Biochemical Pharmacology, Vol. 22, pp. 1581–1588. Pergamon Press, 1973. Printed in Great Britain.

# ENZYMATIC *N*-ACETYLATION OF CARCINOGENIC AROMATIC AMINES BY LIVER CYTOSOL OF SPECIES DISPLAYING DIFFERENT ORGAN SUSCEPTIBILITIES*

GERALD M. LOWER, JR. and GEORGE T. BRYAN†

Division of Clinical Oncology, University of Wisconsin Medical School, Madison, Wis. 53706, U.S.A.

(*Received* 17 *August* 1972; *accepted* 22 *December* 1972)

**Abstract**—The relative capability of *N*-acetyltransferase enzyme systems from liver cytosol of various mammalian species to carry out the transfer of acetyl groups from acetyl-CoA to the amine function of the carcinogens, 2-aminofluorene, 4-aminobiphenyl and 2-aminonaphthalene, was investigated. The carcinogenic arylamines were incubated with liver cytosol in the presence of acetyl-1-$^{14}$C-CoA and enzyme activity was estimated by quantitation of the resulting $^{14}$C-labeled arylacetamides. In hamsters, guinea pigs, mice and rats, 2-aminofluorene was the superior substrate followed in order by 4-aminobiphenyl and 2-aminonaphthalene. Highest enzyme activity was present in liver cytosol from the hamster, followed in order by hepatic cytosol from the guinea pig, mouse and rat. In contrast, dog liver cytosol was incapable of carrying out detectable *N*-acetylation of any of the three carcinogenic arylamines studied. Administration of a variety of carcinogenic arylamines to dogs has resulted in the formation of only urinary bladder tumors, the liver apparently being refractory to this type of carcinogenic insult. In contrast, administration of carcinogenic arylacetamides to dogs has resulted in the unequivocal formation of both urinary bladder tumors and hepatomas. These considerations suggest that *N*-acetylation is not required for carcinogenesis of the urinary bladder and that the expression of urinary bladder or hepatocarcinogenicity by arylamines and arylacetamides in a given experimental system may depend on the quantitative ability of the species being studied to utilize pathways for the interconversion of arylacetamide derivatives and arylamine derivatives.

THE METABOLISM of carcinogenic arylamine derivatives *in vivo* includes ring hydroxylation, *N*-hydroxylation, conjugation of the ring and *N*-hydroxyl metabolites, and *N*-acylation of the amine function. Deacylation reactions are also observed with *N*-acylated arylamines. Unfortunately, there is a deficiency of quantitative data on the relative rates with which these various metabolic processes are carried out in species displaying different organ susceptibilities to carcinogenesis by arylamines and arylacetamides.

One of the major routes for the metabolism of carcinogenic arylamines in a variety of species involves *N*-acetylation of the amine function. This process involves the transfer of the acetyl moiety of acetyl-CoA to arylamines, as catalyzed by *N*-acetyltransferase enzyme systems present in the soluble portion of mammalian liver,[1] and has been considered to be a detoxication mechanism.[2] The present report is a comparative examination *in vitro* of the ability of various species to carry out the *N*-acetyla-

* This research was supported by Public Health Service Research Grants CA 14524 and CA 10017-06 from the National Cancer Institute.

† Career Development Awardee (1-K4-CA-8254-05) of the National Cancer Institute.

tion of 2-aminofluorene, 4-aminobiphenyl and 2-aminonaphthalene in an attempt to clarify the possible role of *N*-acetylation as a determinant of organ susceptibility to these carcinogens.

