use of Deae Sephadex and gel filtration thus demonstrating a chromatographic heterogeneity as well (Aw and Hobbs, 1966).

Urinary isoamylases are separable into 'pancreatic' and 'salivary' according to their electrophoretic mobilities compared with marker pancreatic and salivary enzymes. This organ specificity may, in fact, be oversimplified (see Discussion). It is well known that patients with chronic pancreatitis more often than not have serum and urinary amylase values within normal limits. We were interested in studying urinary isoamylase patterns and in finding out whether patients with exocrine pancreatic deficiency exhibited a decrease in the activity of the pancreatic zone.

MATERIALS

Twenty-four-hour urine collections, preserved with toluene, were obtained from three groups of people.

GROUP 1 Twenty-nine patients with no history or other evidence of exocrine pancreatic disease: this group also included five normal individuals. Total was 34.

GROUP 2 Seven cases of chronic pancreatitis diagnosed on the basis of history, clinical findings, and one or more of the following—pancreatic pseudocyst or calcification on radiographs, abnormal glucose tolerance curves, subnormal pancreatic function tests, and findings at operation. A patient who had total pancreatectomy for carcinoma of the pancreas. Total was eight.

GROUP 3 Four patients with diarrhoea of small bowel origin, three with radiological appearance of Crohn's disease. Two of them had the diagnosis confirmed surgically. A patient with intestinal alactasia. A patient with gross steatorrhoea who also suffered from diabetes mellitus whose pancreatic function tests results were normal. Total was six.

None of the members of the groups had overt kidney or liver dysfunction.

METHOD

Aliquots of the urines were filtered, concentrated by ultrafiltration to about 1/20th their original volumes, and deep-frozen in small plastic thimbles till used.

Urinary amylase was estimated as described by Wootton (1964).

Electrophoresis was carried out in a Wieme type apparatus on agar-coated microscope slides made in veronal buffer of pH 8.6, ionic strength 0.05. Each slide carried two sample slots placed 1 cm. away from the midline towards the side of the anode. The slots were made by inserting the short side of a 6 × 10 mm. strip of filter paper (Whatman no. 3MM) into the agar and allowing the buffer to creep up by 4 to 5 mm. before withdrawing the paper. Urine samples of about 2 μ l. were applied in each slot. Cellulose acetate can be employed as the separating medium though a longer run of two hours is needed to produce a separation comparable to that of agar on the Wieme apparatus.

The electrophoretic run was made at 200 volts, 20 mA per slide, and stopped after exactly one hour. Cellulose acetate strips cut to size were laid on the agar to obtain an imprint of the zymogram pattern and

transferred to starch substrate plate (Aw, 1966) for an hour's incubation at 37°C. The strips were then lifted off carefully and the starch plate was stained in a bath consisting of 50 ml. working iodine solution used in the amylase estimation and 60 ml. of absolute alcohol. When the undigested background was uniformly purple and the digested bands stood out clearly on viewing against a diffuse light source the wash was terminated. After drying a few minutes in air the plates were scanned. We used a Joyce Loebel double-beam automatic recording microdensitometer with an aperture setting of 1 mm. and a magnification of 5 (Model Mark 111B). If the Joyce Loebel Chromoscan, a double-beam recording and integrating densitometer, is used the grey-wedge (optical attenuator) is reversed from the normal position, that is, the dense end is now on the right instead of the left. This enables the optically clear areas of the isoenzyme bands to be scanned by transmission and recorded in the usual way.

The sample holder of the Chromoscan can accommodate starch plates of 2.5 cm. width, that of a microscope slide. But starch gel casts on microscope slides are difficult to handle being easily torn or shed during the wash. This difficulty is avoided by the following procedure which has been used for preparing agar slides. Molten agar is allowed to solidify in Petri dishes. Four microscope slides are placed on the hydrostatically level base so formed. Into the dish is poured the hot degassed starch solution (about 33 ml.) calculated to give a starch gel thickness of about 1 mm. over the slides. Cellulose acetate transfers are carefully positioned over the slides, the whole incubated, and staining carried out with the slides *in situ*. Individual slides are cut out with a scalpel, air-dried, and scanned.

