of older patients include poliomyelitis, spinal tuberculosis, and numerous cases of tabes dorsalis.

The data supplied on death certificates are scanty. Work is in progress to obtain additional information on all cases of the types mentioned, so as to explore further the possibility of excess mortality from bladder cancer among paraplegics and patients with other conditions likely to cause chronic urinary stasis and infection. Particular attention will be paid to histology, which is rarely specified on death certificates, since squamous cell cancers may be relatively common among such patients.

REFERENCES
Kuller L, Lilienfield A & Fisher R
(1967) *Public Health Reports* **82**, 339–346
Melzak J
(1966) *Paraplegia* **4**, 85–96

Dr R M Hicks
(School of Pathology, Middlesex Hospital Medical School, London W1P 7LD),
Dr C L Walters
(Biochemistry Section, British Food Manufacturing Industries Research Association, Leatherhead, Surrey),
Professor I Elsebai, Dr A-B El Aasser, Dr M El Merzabani
(Cairo Cancer Institute, Cairo, Egypt)
and Dr T A Gough
(Laboratory of the Government Chemist, Cornwall House, London SE1 9NQ)

Demonstration of Nitrosamines in Human Urine: Preliminary Observations on a Possible Etiology for Bladder Cancer in association with Chronic Urinary Tract Infections

In any site where nitrates, secondary amines and nitrate-reducing bacteria occur together, N-nitrosation can take place with the formation of nitrosamines (Hill & Hawksworth 1972). In normal urine, nitrates are always present from dietary sources, as also are small amounts of secondary amines. If, in addition, an infection of the urinary tract with nitrate-reducing bacteria is present, conditions are theoretically right for the local production of nitrosamines within the bladder. This was confirmed by Hill & Hawksworth (1972) who demonstrated formation of the nitrosamine N-nitrosopiperidine in the urine of rats with experimentally-induced urinary infections who had been fed the parent amine piperidine. As a class nitrosamines and nitrosamides have been demonstrated to be powerful carcinogens in all animal species in which they have been tested and at least four have been shown to be selective bladder carcinogens (Druckrey *et al.* 1962, Druckrey *et al* 1964, Magee & Barnes 1967, Hicks & Wakefield 1972, Lijinsky & Taylor 1975, Hirose *et al.* 1976).

In man, as in animals, nitrate is always present in the urine from dietary sources, as also are small amounts of secondary amines (Asatoor & Simenhoff 1965). The latter are normally formed by bacterial action on breakdown products of food in the intestine, reabsorbed into the blood stream, then excreted in the urine. The exact amount and chimal species of secondary amines present in the urine will be modified by diet and may be expected to vary in populations in different parts of the world. In patients with bacterial infection of the lower urinary tract it is possible that small amounts of nitrosamines may be produced which could initiate neoplastic or preneoplastic changes in the urothelium. If urinary nitrosamines play any part in the initiation of human bladder cancer, then nitrosamines should be detectable in infected human urine and, furthermore, bladder cancer incidence should be higher in populations subject to chronic urinary tract infections.

A preliminary study has been made of the nitrosamine content of urines from three groups of patients:

Group A: Patients under treatment for neoplastic bladder disease in the Middlesex Hospital. Urine samples were obtained with the collaboration of Mr E Milroy and his colleagues and Dr J Newman.
Group B: Hemiplegic and paraplegic patients attending Stoke Mandeville Hospital. Bacteriological information and urine samples were supplied by Dr J Harris and his colleagues.
Group C: Egyptian patients hospitalized for treatment of neoplastic bladder disease superimposed on bilharziasis of the urinary bladder.

Method

N-nitroso compounds can vary from simple, low molecular weight compounds to large complex molecules, and although many may be extracted from urine with the solvent dichloromethane some are unextractable and remain behind in the urine. At the present time no satisfactory method is available for the separation and characterization of individual unextractable urinary N-nitroso compounds, though they can be detected by a group test which is not yet proved to be completely specific (Preussman 1972). Total amounts of volatile plus nonvolatile extractable nitrosamines in dichloromethane extracts of urines were determined by the Eisenbrand & Preussman method

(1970) which is specific for N-nitroso compounds. The volatile nitrosamines in the dichloromethane extracts were further positively identified by gas chromatography coupled with high resolution mass spectrometry either by the method of Crathorne *et al.* (1975) or that of Gough & Webb (1973). The equipment was set to detect the presence of six common volatile N-nitrosamines. The total nitrosamine in urine was calculated as if it were all present in the form of a compound with molecular weight of 100 which is the molecular weight of N-nitrosopyrrolidine.