## MATERIALS AND METHODS

*Chemicals.* 2-Aminonaphthalene and 2-acetylaminonaphthalene (Aldrich Chemical Co., Milwaukee, Wis.), 2-aminofluorene (K & K Labs. Inc., Plainview, N.Y.), 2-acetylaminofluorene (Mann Research Labs., New York), and 4-aminobiphenyl (Eastman Organic Chemicals, Rochester, N.Y.) were obtained commercially. 2-Aminonaphthalene and 4-aminobiphenyl were purified before use by recrystallization from ethanol and distillation *in vacuo* from zinc dust respectively.[3] 4-Acetylaminobiphenyl was a generous gift from Dr. James Miller. Unlabeled acetyl-CoA as the lithium salt was purchased from P-L Biochemicals, Inc. (Milwaukee, Wis.) and acetyl-1-$^{14}$C-CoA (spec. act. 59·2 mCi/m-mole) was purchased from New England Nuclear Corp. (Boston, Mass.). All other chemicals were reagent grade.

*Preparation of liver cytosol.* Fresh liver tissues were obtained from 150–200 g female Sprague–Dawley rats (Sprague–Dawley Co., Madison, Wis.), 30–35 g female Swiss albino mice (Rolfsmeyer Co., Madison, Wis.), 50–60 g female Syrian golden hamsters (A. R. Schmidt Co., Madison, Wis.), 250–300 g female guinea pigs (Marvin O'Brian, Oregon, Wis.), and female mongrel dogs (Laboratory of Experimental Surgery, University of Wisconsin Medical School). Rodents were sacrificed by cervical dislocation whereas dogs were anesthetized with pentobarbital prior to the removal of a liver biopsy. All liver samples were immediately chilled in ice-cold 0·25 M sucrose, blotted, weighed and homogenized at 0° in 10 volumes of 0·25 M sucrose in a Teflon-glass homogenizer. Homogenates were centrifuged at 105,000 *g* for 60 min in a Beckman L-265B ultracentrifuge with a No. 40 rotor. The resulting supernatant was utilized in the enzyme assay which allowed the estimation of total *N*-acetyltransferase activity in the absence of competing microsomal enzymes responsible for the ring and *N*-hydroxylation of arylamines.

*Incubation procedures.* The method of Lotlikar and Luha[4] was modified so as to make it suitable for the measurement of liver *N*-acetyltransferase activity. A typical incubation mixture contained the following in a final volume of 1·0 ml: 4·0 $\mu$moles of arylamine substrate dissolved in 0·1 ml of dimethyl sulfoxide, 1·0 $\mu$mole of acetyl-CoA containing acetyl-1-$^{14}$C-CoA (0·25 $\mu$Ci), 100 $\mu$moles of Tris–HCl buffer (pH 7·5 at 37°), and liver cytosol equivalent to 50 mg wet wt of liver. The reaction was initiated by the addition of liver cytosol and was allowed to proceed with gentle shaking at 37° for 5 min or longer in certain instances. Termination of the reaction was accomplished by the addition of 2·5 ml of 0·5 N HCl.

*Assay procedures.* Enzyme activity was estimated by quantitating the amount of arylacetamide produced in the incubation mixtures. The acidified incubation mixtures were extracted with 10 ml of benzene and the benzene extract was washed twice with equal volumes of distilled water. Radioactive arylacetamides in samples of the washed benzene extracts were quantitated using liquid scintillation techniques described previously.[5] Corrections in enzyme activity were made for trace amounts of benzene-extractable radioactivity (0·33 $\pm$ 0·06 nmoles of acetamide/50 mg wet wt of liver/5 min) in appropriately treated blanks incubated in the absence of arylamine substrates. Identification of the arylacetamides was effected by determination of the u.v. absorp-

tion spectra of the reaction product in benzene extracts using a Beckman DB-G recording spectrophotometer. A portion of the benzene extracts was concentrated under a stream of air and chromatographed on 0·25 mm Silica gel thin-layer plates (EM Labs. Inc., Elmsford, N.Y.) with the solvent system of Boyland and Manson[6] consisting of benzene–ethanol (95:5, v/v). Arylacetamides were visualized under u.v. light and radioactivity was monitored with a Varian Series 6000 radiochromatogram scanner. Mass spectra of arylacetamides present in concentrated benzene extracts were obtained with a Hewlett Packard model 5930A mass spectrometer interfaced to a Hewlett Packard model 5700A gas chromatograph (6 ft stainless steel columns, UCW 982 (10%) on Chromosorb W-HP, 250° isothermal).

