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False negative hyperglucosuria test-strip reactions in laboratory mice

D. M. TAYLOR & D. L. NEAL

Agricultural Research Council and Medical Research Council, Neuropathogenesis Unit, West Mains Road, Edinburgh, EH9 3JQ, United Kingdom

Summary

Inhibition of a variety of commercial test strips for hyperglucosuria was experienced in laboratory mice. All mouse strains tested were found to have sufficiently high levels of ascorbic acid to cause inhibition, and male levels were higher than those of females. A regime to obtain optimum detection of positive results is discussed.

Glucose-oxidase test strips were developed as a quick and simple method for detecting human hyperglucosuria (Free, Adams, Kercher, Free & Cook, 1956), their specificity and general usefulness being established by clinical trial (Free & Fonner, 1958). Despite a limited quantitative ability (Jablokow, Hutchins & Knights, 1957) these systems are very sensitive, being able to detect glucose at concentrations as low as 0·55 mmol/l or even less (Free *et al.*, 1957).

The test was used with small animals by Free & Free (1963) and has subsequently been used with a wide variety of mammals, for example cats (Palmore, Gaskin & Nielsen, 1978), dogs (Watts, 1968), hamsters (Dulin & Gerritsen, 1972; Gerritsen & Dulin, 1972), mice (Nakamura & Yamada, 1967; Craighead & Higgins, 1974), rats (Tarrant & Ashmore, 1965; Like, Rossini, Guberski, Appel & Williams, 1979), and squirrels (Stuhlman, Wagner, Garro & Musacchia, 1977).

In the test, glucose oxidase in the presence of oxygen and water catalyses the oxidation of glucose to gluconic acid with the formation of hydrogen peroxide. The peroxide is reduced by peroxidase catalysis to water and nascent oxygen which, in turn, oxidises the chromogen to produce a colour reaction. However, other potent reducing substances in the urine, such as ascorbic acid and certain drug metabolites, can compete for the nascent oxygen, causing the chromogen to remain in a reduced form which results in false negative reactions (Nakamura, Reilly, Kay Jujita, Brown & Kunitake, 1965; Feldman, Kelley & Lebovitz, 1970; Feldman, Lebovitz & Durham, 1973). Although ascorbic acid inhibits glucose-oxidase test strips at levels as low as 10 mg% (Mayson, Schumaker & Nakamura, 1972), the human urinary rate of excretion is below this level, being 5–55 mg daily (Cockburn, 1961) except where the vitamin is being ingested in large amounts, for example in self medication (Pauling, 1970). In a clinical trial Mayson *et al.* (1972) detected a relatively modest false negative rate of 1·2%.

In contrast to the human situation, there seems to be little evidence of similar evaluations having been made in animals. A notable exception is the work of Watts (1968), who observed false negative results in the presence of as little as 5 mg % ascorbic acid in canine urine, and concluded that because normal canine values exceed this level the test had no place in canine urology. Lang, Munger & Rapp (1977) too, in their work with diabetic guineapigs, stated their awareness of the potential problem. Considering the widespread use of glucose-oxidase test strips in animal work and lack of publications on test inhibition, one concludes that much research involving these test strips for hyperglucosuria is done without recognising their potential limitations. For example, the inexplicable poor correlation between blood and urine glucose levels in 10% of the urine samples from diabetic mice studied by Boucher & Notkins (1973) might well be explained by test strip inhibition. The manufacturers of all the most widely-used systems do warn that high levels of ascorbic acid can inhibit the test, and at least one major manufacturer does point out that there may be large amounts of ascorbic acid naturally present in the urine of all animal species except the guineapig and primates (species which require an exogenous source of the vitamin). It seems, however, that many researchers are unaware of the actual excretion levels of urinary ascorbic acid of the species which they are handling since this information is not always readily available. Furthermore, there are other unidentified substances in urine which, in man, are the most significant cause of glucose-oxidase test strip inhibition (Feldman *et al.*, 1970).