RESULTS

The isoamylases of the urinary zymograms are numbered from the slowest, nearest the cathode, to the fastest in the order P1, P2, P3 (pancreatic group) and S1, S2, S3 (salivary group). Three basic patterns are seen according to the location and number of the pancreatic bands. The salivary patterns usually contain S1 or S1 and S2. Three representative scans are shown in Figure 1. Of the total 40 control urines examined, 35 were type 1, three were type 2, and two were type 3. The proportions of pancreatic and salivary isoamylases were measured from the scans and the absolute values of the isoenzymes calculated from the urinary amylase values. The ratios of pancreatic to salivary enzymes of the three groups were then calculated. The results are set out in Table I.

Inspection alone often suffices to reveal whether a zymogram is deficient or not in pancreatic amylase (Fig. 2). This is especially so with type 1 urines which form the majority. Urine 10 (Table I) presented an equivocal zymogram on inspection which on scanning gave a ratio of 0.9. The urines of patients

FIG. 1. Scans of three representative normal zymograms of urinary isoamylases. Vertical line marks off pancreatic (P) from salivary (S) isoamylases, which are numbered from the slowest isoenzyme. About 90% of normal urines are type 1 in their isoamylase pattern.

TABLE I
TABULATED RESULTS OF SURVEY OF URINARY AMYLASE
ON 24-HOUR URINE COLLECTIONS IN GROUP 1

No.	Amylase/100 ml.			Pancreatic/ Salivary
	Urinary	Pancreatic	Salivary	
1	200	155	45	3.4
2	121	76	45	1.7
3	140	77	63	1.2
4	218	138	80	1.7
5	150	95	55	1.7
6	275	187	88	2.1
7	103	78	25	3.1
8	110	67	43	1.5
9	133	81	52	1.6
10	325	157	168	0.9
11	85	62	23	2.7
12	143	89	54	1.6
13	122	81	41	2.0
14	170	110	60	1.8
15	400	250	150	1.7
16	145	82	63	1.3
17	200	116	84	1.4
18	147	91	56	1.7
19	109	71	38	1.9
20	282	216	66	3.3
21	61	41	20	2.0
22	50	35	15	2.3
23	180	106	74	1.4
24	222	163	59	2.8
25	85	47	38	1.2
26	262	159	103	1.5
27	266	144	122	1.2
28	88	48	40	1.2
29	78	51	27	1.9
30	206	134	72	1.9
31	170	116	54	2.1
32	200	125	75	1.7
33	103	64	39	1.6
34	113	73	40	1.8
Mean	165	105	61	
S.D.	77	48	53	
Range	50-400	35-250	15-168	0.9-3.4

TABLE II
FINDINGS FROM EIGHT PATIENTS WITH KNOWN
PANCREATIC DISEASE (GROUP 2) AND CASES OF
GROUP 3

No.	Patient	Nature of Disease	Urinary Amylase	Pancreatic Isoamylase	Salivary Amylase	Pancreatic/Salivary
Group 2						
1	J. M.	Carcinoma of pancreas (pancrea-tectomy)	111	31	80	0.4
2	E.H.	Chronic pancreatitis	88	15	73	0.2
3	J.I.	Chronic pancreatitis	290	101	189	0.5
4	C.P.	Chronic pancreatitis	90	21	69	0.3
5	M.M.	Chronic pancreatitis	222	86	136	0.6
6	E.M.	Chronic pancreatitis	60	0	60	0
7	L.W.	Chronic pancreatitis	121	81	40	2.0
8	W.R.	Chronic pancreatitis	88	27	59	0.5
Group 3						
1	G.S.	Alactasia	282	173	109	1.6
2	E.L.	Crohn's disease	120	73	47	1.5
3	M.B.	Crohn's disease	155	109	46	2.3
4	N.B.	Crohn's disease	127	77	50	1.5
5	C.B.	Idiopathic steatorrhoea	160	98	62	1.6
			160	98	62	1.6
6	H.S.	Idiopathic steatorrhoea	155	96	59	1.6