The relative efficiency of extraction of the arylacetamides was determined by replacing arylamine substrates in acidified incubation mixtures with 1·0 $\mu$mole of the respective arylacetamides. The arylacetamides were extracted with benzene as described above and quantitated using spectrophotometric measurements at their respective u.v. absorption maxima. The recoveries of 2-acetylaminofluorene (95·3 $\pm$ 0·6 per cent), 4-acetylaminobiphenyl (97·2 $\pm$ 1·5 per cent), and 2-acetylaminonaphthalene (88·4 $\pm$ 1·6 per cent) in four replicate assays proved to be sufficiently precise and quantitative to allow comparisons of the relative rates of *N*-acetylation of the respective arylamine substrates.

## RESULTS

The effect of pH on the activity of the *N*-acetyltransferase enzyme system in mouse liver cytosol was investigated using 2-aminofluorene as substrate. Employing Tris–HCl buffers, taken to various pH values at 37°, optimum *N*-acetyltransferase activity was evident at about pH 7·5 (Fig. 1), an observation similar to those reported by other workers.[1] Although Lotlikar and Luha[4] reported optimum *N*-acetylation of 2-aminofluorene at pH 8·0 with rat liver cytosol, the present experiments were conducted at

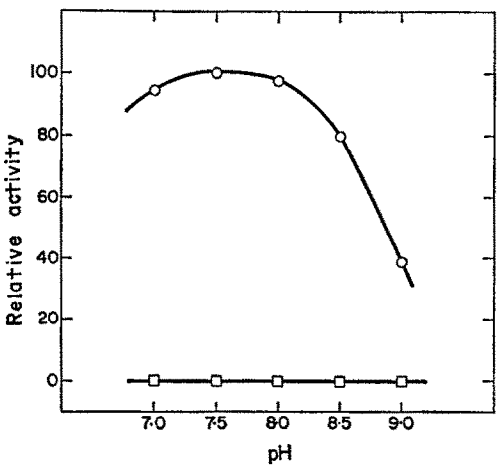


FIG. 1. pH Profile of the *N*-acetyltransferase enzyme system present in mouse and dog liver cytosol as determined by using 2-aminofluorene as substrate and 5-min incubation periods. Mouse liver cytosol = ○——○; dog liver cytosol = □——□.

pH 7·5 because of its more central position on the plateau of the *N*-acetyltransferase pH profile.

To determine the appropriate conditions for a valid estimation of enzyme activity, the formation of 2-acetylaminofluorene from 2-aminofluorene was measured with varying times of incubation (Fig. 2). The formation of 2-acetylaminofluorene was a linear function and directly proportional with time indicating that, with up to 7·5-min incubation periods, the reaction was zero order and independent of substrate concentrations. This finding casts some doubt upon the validity of the enzyme assay of Lotlikar and Luha[4] in which 100 mg equivalents of liver cytosol were incubated with 2-aminofluorene for periods of 30 min. Under these conditions, the formation of 2-acetylaminofluorene rapidly depleted both substrate and acetyl-CoA, such that the reaction had nearly halted at equilibrium after 10 min.

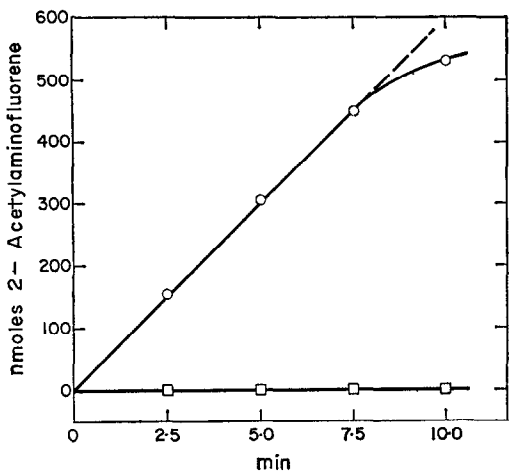


FIG. 2. Formation of 2-acetylaminofluorene with time by mouse and dog liver cytosol. 2-Aminofluorene (4·0 μmoles) and 1·0 μmole of acetyl-1-$^{14}$C-CoA were incubated with 50 mg equivalents of liver cytosol at pH 7·5 and 37°. Mouse liver cytosol = ○——○; dog liver cytosol = □——□.