We had used test strips for some time before realising their limitations. Typically, fresh mouse urine was tested, but when one batch of samples was not examined until 24 h after collection, an

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unexpectedly high proportion of positive results occurred. Further fresh samples from the same animals yielded negative results, which also became positive when tested after 24 h. In terms of our own research, the detection of this inhibtion was fortuitous since it revealed previously unsuspected diabetes in one of our inbred strains of mice. This paper describes the studies of the test strip inhibitions.

Materials and methods

Inbred mice

The strains studied were C3H/fLac, VM/Dk, C57BL/FaDK, MM/Dk and crosses and back crosses of the last 2. These were all bred and maintained within our barrier-maintained, closed colony. Details of the husbandry methods have been previously described (Taylor & Fraser, 1973).

Urine sampling

Cleanly voided specimens were collected in sterile autoanalyser cups (Medfor Products, Fleet, UK).

Specific tests for hyperglucosuria

The glucose-oxidase strips used initially were 'Clinistix' (Ames Company, Stoke Poges, UK), but subsequently 'Diastix' (Ames Company), 'Tes-Tape' (Eli Lilly, Basingstoke, UK) and 'BM-Test-Glucose' (Boehringer Corporation (London) Ltd, Lewes, UK) were tested. When urine samples were tested more than once, or with more than one type of strip, aliquots were removed for testing to prevent contamination of the sample with enzyme.

Urinary ascorbic acid titration

Dichlorophenolindophenol (DCPIP), an oxidising dye, is widely used for the clinical titrimetry of ascorbic acid (Wootton, 1964), the blue compound becoming colourless when it is reduced by ascorbic acid. Although the DCPIP method is not wholly specific for ascorbic acid, this procedure was chosen because it presented the possibility of detecting other reducing metabolites which might also interfere with glucose-oxidase test-strip reactions. Therefore results from these titrations will be presented as 'total DCPIP reactants'. To establish what proportion of the total DCPIP reactants was ascorbic acid, a number of urine samples were retitrated following treatment with ascorbate oxidase which catalyses the oxidation of ascorbic acid, in which form it does not react with DCPIP. To minimise degradation of reagent and urinary reactants, urine samples were tested within minutes of collection and fresh DCPIP (BDH Chemicals Ltd, Poole, UK) solution was not used beyond 2 h after preparation.

Preparation of ascorbate oxidase

Ascorbate oxidase of varying purity can be extracted from a variety of plants; cucumber is a convenient indigenous source (Booth & Constable, 1960; Nakamura, Makino & Ogura, 1968). Our method was to prepare a crude extract by homogenising 1 kg fresh cucumber in a domestic liquidiser. The wet pulp was filtered through gauze and the fluid stored at -30°C.

Titration established that the extract contained no substances which react with DCPIP and it was found that by the arbitrary addition of 1 ml extract to 2·5 ml 0·1% ascorbic acid solution at room temperature, no ascorbic acid could be detected in a DCPIP titration carried out 5 min later. The activity of the extract did not diminish after 2 weeks storage at -30°C, nor was enzyme activity lost over a 2 h period at bench temperature. The pH of the extract was found to be 6·5, which is within the optimal range for activity of ascorbate oxidase at 25°C (Nakamura *et al.*, 1968). Conveniently, the pH of mouse urine is also within this optimal range.

Ascorbic acid solution

Aqueous 0·1% ascorbic acid sodium salt (Sigma London Chemical Company, Poole, UK) was prepared volumetrically and standardised by titration against DCPIP. The reagent was always freshly prepared and used immediately, because of its lability.