from groups 1 and 3 show a preponderance of pancreatic over the salivary enzyme which was confirmed by the scans subsequently. From group 2, however, seven of eight zymograms revealed a decrease of pancreatic amylase activity in relation to the salivary. We were not able to obtain another urine specimen from patient L. W. for a repeat experiment. Figure 3 shows five scans from group 2 patients. Figure 4 A and B illustrate two types of urines which could be mistaken for one of the urines from group 2 shown in the same Figure 3. Figure 4A is a scan of the urinary isoamylase pattern

FIG. 2. Urinary zymograms of a normal individual and that of a patient (C.P.) showing decrease of pancreatic amylase (P) in the latter in comparison with salivary amylase (S).

FIG. 3. Scans of urinary zymograms from five patients with deficient exocrine pancreatic function.

FIG. 4. Scans of urinary zymograms from (A) 6-month old infant and (B) 4-year-old boy with mumps.

from a 6-month-old infant. Pancreatic function is not well-established in the first year of life (Janowitz and Dreiling, 1959). Figure 4B is a scan for 4-year-old boy with mumps. In both these urines, for different reasons, the pancreatic isoamylase appears to be relatively less than the salivary.

In group 1 the correlation coefficients between pancreatic and total amylase is 0.96, that between salivary and total amylase is 0.91. Distribution histograms show an increase in skewness in the order salivary, pancreatic, and total amylase. A plot of pancreatic against salivary amylase values

FIG. 5. Plot of pancreatic against salivary isoamylase values in urines of group 1 (●) and group 2 (○).

also demonstrates a scatter arising from greater variation in pancreatic values than salivary (Fig. 5).

DISCUSSION

The values of urinary amylases obtained are as expected for an enzyme in which variations between individuals are large but those exhibited by single individuals are small (Somogyi, 1941; Budd, Walter, Harris, and Knight, 1959). The mean enzyme concentration per 100 ml. urine was 165 Somogyi units with a S.D. of 77. On the basis of a two-hour collection from 100 normal people Gambrill and Mason (1964) found the mean value of urinary excretion per hour to be 127 Roe units with a S.D. of 64 (Smith and Roe units $\times 1.1$ = Somogyi unit).

With such a wide range of values for the total amylase, diagnosing pancreatic deficiency is rarely possible. In fact all five cases of advanced chronic pancreatitis with steatorrhoea investigated by Kirshen, Gambrill, and Mason (1965) had normal serum and urinary amylase levels. In our series if only the total urinary amylase were considered it would not be possible to diagnose any of the eight cases; if the pancreatic fraction alone were considered it would implicate five patients (J. M., E. H., C. P., E. M., and W. R.). When the ratio of the amounts of pancreatic to salivary amylase is the criterion it is possible to diagnose a deficiency in seven of the eight cases.