Utilization of a 5-min incubation period for the estimation of enzyme activity revealed that liver cytosol from a variety of species is capable of *N*-acetylating carcinogenic arylamines. With each arylamine substrate examined, *N*-acetylation by liver cytosol enzyme gave, as the only radioactive product, the corresponding arylacetamide. After incubating 2-aminofluorene, 4-aminobiphenyl or 2-aminonaphthalene with liver cytosol from rats, mice, hamsters or guinea pigs, the mass spectrum ($M^+$ = 223,211,185), the gas chromatographic retention times, the $R_f$ value on thin-layer chromatograms and the u.v. absorption spectrum of each reaction product were identical to that observed with authentic 2-acetylaminofluorene, 4-acetylaminobiphenyl or 2-acetylaminonaphthalene respectively. In addition, all detectable radioactivity on chromatograms corresponded exactly with the elution position of the authentic arylacetamides (Fig. 3). In the absence of enzyme, none of the arylamine substrates were *N*-acetylated by acetyl-CoA.

The ability of liver cytosol from various species to *N*-acetylate carcinogenic arylamines is summarized in Table 1. While there were large and significant differences

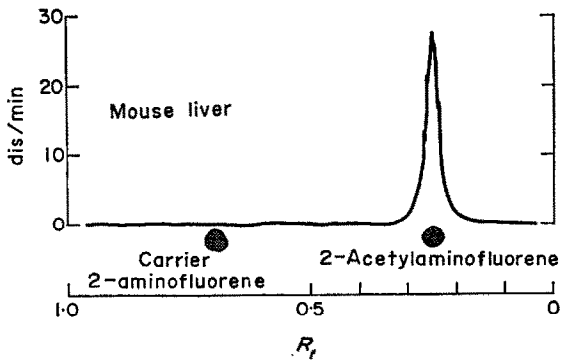


FIG. 3. Typical radiochromatogram of benzene extracts of incubations of 2-aminofluorene and acetyl-CoA with mouse liver cytosol. With all species except the dog, the only detectable radioactive product was $^{14}$C-labeled 2-acetylaminofluorene.

among the species studied, the degree of variation within animals of the same species, as reflected in the standard deviations, was generally quite small. In all instances, 2-aminofluorene proved to be the superior substrate, usually followed in order by 4-aminobiphenyl and 2-aminonaphthalene.

TABLE 1. ENZYMATIC *N*-ACETYLATION OF CARCINOGENIC AROMATIC AMINES BY LIVER CYTOSOL FROM VARIOUS SPECIES*

| Species | No. of animals | Enzyme activity (nmoles acetamide/50 mg wet wt liver/5 min) | | |
|---|---|---|---|---|
| | | 2-Aminofluorene | 4-Aminobiphenyl | 2-Aminonaphthalene |
| Hamster | 4 | 618 ± 49 | 506 ± 35 | 469 ± 23 |
| Guinea pig | 4 | 408 ± 12 | 351 ± 19 | 289 ± 22 |
| Mouse | 6 | 332 ± 25 | 215 ± 15 | 209 ± 9 |
| Rat | 4 | 38·6 ± 7·9 | 20·5 ± 3·3 | 33·2 ± 6·1 |
| Dog | 5 | 0 | 0 | 0 |

* With all substrates, statistically significant differences in enzyme activity were observed with all species. With 2-aminofluorene as substrate, for example, calculated P values were: hamsters vs guinea pigs ($P < 0{\cdot}001$), guinea pigs vs mice ($0{\cdot}001 < P < 0{\cdot}01$) and mice vs rats ($P < 0{\cdot}001$). Similarly, 2-aminofluorene was a better substrate than 4-aminobiphenyl with hamsters ($0{\cdot}001 < P < 0{\cdot}01$), guinea pigs ($0{\cdot}001 < P < 0{\cdot}01$), mice ($P < 0{\cdot}001$) and rats ($0{\cdot}001 < P < 0{\cdot}01$).