Results

Glucose-oxidase test-strips

Table 1 demonstrates that delayed testing considerably increases the detection rate of positive 'Clinistix' results in our diabetes-susceptible, MM male mice. The inhibitory effect was such that in males 83% of subsequently confirmed positives were undetectable using fresh urine. The inclusion of VM mice, which often have hyperglucosuria as a consequence of renal amyloidosis, enabled a similar phenomenon to be detected in that strain, demonstrating that the inhibition was not confined to the MM. Subsequently urine from 116 male C57 and MM crosses and back crosses, aged 102–287 days (mean 194), was tested using a wider range of commercial test-strips. Positive results were detected by at least 1 brand in 72 instances and an analysis of the data is given in Table 2. However, in only 23 cases were positives identified by all 4 strips. There are a number of important differences between brands: maximum detection of positives was achieved by 'BM-Test-Glucose' (65/72) in contrast to 'Clinistix' (23/72). Table 3 shows differences in the approximate degree of positivity detected, with 'Diastix' giving readings of >28

Table 1. Effect of delayed testing upon 'Clinistix' test-strip results with urine from mice of the MM and VM strains

	Sex	*Days old (mean)*	*No. tested*	*Positive results*	
				fresh	*24 h*
MM	♂	27–496 (199)	94	9 (10%)	54 (57%)
VM	♀	86–269 (131)	9	0	2 (22%)
	♂	55–502 (195)	11	0	2 (18%)

Table 2. Analysis of 72 positive hyperglucosuria results from 116 male C57 and MM mouse crosses and back crosses tested with 4 brands of test strip

	Positive 1st detectable at different times after collection (% of all positives)											
Degree	*'BM-Test-Glucose'*			*'Clinistix'*			*'Diastix'*			*'Tes-tape'*		
+ve	*fresh*	*6 h*	*24 h*	*fresh*	*6 h*	*24 h*	*fresh*	*6 h*	*24 h*	*fresh*	*6 h*	*24 h*
< 500 mg%	4 (6)	14 (19)	28 (39)	3 (4)	2 (3)	5 (7)	1 (1)	1 (1)	0	4 (6)	0	15 (21)
⩾ 500 mg%	19 (26)	0	0	6 (8)	5 (7)	2 (3)	22 (31)	0	0	20 (28)	2 (3)	1 (1)
Total	23 (32)	14 (19)	28 (39)	9 (12)	7 (10)	7 (10)	23 (32)	1 (1)	0	24 (34)	2 (3)	16 (22)

Table 3. Relative degree of positivity and effect of delayed testing with commercial test strips using 23 hyperglucosuric urines from C57 and MM mouse crosses and back crosses

	Positive 1st detectable at different times after collection (%)											
Degree	*'BM-Test-Glucose'*			*'Clinistix'*			*'Diastix'*			*'Tes-tape'*		
+ve	*fresh*	*6 h*	*24 h*	*fresh*	*6 h*	*24 h*	*fresh*	*6 h*	*24 h*	*fresh*	*6 h*	*24 h*
< 28 mmol/l	17	4	0	12	9	22	4	4	0	13	0	0
⩾ 28 mmol/l	79	0	0	26	22	9	92	0	0	87	0	0
Total	96	4	0	38	31	31	96	4	0	100	0	0

mmol/l in 92% of the samples, 'Clinistix' doing so in 57%. All of the positives in Table 3 were detectable in fresh urine with 'Tes-tape', but with 'Clinistix' further testing at 6 and 24 h was required to reveal 62% of positives. With all strips there was a marked tendency for the degree of positivity to increase with delayed testing.

'DCPIP reactant' levels in mouse urine

Details of the mice studied (Table 4) show that average male levels were higher than female ($P = <0{\cdot}001$). In the MM strain the average levels were considerably higher than in the other strains ($P = <0{\cdot}001$), but all groups tested had sufficiently high levels potentially to inhibit glucose-oxidase test strips. Variation between individuals was considerable, the lowest level recorded being 2 mg % in a VM male and the highest being 187 mg % in an MM male. As Table 4 shows, the mean age of the MM mice was higher than the other strains. To demonstrate that the higher levels of DCPIP reactants found in the MM mice was not simply a function of their greater age, the MM data was subjected to analysis of age groups (Table 5).