Results

Group A: The patients in Group A included both men and women with neoplastic bladder disease of industrial or unknown etiology. Urine samples were routinely examined for the presence of bacterial infection and samples were analysed for their nitrite and nitrosamine content. Urines from two normal adults were analysed as controls (Table 1). The only urine in which a trace amount of nitrosamine was detectable was from a woman with a large bladder tumour and a heavy urinary infection. In 3 others, although nitrite was present, active bacterial infection of the urine was absent and no nitrosamines were detectable.

Group B: The patients in this group were all men with spinal lesions resulting in paraplegia or hemiplegia. The duration of the lesion varied from 3 months to 32 years, and all had a history of recurrent bacteriuria. However, not all urine samples analysed for nitrosamines were actually infected at the time of sampling. None of these patients had neoplastic bladder disease. The nitrite and nitrosamine content of the urines from 11 patients is shown in Table 2. Significant amounts of nitrosamines were detected in the urine of 3 patients who also had active bacterial infections of the bladder. Although the urine of Patient 8 was infected and had quite a high nitrite content, no nitrosamine was detectable suggesting either that no secondary amine precursors were available for their synthesis or that conditions were not conducive to bacterial nitrosation. Large amounts of nitrosatable precursors were found in 2 normal control subjects.

Group C: The patients in this group included men and women with a history of chronic *Schistosoma hæmatobium* infestation with resultant bilharzial bladder disease plus chronic secondary bacteriuria. All had developed bladder cancer and were hospitalized for treatment. Three sets of urine samples were collected in the Cairo Cancer Institute, and analysed in England. The first set was analysed without extraction, to give an estimate of the total amounts of N-nitroso compounds present (Table 3). The method used (Walters *et al.* 1974) has not been proved to be entirely specific and not all compounds detected were necessarily nitrosamines. The second set of urines was extracted with dichloremethane, and the extracts analysed for

Table 1

Patients with neoplastic bladder disease (Group A)

Patient No.	*Urine culture*	*Nitrite in urine (parts/10^6)*	*Nitrosamine in urine● (parts/10^9)*
1	No growth	0	Not detectable
2	No growth	0	Not detectable
3	No growth	0	Not detectable
4	No growth	0	Not detectable
5	No growth	0	Not detectable
6	No growth	0	Not detectable
7	No growth	20	Not detectable
8	No growth	1	Not detectable
9	No growth	5	Not detectable
10	Fæcal strepto-cocci, coliforms & staphylococci	5	0.4
Control 1	No growth	0	Not detectable
Control 2	No growth	0	Not detectable

● Calculated as N-nitrosopyrrolidine (mol. wt. 100)

Table 2

Patients with hemiplegia or paraplegia (Group B)

Patient No.	*Duration of lesion (years)*	*Urine culture*	*Nitrate in urine (parts/10^6)*	*Nitrosamine in urine● (parts/10^9)*
1	0.25	No record available	0	Not detectable
2	0.5	*E. coli*	0	Not detectable
3	3.5	No record available	15	Not detectable
4	8	No growth	5	Not detectable
5	9	No record available	3	Not detectable
6	13	No growth	<1	Not detectable
7	22	No growth	20	Not detectable
8	1	Heavy mixed growth	10	Not detectable
9	22	Heavy mixed growth	0	6.6
10	29	Mixed growth	5	3
11	17	Heavy mixed growth	20	4.6

● Calculated as N-nitrosopyrrolidine (mol. wt. 100)

Table 3

Egyptian bladder cancer patients (Group C)
Whole urine analyses

Patient No.	*Total N-nitroso compounds in unextracted urine● (parts/10^9)*
1	Not detectable
2	100 000
3	43 000
4	100 000
5	230 000
Control	Not detectable

● Method used not specific for nitrosamines and these totals may include other compounds; calculated as N-nitrosopyrroldine (mol. wt. 100)

their total extractable nitrosamine content (Table 4). The third set was prepared in three ways. Direct dichloromethane extracts of some samples were made for total (volatile plus nonvolatile) extractable nitrosamine estimation, while others were distilled, and the distillates extracted for estimation of volatile nitrosamines. In addition, some urines were chemically nitrosated before extraction, for estimation of the total precursors present which were available for conversion to volatile plus nonvolatile nitrosamines (Table 5). Four control urine samples were collected from staff members of the Cairo Cancer Institute who had no history of bilharziasis. No detailed bacteriological data are available for these urine samples, but in general, the bilharzial bladder cancer patients also had bacteriuria and the controls were not known to be infected. The urines of 2 noninfected English controls were also analysed.