Of the species studied, hamster liver cytosol displayed the greatest enzyme activity followed by liver cytosol from the guinea pig, mouse and rat. Of interest, however, was the apparent inability of dog liver cytosol to carry out the *N*-acetylation of any of the carcinogenic arylamines studied. Indeed, incubation of arylamines with dog liver cytosol for periods of up to 60 min at pH values from 7–9 failed to yield detectable benzene-extractable radioactivity (Fig. 1) or chromatographic evidence for the presence of arylacetamides (Fig. 3), despite the fact that the present assay method should allow the measurement of enzyme activity as low as 0·1 nmole of acetamide/50 mg wet wt of liver/5 min.

## DISCUSSION

Carcinogenic arylamines, arylacetamides and arylnitro compounds seldom induce tumors at the site of administration. Indeed, most tumors induced by these compounds are found on the routes of excretion, i.e. the liver, biliary tract, intestinal tract or urinary tract, and data have been presented[7,8] strongly supporting the postulate that these carcinogens require metabolic activation to express their carcinogenicity.

The proximate carcinogenic metabolites of carcinogenic arylamines, arylacetamides and arylnitro compounds would appear to be the corresponding *N*-hydroxyarylamines (arylhydroxylamines) or the corresponding *N*-hydroxyarylacetamides (arylhydroxamic acids).[7,8] Formation of both *N*-hydroxyarylamines and *N*-hydroxyarylacetamides from carcinogenic arylamines, arylacetamides and arylnitro compounds seems probable in view of the possible metabolic interconversion of arylamine and arylacetamide derivatives. Thus, while *N*-acetylations, deacetylations and transacetylations of free and acylated arylamines have been described,[9,10] there is also evidence *in vivo*[11] and *in vitro*[4] for *N*-acetylation of *N*-hydroxyarylamines and considerable evidence *in vitro* for the deacetylation of *N*-hydroxyarylacetamides.[12,13]

An essential role of *N*-hydroxyarylacetamides in hepatocarcinogenesis in rodents by arylamines and arylacetamides has been advocated.[7,8] Thus, proximate *N*-hydroxyarylacetamides are more strongly hepatocarcinogenic than the parent compounds, and they are also active at sites where the parent arylacetamides are inactive. In contrast, carcinogenesis of the urinary bladder in dogs appears to be directly related to the formation of *N*-hydroxyarylamines,[14] with these compounds demonstrating carcinogenic activity by direct local application to the dog bladder epithelium.[15]

The importance of *N*-hydroxyarylamines in carcinogenesis of the urinary bladder is further supported by the observation that a large number of carcinogenic arylamines induce bladder cancer in the dog despite the present study *in vitro* indicating that the dog is incapable of *N*-acetylating the aromatic amine group of known urinary bladder carcinogens (Table 1). Similarly, studies *in vivo* have suggested that the dog is apparently incapable of *N*-acetylating 4-aminobiphenyl,[3] 2-aminofluorene[16] and 2-aminonaphthalene.[17] Thus, oral administration of carcinogenic arylamines or arylnitro compounds to dogs has resulted in the formation of primarily urinary bladder tumors with tumors of the liver seldom, if ever, being observed (Table 2, Section A). In one exception, tumors of the liver appearing in dogs fed *o*-aminoazotoluene were believed to have originated in the gall bladder[18] (discussed in Ref. 19), an organ in the dog also appearing to be susceptible to carcinogenesis by arylamine and arylnitro compounds. In sharp contrast, administration of carcinogenic arylacetamides to dogs has led to the unequivocal formation of both urinary bladder tumors and hepatomas (Table 2, Section B).