Table 4. 'DCPIP reactant' levels (mean ± se) in the urine of mice of C3H, C57BL, MM and VM strains

	Days old (mean)		*No. mice*		*Reactant levels (mg %)*	
	♀	♂	♀	♂	♀	♂
C3H	12–266 (136)	35–235 (145)	28	43	17 (2·5)	35 (2·7)
C57BL	23–264 (157)	36–266 (161)	34	38	13 (1·7)	39 (4·2)
MM	29–803 (276)	45–375 (215)	56	44	24 (2·3)	70 (7·1)
VM	55–348 (150)	55–360 (150)	35	51	16 (2·3)	23 (3·3)

Table 5. Age-group analysis of the MM data presented in Table 4

Days old (mean)		*No. mice*		*Mean levels of reactants (mg %)*	
♀	♂	♀	♂	♀	♂
29–60 (48)	(45–59) 49	10	10	22	96
119–196 (148)	112–188 (139)	13	16	16	73
200–803 (468)	210–532 (375)	33	18	27	53

Levels of ascorbic acid and non-specific test-strip inhibitors in mouse urine

To assess if inhibitors other than ascorbic acid were reacting in the DCPIP titration, urine samples from some of the mice shown in Table 4 were titrated before and after treatment with cucumber extract (1 : 1 for 5 min at room temperature). Originally, this was an ad hoc procedure, but the results in Tables 4, 5 and 6 confirm that the ascorbate oxidase in the extract was effective. Fresh aliquots of extract were removed from cold storage each day and were not used beyond 2 h; all tests were carried out within 2 weeks. As a prelude to the experimental work it was established that no significant natural degradation of urinary ascorbic acid occurred in untreated urines during the 5 min in which they would be in contact with cucumber extract. This was achieved by titrating urine samples fresh and after a 5 min holding period when diluted 1 : 1 with distilled water.

Table 6 demonstrates that ascorbic acid accounted for the highest proportion of DCPIP reactants. Nevertheless, unidentified reactants represented a notable proportion in all groups and again there was considerable variation between individuals. In 1 C57 male, none of the 47 mg % total DCPIP reactivity was due to ascorbic acid, whereas in another all of the 35 mg % of reactivity was identifed as ascorbic acid.

Discussion

Our interest in glucose-oxidase test-strip inhibition was prompted by the unexpected discovery of positive reactions from stored, but not fresh, urine samples, and at that time we were unaware of the extent and nature of test-strip inhibition. It is hardly surprising, therefore, that initially we failed to recognize an interesting, spontaneous diabetes in male MM mice, particularly since the data show that of all strains tested MM has the highest levels of urinary ascorbic acid and other potential test-strip inhibitors. In all strains, males had higher levels than females. All strains had levels sufficiently high to cause inhibition of glucose-oxidase test strips, but the effect could be considerably reduced by testing urines 24 h after collection, by which time some degradation of ascorbic acid will have occurred (Watts, 1968).

In practice we have found that this procedure reveals many, but not all, cases of hyperglucosuria, but it is purely an arbitrary arrangement which takes no account of degradation of urinary glucose, especially by bacteria, nor is it tenable when quick results are required. Although a small pilot study indicated that even more positive results might be obtained by extending the storage period to 96 h, this is even more impracticable. Furthermore, the detailed evidence indicated that this would only be true for samples with extremely high levels of glucose.

The comparison of the various commercial test strips demonstrates that our original choice ('Clinistix') was least suited to the work, but we are now aware that the manufacturers recommend their alternative product ('Diastix') for work with diabetic animals. With the others, our data relating to the detection of positives with an approximate glucose value of less than 28 mmol/l suggest either a hypersensitivity of 'BM-Test-Glucose' and 'Testape' or a hyposensitivity of 'Diastix'.