Deliberate nitrosation of Egyptian urines from both bladder cancer patients and a control individual yielded large amounts of extractable (volatile plus nonvolatile) nitrosamines (Table 5). This indicates a plentiful supply of secondary amine precursors which are available for nitrosation if the urine becomes infected with appropriate strains of bacteria. Similar amounts of nitrosatable amine precursors leading to extractable N-nitroso derivatives were found in the urines of 2 normal persons on typical British diet. The amounts detected ranged from 2900 to 7100 parts/10^9 based on a molecular weight of 100. Table 3 shows large quantities of unidentified nitroso compounds in unextracted urines from Egyptian bladder cancer patients, and when those which could be extracted by dichloromethane were further examined, the bulk of the nitrosamines were found in the nonvolatile fraction (Table 5). These nonvolatile nitrosamines could not be further characterized by the gas chromatography and mass spectrometry method used which will characterize only volatile

Table 4

Egyptian bladder cancer patients (Group C). Dichloromethane extracts of urine analysed for volatile nitrosamines

Patient No.	*Volatile nitrosamines in urine●* (*parts/10^9*)
1	880■
2	Trace
3	Not detectable
4	660
5	Not detectable
6	Not detectable
7	Not detectable
8	Not detectable
9	230
10	Not detectable

● Calculated as if mol. wt. = 100
■ All nitrosamine positively identified as dimethylnitrosamine by gas chromatography coupled with mass spectrometry.

Table 5

Egyptian bladder cancer patients (Group C)

Patient No.	*Total extractable nitrosamines in urine●* (*parts/10^9*)	*Volatile nitrosamines in urine●* (*parts/10^9*)	*Total precursors available for nitrosation●* (*parts/10^9*)
1	2000	140■	3600
2	1800	480■	8900
3	Not detectable	Not detectable	Not analysed
4	Not detectable	Not detectable	9100
5	Not analysed	110■	1200
Egyptian controls:			
1	Not detectable	Not detectable	Not analysed
2	Not detectable	Not analysed	6800
3	300	Not detectable	Not analysed
4	Not analysed	Not detectable	Not analysed
English controls:			
1	Not detectable	Not detectable	2800
2	Not detectable	Not detectable	7100

● Calculated as if mol. wt. = 100
■ Dimethylnitrosamine was positively identified in the volatile fraction by gas chromatography coupled with mass spectrometry

compounds. Relatively small amounts of nitrosamines were found in the volatile fraction of some urines (Tables 4 & 5) and in these, N-nitrosodimethylamine was positively identified by gas chromatography coupled with high resolution mass spectrometry. The method used was capable of identifying six nitrosamines only, but the other five, N-nitroso-diethylamine, -di-*n*-propylamine, -di-*n*-butylamine, -pyrrolidine and -piperidine, were not present in detectable amounts.

Discussion

This is the first time that nitrosamines have been positively identified in infected human urines. An earlier report of N-nitrosodimethylamine in the urine of 2 women with *Proteus mirabilis* infections of the urinary tract (Brooks *et al.* 1972) was almost certainly correct, but the method used, namely gas chromatography, is not completely specific without confirmatory identification of the peaks by mass spectrometry. The results reported here indicate that in addition to N-nitrosodimethylamine, other species of N-nitrosamines, particularly nonvolatile compounds, are formed in some infected human urines.

The analyses of Egyptian urines are preliminary results and form part of an ongoing research project in collaboration with the Cairo Cancer Institute. Comparison with the infected and uninfected English urines illustrates marked difference in the total amounts of nitrosamines found in infected urines in the two countries. The Egyptian patients were all fellahin, agricultural workers from a low socio-economic group with a poor standard of hygiene, whose diet differs considerably from that of the English subjects studied. Although nitrosatable precursors were available in

considerable quantities in both Egyptian and English individuals even when bacteria are present, the amounts of nitrosamines actually formed are far greater in Egyptians than in English subjects, suggesting that some other unidentified factor has a controlling influence on nitrosamine production in the urine.

It is not known whether nitrosamines in human urine are of any etiological significance for carcinoma of the bladder or of any other organ. In animals, individual nitrosamines are highly organ specific and the only one which has been positively identified in human urine so far, N-nitrosodimethylamine, is a liver carcinogen in animals and does not affect the bladder when given by mouth or by subcutaneous injection. Of the nitroso compounds demonstrated to be animal bladder carcinogens, N-nitroso-di-*n*-butylamine, -butylbutanolamine, -ethylbutanolamine, -methyldodecylamine and -methylurea, only the first could have been positively identified if present in the human urine samples studied, because of the technical limitations of the analytical methods used. Such compounds could, however, have contributed to the total nitrosamines found in the volatile plus nonvolatile fractions.