Thus, it would appear that while *N*-acetylation may be required for carcinogenesis of the liver,[7,8] it is probably not involved in carcinogenesis of the urinary bladder. These considerations and the data reported in the present study lend considerable support to the proposition first suggested by Poirier *et al.*[16] that one of the proximate carcinogens involved in urinary bladder carcinogenesis may be an *N*-hydroxyarylamine, whereas in liver an *N*-hydroxyarylacetamide may be required for carcinogenesis, with both of these proximate carcinogenic metabolites being derived from carcinogenic arylamines, arylacetamides and arylnitro compounds by virtue of interconvergent pathways of metabolic activation. While overlap of organ specificity

TABLE 2. AROMATIC AMINES, AROMATIC ACETAMIDES AND NITRO COMPOUNDS TESTED FOR CARCINOGENIC ACTIVITY BY ORAL ADMINISTRATION TO DOGS

| Carcinogen | No. dogs tested | No. with bladder tumors | No. with liver tumors | Other tumors | References |
|---|---|---|---|---|---|
| A. Aromatic amines and nitro compounds | | | | | |
| 4-Aminobiphenyl | 2 | 2 | 0 | None | Walpole *et al.*[20] |
| 4-Aminobiphenyl | 4 | 4 | 0 | None | Deichmann *et al.*[21] |
| 2-Aminonaphthalene | 16 | 8 | 0 | None | Hueper *et al.*[22] |
| 2-Aminonaphthalene | 4 | 3 | 0 | None | Bonser[23] |
| 2-Aminonaphthalene | 4 | 2 | 0 | None | Bonser *et al.*[24] |
| 2-Aminonaphthalene | 5 | 4 | 0 | None | Deichmann and Radomski[25] |
| 2-Aminonaphthalene | 3 | 2 | 0 | None | Gehrmann *et al.*, quoted in Ref. 25 |
| 4-4′-Diaminobiphenyl | 6 | 1 | 0 | None | Spitz *et al.*[26] |
| 4-4′-Diaminobiphenyl | 7 | 3 | 0 | None | Spitz, quoted in Ref. 27 |
| 4-Nitrobiphenyl | 4 | 3 | 0 | None | Deichmann *et al.*[28] |
| *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide | 4 | 4 | 0 | 4-gall bladder | Ertürk *et al.*[29] |
| *o*-Aminoazotoluene | 4 | 2 | 2 (gall bladder metastases) | 2 gall bladder | Nelson and Woodard[18] |
| B. Aromatic acetamides | | | | | |
| 4-Acetylamino-biphenyl | 3 | 3 | 1 | None | Jabara[30] |
| 2-Acetylamino-fluorene | 2 | 0 | 1 | None | Jabara[30] |
| 2-Acetylamino-fluorene | 5 | 4 | 4 | None | Morris and Eyestone[31] |

cannot be ruled out, the expression of hepatocarcinogenicity or urinary bladder carcinogenicity by an arylamine, arylacetamide or arylnitro compound in a given experimental system might, therefore, tend to depend on the quantitative ability of the species being studied to utilize pathways for the interconversion of arylacetamide derivatives and arylamine derivatives.

Obviously, clarification of the precise role of *N*-acetylation as a possible determinant of liver and urinary bladder susceptibility to carcinogenic arylamines and arylacetamides will require further detailed examinations of the activity of mammalian *N*-acetyltransferase and deacetylase enzyme systems and their respective functions in defining the urinary excretion patterns of arylamine and arylacetamide derivatives.

*Acknowledgements*—The authors would like to thank Miss Gail Comer for technical assistance and Mrs. Susanne Pertzborn and Mrs. Kathleen Deighton for assistance in the preparation of the manuscript.