Since the completion of this work new compound strips have become available in this country.

Table 6. Effects of ascorbate oxidase treatment on the mean levels of 'DCPIP reactants' in the urine of mice of C3H, C57BL, MM and VM strains

	Days old (mean)	*Sex*	*No.*	*Total reactant levels (mg%)*	*Ascorbic acid (mean %)*	*Unidentified reactants (mean %)*
C3H	121–199 (160)	♀	2	38	89	11
	90–257 (190)	♂	10	39	79	21
C57BL	138–274 (215)	♂	11	36	66	34
MM	375–525 (479)	♀	5	33	77	23
	121–521 (242)	♂	11	56	61	39
VM	68–348 (173)	♂	8	17	78	22

These are 'Medi-Test Glucose 2' (Machery-Nagel, Düren, GDR, marketed in the UK by Camlab Ltd, Cambridge). These have 2 urine test areas, one for glucose the other for ascorbic acid. In use with human urine it is recommended that a negative glucose reaction should be disregarded (and a further sample tested) if the ascorbic acid test area reacts. In view of the results already presented in this paper, it is not surprising that all 72 male mice (C57BL and MM back crosses) showed maximum positivity (++) for ascorbic acid ($\geqslant$ 20 mg%). Of 18 females tested, 16 gave ++ reactions, 1 +, and 1 was negative.

Like Watts (1968), we found no practical means of removing inhibitors from fresh urine to permit valid testing. Unfortunately, in addition to ascorbate oxidase, cucumber extract – which might have proved useful – contains large amounts of glucose. With species where larger volumes of urine are available it may be practical to remove ascorbic acid by anion resin treatment (Brandt, Guyer & Banks, 1974). However, even such treatment may not be effective since a significant proportion of potential inhibitors can be substances other than ascorbic acid. In man, Feldman *et al.* (1973) found that 78% of test inhibitions were due to these other substances, and in canine urine Watts (1968) found that up to 29% could be. Our mouse data show that the proportion of potential non-specific inhibitors is appreciable and highly variable between individuals. Removal of these substances can be achieved by cationic exchange resin (Hughes, 1964), but again the procedure is only practical where reasonable volumes of urine are available. For the veterinary clinician, warning of the inhibitory nature of a urine sample can be obtained by challenging test strips giving negative results with a drop of 0·5% glucose solution (Mayson *et al.*, 1972). Continued failure of the strip to change colour demonstrates the inhibitory nature of the urine and signals the possibility that a positive result has been missed, even if there may be no remedial measure available. Our pilot study also indicates that, although perhaps not practically or economically feasible, the detection of positives would be enhanced by the use of more than 1 type of test strip, because not all types were inhibited by the same urines.

Animals which synthesise ascorbic acid (the majority of species) tend to excrete larger amounts of the vitamin than those which require an exogenous source (primates and guineapigs). In view of this, and the evidence that glucosuria test strips are widely used in animals, it is somewhat surprising that reports on test inhibition seem scant, raising the possibility that a significant proportion of positive cases have gone undetected.

This work was carried out to find the reasons for some unusual results and although it is not intended to extend the study, it is hoped that the findings will prove useful in explaining or preventing anomalous findings elsewhere.

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Falsch negative Hyperglukosurie Test-Streifen Reaktion bei Labormäusen

D. M. TAYLOR & D. L. NEAL

Zusammenfassung

Bei Labormäusen wurde die Erfahrung gemacht, daß eine Reihe von kommerziellen Test-Streifen zur Bestimmung der Hyperglukosurie gehemmt wird. Bei allen Mäusestämmen wurden Ascorbinsäure-Spiegel gefunden, die hoch genug waren, um die Hemmung zu verursachen. Bei männlichen Tieren waren die Spiegel höher als bei weiblichen. Es wird eine Diät diskutiert, mit der positive Ergebnisse optimal gefunden werden können. (G)