In man there are at least five established sources of α -amylase, namely, the salivary glands, pancreas, the intestinal epithelium (Thomson, 1965), the Fallopian tube and 'tubelike' epithelium of Mullerian and mesonephric origin (Green, 1957), and the lactating mammary gland (Kuttner and Somogyi, 1934). There is evidence that the liver may regulate the serum level of the enzyme (Gray, Probst, and Heifetz 1941) but this has not been confirmed (Cummins and Bockus, 1951). Franzini and Moda (1965) have reported an alteration of urinary isoamylase pattern with paper electrophoresis in acute hepatitis. Whether this is expressive of a derangement in a control mechanism normally exercised by the liver or an efflux of abnormal amounts of a liver enzyme remains to be determined. A search for the liver enzyme has produced negative results, at least in post-mortem tissues (Somogyi, 1941; Aw and Hobbs, 1966). More recently Joseph, Olivero, and Ressler (1966) detected an 'amylase' band whose electrophoretic mobility in 0.4% agar at pH 7.25 was even slower than pancreatic amylase. The enzyme was found in human liver extracts and serum. It was stainable with o-dianisidine in a ternary enzyme system consisting of maltase, glucose oxidase, and peroxidase. Unlike pancreatic and salivary amylase it failed to prevent iodine-starch colour reaction. These findings are interesting in relation to a report by Rosenfeld (1964) of the isolation of γ -amylase from human liver and kidneys. This enzyme is an exopolylglucosidase which splits glycogen and starch with the formation of high molecular weight dextrans and glucose. Thomson (1965) isolated small amounts of a similar enzyme from human small intestine mucosa besides the more abundant α -amylase. Our electrophoresis experiments with jejunal mucosal extracts yielded the P2 isoenzyme. In several ovarian cyst fluids examined amylolytic activity with slightly greater mobility than S1 was found (unpublished observations). Milk contains mainly salivary type enzyme with a small amount of activity in the P2 region (Aw and Hobbs, 1966).

It is probable, therefore, that in normal circumstances the pancreas, salivary glands, and the gut constitute the main sources of serum α -amylase activity. The behaviour of the urinary pancreatic complex in acute pancreatitis and the chromatographic isolation of P1 and P2 from extracts (of pancreas) offer strong corroborative evidence that the urinary isoamylases P1 and P2 are largely pancreatic in origin.

Of 120 children examined, serum amylases corresponding to P2 and S1 were found in 109 (90.9%) (Kamarýt and Laxová, 1965). The proportion in our smaller series is 87.5%. In the remaining 11

children, the pancreatic fraction was doubled. The same authors obtained the interesting results with a group of 14 diabetic children aged from 10 to 15 years where in 10 a considerable increase of activity of the salivary fraction was noted. We investigated three diabetic patients not in the series. Two of them had the disease as adults and their amylase ratios were within normal limits. The third, who had suffered from the disease since the age of 5, had the ratio 0.8. Advanced dysfunction of the islet or acinar tissue has adverse effects on each other and the association of frank diabetes with chronic pancreatitis is well documented.

Wieriks (1964) demonstrated that the buccal application of bacterial α -amylase to rabbits can result in the production of precipitating antibodies in the sera. Furthermore it has also been observed that patients with chronic pancreatitis occasionally develop an avidity in the intestinal absorption of various substances (Dawson, 1964). If in fact the intestinal epithelium becomes abnormally permeable to large molecules the ingestion of pancreatic supplements may interfere with the urinary amylase pattern. Two commercial preparations we tested contain amylases in the region of the normal isoamylases. Crystalline hog pancreatic amylase gives a zymogram pattern very similar to that of a type 1 urine. As a precaution patients should abstain from taking supplements for a few days before the collection of the urine. The test will only be of value where the total urinary amylase is within the normal limits.

Amylase is cleared from the blood by simple filtration and is not reabsorbed in the tubules (McGeachin and Hargan, 1956). Impaired renal clearance has been found to cause elevated amylase values in the serum and lowered values in the urine *e.g.*, Budd *et al.*, 1959). This is an uncommon finding (Gross, Parkin, Maher, and Power, 1960). However, it is highly unlikely that the filtration of two species of molecules so similar as pancreatic and salivary amylase could be separately affected. The ratio of the two is likely to remain the same as that of the parent plasma even when poor renal function lowers the amount of urinary enzyme.

SUMMARY

A simple method is described for the electrophoretic separation of urinary pancreatic and salivary iso-enzymes of α -amylase and this offers a convenient screening test. The ratio of the amounts of pancreatic to salivary isoamylases in the urine is proposed as an index of deficient exocrine pancreatic function. Visual assessment of the urinary zymogram is

frequently sufficient for a diagnosis. Studies were made on 40 controls and eight patients with known pancreatic deficiency. Seven of the eight patients had abnormal zymograms and low ratios. The results and possible factors influencing the zymogram pattern are discussed.

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