Animal experiments have shown that exposure of the rat bladder to a subcarcinogenic dose of the nitrosamide N-nitrosomethylurea, produced preneoplastic changes within the urothelium which can be promoted to tumour growth either by other carcinogenic or cocarcinogenic stimuli (Hicks *et al.* 1975) or by further doses of the same carcinogen (Hicks & Wakefield 1972). It is possible that a similar mechanism could operate in man. Low doses of carcinogenic nitrosamines produced in the urine as a result of chronic or recurrent bacterial infections may induce preneoplastic changes in the urothelial cells. These may then be promoted to tumour growth either by exposure to other urine borne carcinogens or by other factors including mechanical irritation from urinary calculi or repeated ulceration such as occurs in the bilharzial bladder. Judging from the preliminary findings, the amounts of nitrosamines found in the Egyptian urines suggest that such a mechanism could well contribute to the exceptionally high incidence of bladder cancer found in association with bilharziasis in the fellahin population, compared with the low incidence of bladder cancer in the noninfected city workers and professional classes in Cairo and Alexandria. In England, the relatively low incidence of chronic urinary tract infection and the much lower amount of nitrosamines found in some English patients with bacteriuria, suggests a quantitatively less important role for urinary nitrosamines in the etiology of bladder cancer in this country. As in other Western industrialized countries, such factors as cigarette smoking, exposure to industrial chemicals, food additives &c. may be expected to be of greater importance. Thus, although women are more susceptible to bacterial cystitis than men, in England and Wales the bladder cancer incidence is higher in men than in women. In paraplegics and hemiplegics, on the other hand, who as a group are particularly subject to bladder infections, there appears to be a slightly elevated mortality from bladder cancer among men under 47 (Davies 1977). A link between urinary tract infection and bladder cancer in paraplegics was suggested by Melzak (1966). He observed 12 cases of urothelial cancer (11 bladder plus 1 ureter) by comparison with only 5 cases of lung cancer, in 3800 patients with paraplegia attending the National Spinal Injuries Centre, Stoke Mandeville Hospital, between 1944 and 1966, and noted that the only common factor between them was long-standing, chronic bladder infection.

The various possibilities discussed in this preliminary report are being investigated further both here and in Cairo.

Acknowledgments: The high cost of analyses has been funded by the International Agency for Research on Cancer at Lyon, France. R M H and C L W are also supported by grants from the Cancer Research Campaign.

REFERENCES

Asatoor A M & Simenhoff M L
(1965) *Biochimica et biophysica acta* **111**, 384–392
Brooks J B, Cherry W B, Thacker L & Alley C C
(1972) *Journal of Infectious Diseases* **126**, 143–153
Crathorne B, Edwards M W, Jones N R, Walters C L & Woolford G
(1975) *Journal of Chromatography* **115**, 213–217
Davies J M
(1977) *Proceedings of the Royal Society of Medicine* **70**, 411
Druckrey H, Preussman R, Schmähl D & Müller M
(1962) *Naturwissenschaften* **49**, 1924
Druckrey H, Preussman R, Ivankovic S, Schmidt C H, Mennel H D & Stahl K W
(1964) *Zeitschrift für Krebsforschung* **66**, 280–290
Eisenbrand G & Preussman R
(1970) *Arzneimittel-Forschung* **20**, 1513–1517
Gough T A & Webb K S
(1973) *Journal of Chromatography* **79**, 57–63
Hicks R M & Wakefield J St J
(1972) *Chemico-Biological Interactions* **5**, 139–152
Hicks R M, Wakefield J St J & Chowaniec J
(1975) *Chemico-Biological Interactions* **11**, 225–233
Hill M J & Hawksworth G
(1972) In: N-Nitroso Compounds, Analysis and Formation. Ed. P Bogovski, R Preussman & E A Walker. Scientific Publications No. 3, IARC, Lyon; pp 127–129
Hirose M, Fukushima S, Hananouchi M, Shirai T, Ogiso T, Takahashi M & Ito N
(1976) *Gann: the Japanese Journal of Cancer Research* **67**, 175–189
Lijinsky W & Taylor H W
(1975) *Cancer Research* **45**, 958–961
Magee P N & Barnes J M
(1967) *Advances in Cancer Research* **10**, 163–246
Melzak J
(1966) *Paraplegia* **4**, 85–96