## REFERENCES

1. W. W. WEBER and S. N. COHEN, *Molec. Pharmac.* **3**, 266 (1967).
2. R. T. WILLIAMS, *Ann. N.Y. Acad. Sci.* **179**, 141 (1971).
3. J. A. MILLER, C. S. WYATT, E. C. MILLER and H. A. HARTMANN, *Cancer Res.* **21**, 1465 (1961).

4. P. D. LOTLIKAR and L. LUHA, *Biochem. J.* **123,** 287 (1971).
5. G. M. LOWER, JR. and G. T. BRYAN, *Cancer Res.* **29,** 1013 (1969).
6. E. BOYLAND and D. MANSON, *Biochem. J.* **101,** 84 (1966).
7. E. C. MILLER and J. A. MILLER, *Ann. N.Y. Acad. Sci.* **163,** 731 (1969).
8. J. A. MILLER and E. C. MILLER, *Prog. exp. Tumor Res.* **11,** 273 (1969).
9. J. H. PETERS and H. R. GUTMANN, *J. biol. Chem.* **216,** 713 (1955).
10. J. BOOTH, *Biochem. J.* **100,** 745 (1966).
11. J. H. WEISBURGER, P. H. GRANTHAM and E. K. WEISBURGER, *Biochem. Pharmac.* **15,** 833 (1966).
12. C. C. IRVING, *Cancer Res.* **26,** 1390 (1966).
13. M. JÄRVINEN, R. S. S. SANTTI and V. K. HOPSU-HAVU, *Biochem. Pharmac.* **20,** 2971 (1971).
14. J. L. RADOMSKI and E. BRILL *Science, N.Y.* **167,** 992 (1970).
15. J. L. RADOMSKI, E. BRILL, W. B. DEICHMANN and E. M. GLASS, *Cancer Res.* **31,** 1461 (1971).
16. L. A. POIRIER, J. A. MILLER and E. C. MILLER, *Cancer Res.* **23,** 790 (1963).
17. E. BOYLAND, C. H. KINDER and D. MANSON, *Biochem. J.* **78,** 175 (1961).
18. A. A. NELSON and G. WOODARD, *J. natn. Cancer Inst.* **13,** 1497 (1953).
19. G. M. BONSER, *J. natn. Cancer Inst.* **43,** 271 (1969).
20. A. L. WALPOLE, M. H. C. WILLIAMS and D. C. ROBERTS, *Br. J. ind. Med.* **11,** 105 (1954).
21. W. B. DEICHMANN, J. L. RADOMSKI, W. A. D. ANDERSON, M. M. COPLAN and F. M. WOODS, *Ind. Med. Surg.* **27,** 25 (1958).
22. W. C. HUEPER, F. H. WILEY and H. D. WOLFE, *J. ind. Hyg. Toxicol.* **20,** 46 (1938).
23. G. M. BONSER, *J. Path. Bact.* **55,** 1 (1943).
24. G. M. BONSER, D. B. CLAYSON, J. W. JULL and L. N. PYRAH, *Br. J. Cancer* **10,** 533 (1956).
25. W. B. DEICHMANN and J. L. RADOMSKI, *Ind. Med. Surg.* **32,** 161 (1963).
26. S. SPITZ, W. H. MAGUIGAN and K. DOBRINER, *Cancer, N.Y.* **3,** 789 (1950).
27. S. SPITZ, quoted by G. M. BONSER, *Acta Un. int. Cancer.* **18,** 538 (1962).
28. W. B. DEICHMANN, W. M. MACDONALD, M. M. COPLAN, F. M. WOODS and W. A. D. ANDERSON, *Ind. Med. Surg.* **27,** 634 (1958).
29. E. ERTÜRK, S. A. ATASSI, O. YOSHIDA, S. M. COHEN, J. M. PRICE and G. T. BRYAN, *J. natn. Cancer Inst.* **45,** 535 (1970).
30. A. G. JABARA, *Cancer Res.* **23,** 921 (1963).
31. H. P. MORRIS and W. H. EYESTONE, *J. natn. Cancer Inst.* **13,** 1139 (1953).