Preussman R
(1972) In: N-Nitroso Compounds, Analysis and Formation. Ed. P Bogovski, R Preussman & E A Walker. Scientific Publications No. 3, IARC, Lyon; pp 6–9
Walters C L, Fueggle D G & Hunt I G
(1974) In: N-Nitroso Compounds in the Environment. Ed. P Bogovoski & E A Walker. Scientific Publications No. 9, IARC, Lyon; pp 22–25

Miss C M Evans
(Urology Department, Nottingham City Hospital, Nottingham, NG5 1PB)
and Dr J G Bowen
(Cancer Research Campaign Laboratories, Nottingham University, Nottingham, NG7 2RD)

Immunological Tests in Carcinoma of the Prostate

It has been well established that in animal tumour systems, the tumours carry antigens which are immunogenic in the autochthonous host (Baldwin 1973, Basombrío & Prehn 1972, Hellström & Hellström 1969). In man, however, the evidence is more circumstantial and age distribution of tumours, for example, has been cited as being indicative of an immune surveillance mechanism, as has the higher incidence of tumours in immunosupressed or immunodeficient patients. The fact that there appears to be a correlation between lymphoid cell infiltration into the tumour, and prognosis has also been interpreted as showing the existence of an immune response directed against the tumour. *In vitro* evidence for the existence of an ongoing immune response, particularly in terms of the cytotoxicity of lymphoid cells for cultured tumour cells, is currently an area of much controversy (Baldwin 1975, Herberman & Oldham 1975), although other systems such as leukocyte migration testing (Char *et al.* 1973, Cochran *et al.* 1974, Black *et al.* 1974*a*, Black *et al.* 1974*b*) or leukocyte adherence inhibition testing (Halliday *et al.* 1974, Halliday *et al.* 1975, Marti & Thomson 1976) have suggested the existence of antigens which crossreact between tumours of the same histological type but not with tumours which arise in other tissues. These latter two tests have shown that reactivity is present in melanoma, breast and colorectal carcinoma patients. However, in patients with a heavy tumour load, i.e. Stage IV tumour, reactivity can be depressed. For example, with the leukocyte migration (LM) test, Black *et al.* (1974*a*), examining mammary carcinoma, and Elias & Elias (1975), with colorectal carcinoma patients, concluded that the lower the staging the greater the percentage of patients who were capable of reacting to homologous tumour tissue. Similarly, Marti & Thomson (1976) examined melanoma patients using the leukocyte adherence inhibition (LAI) test and showed that 80% of these patients could react against a melanoma extract whereas only 4.5% of healthy donors responded against the same extract. They also showed that the response was decreased for a short period following surgery in patients with disseminated melanoma or in patients being treated by cytotoxic drugs. Furthermore, they concluded that residual or recurrent tumour caused a persistent or recurrent LAI reactivity, results which are similar to those of O'Toole *et al.* (1973) who maintained that the reappearance of lymphocytes cytotoxic for the bladder tumour cell line T24 indicated the recurrence of tumour.

Delayed dermal hypersensitivity has been used by a number of investigators to examine the *in vivo* immunological response of cancer patients. The results using recall antigens such as paraphenylenediamine (PPD) (Thomas *et al.* 1976), primary reactive antigens e.g. dinitrochlorobenzene (DNCB) (Simo-Camps *et al.* 1976) or homologous tumour extracts (Oren & Herberman 1971) have been rather disappointing in terms of determining a patient's prognosis, the major correlation being with a patient's age (Gross 1965). However, although poor reactivity has been detected in a group of morbid patients in the terminal stages of disease good reactivity is not found in patients with minimal disease. In general, these tests have proven uninformative when applied at the individual patient level.

On the hypothesis that two tests may give more information than either alone, a pilot study was begun using patients with prostatic cancer who were examined by the leukocyte migration test or the leukocyte adherence inhibition test and by dinitrochlorobenzene delayed dermal hypersensitivity testing.

Materials and Methods

Heparinized blood samples were obtained from patients attending the City Hospital, Nottingham. Diagnosis of prostatic carcinoma was confirmed histologically from biopsy specimens during transurethral resection of the prostatic mass and patients were staged according to the Veterans Administration Cooperative Urological Research Group (VACURG) system (1967). Control blood was obtained from a variety of age and sex-matched hospital patients undergoing treatment for nonmalignant disease as well as from a panel of male laboratory workers.

Antigen extract preparation: Tissue obtained during transurethral resection of a prostatic mass was classed on the basis of histology by an experienced pathologist as prostatic carcinoma (PC) or benign prostatic hypertrophy (BPH). Both tissues were