4 Perhaps the greatest difference between our conclusions and those of Edmunds is his claim that synergy may be important where pus is present *in vivo*. Even if synergy had been demonstrated *in vitro*, the relative amounts of sulphadiazine (20 µg) to trimethoprim (1 µg) may be far from the levels of these agents in purulent tissues in man.

It is not clear whether Edmunds believes his findings are relevant to the prescribing of co-trimoxazole (this contains sulphamethoxazole rather than sulphadiazine); the actual tissue or urine levels of active sulphamethoxazole are so low, on account of hepatic acetylation, that there is virtually no prospect of synergy *in vivo*. We have demonstrated this with urine and consider that, for many infections, the level of sulphamethoxazole is subinhibitory.

5 One point that we agree upon is that organisms highly resistant to either sulphonamide or trimethoprim never show synergy with the combination. Because of (1) the high frequency of sulphonamide resistance in many pathogens and (2) the possibility of serious toxicity associated with the sulphonamide moiety, the case for preferring trimethoprim alone (when available) to the combination co-trimoxazole for many infections must be overwhelming.

R. W. LACEY and ANNE STOKES
West Norfolk and King's Lynn
General Hospital,
London Road,
King's Lynn,
Norfolk, PE30 5QD

The author comments as follows:

1 In my experience, distortion of zones of inhibition due to additive effects are minimal, and I have seen many such instances over my 28 years as a bacteriologist. They never show the obvious and marked increase shown in the Figure in my article (Edmunds, 1978). I agree that MIC tests should be performed; the fact that they have not been done is due to lack of suitable pus, time, and facilities. I hope that someone with access to more of these commodities will take this matter up. There can, however, be no doubt whatever that synergy occurred under the conditions of my experiments, using basal minimal

salts agar medium with pus in up to 50% concentration.

2 I have found only minor degrees of bactericidal synergy in Davis-Mingoli minimal salts broth (Davis and Mingoli, 1950), and I make no claims on this score. However, Bushby (1973, 1975) has shown this to occur *in vitro*.

3 There were in fact sufficient antagonists present even in the filtered pus extract at 10% concentration to abolish zones of inhibition of well-separated disks of sulphonamide (SU) and trimethoprim (TM). The same effect of antagonism, and synergy despite antagonists, was observed in plates containing up to 50% unfiltered pus, whose only treatment was autoclaving. How much more antagonists do Dr Lacey and Miss Stokes want? If they wish, I will be happy to provide them with a photograph of the 50% pus plate, although this does not show enough contrast for publication.

4 I do not know whether synergy is important *in vivo*. According to Bushby (1975), it has been established in animal experiments. I merely point out that synergy occurred in my experiments in the presence of large amounts of pus and that this may be of significance in treatment. As far as urine is concerned, I hold no particular brief for using co-trimoxazole in lower urinary tract infections, except that one is not always sure that the infection has no renal tissue element. My arguments are partly directed to the small concentrations likely to be found in the tissues and also to a possible protective effect of the combination of sulphonamide and trimethoprim against the long-term development of resistance to these two drugs, particularly trimethoprim.

5 Dr Lacey and I agree that co-trimoxazole should not be used against an organism which is completely resistant to sulphonamide. The effect would then be that of trimethoprim alone but with the disadvantage of added sulphonamide toxicity. On the other hand, if trimethoprim is used extensively as a single-drug preparation, any protective effect of sulphonamide for sensitive strains will be lost. At present we in Fife enjoy the favourable position where trimethoprim resistance occurs in 1% of hospital *Escherichia coli* strains and 2% of all urinary coliforms combined, excluding *Pseudomonas aeruginosa*. I fear that this position will be eroded and trimethoprim resistance will rise to or exceed the 8.2% reported in London by Grüneberg (1976).

P. N. EDMUNDS

Department of Medical Microbiology,
Fife Area Laboratory,
Scotland

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A non-carcinogenic substrate for immunoperoxidase procedures

3,3'-Diaminobenzidine tetrahydrochloride (DAB) is used in immunoperoxidase procedures to render the horseradish peroxidase labelled antibody-antigen complex visible at the light microscope level (Burns, 1978). The recent decrease in availability of good quality DAB could be due to its borderline carcinogenic properties (Hanker *et al.*, 1977). An alternative non-carcinogenic DAB substrate was described by these authors

7.5-15 mg *p*-phenylenediamine dihydrochloride (1 part) + pyrocatechol (2 parts)-premixed reagent (PDP) (Polysciences) dissolved in
10 ml Tris buffer (0.1 M) pH 7.6
0.1 ml H₂O₂ (1%)

for the demonstration of injected horseradish peroxidase in tissue sections.

I wish to report that this substrate can also be substituted for the DAB substrate in immunoperoxidase procedures for demonstrating antigens in paraffin sections (Figure). Sections are incubated for 5-20 minutes. The end product, like that of DAB, is insoluble and osmophilic, can be counterstained with routine histological methods, withstands pro-

Figure Formalin paraffin section of tonsil stained by the immunoperoxidase-PDP method for intracellular immunoglobulin (Haematoxylin $\times 800$).

concentration is frequently reduced in B12 deficiency (Hoffbrand *et al.*, 1966); it should be made clear how many of the patients with a reduced red cell folate concentration were in fact B12 deficient. Certainly their statement that 'A low red cell folate is considered unequivocal evidence of folate deficiency' cannot be supported. Significant B12 and folate deficiency is a rare finding in patients with a normal MCV unless there is accompanying iron deficiency, chronic inflammatory disease, or malignancy. No mention of these factors is made by Raper and Choudhury in their patients with normal MCVs and a low red cell folate or B12 concentration, and hence any significance of their findings is masked.

The fact that 18 out of 40 patients with normal red cell folate concentrations and raised MCVs 'developed folic acid deficiency during the following six months' is not surprising, and an appraisal of blood film morphology and serum folate results together with bone marrow examination would have been appropriate. Perhaps more surprising was the finding of a normal B12 concentration in a patient with pernicious anaemia. There would certainly appear to be little justification for repeated red cell folate assays in these patients as seems to be suggested by the authors.

There is still no substitute for a careful evaluation of the clinical and haematological findings in an individual case before requesting B12 or folate assays.

R. D. HUTTON

Department of Haematology
University Hospital of Wales
Heath Park
Cardiff CF4 4XN

The author comments as follows:

This laboratory is a regular contributor to the DHSS and BCSH Haematology Quality Assessment Trial and during the period of our study we submitted results on 40 blood samples supplied by the DHSS. On 38 out of the 40 samples our MCV result was within 1.8 standard deviations of the overall national mean obtained from the results submitted by all the laboratories. This means that our MCV is usually no more than 2 fl different from the national average.

Five of the 156 patients with a red cell folate of <170 ng/ml had a subnormal serum B12 level. We agree that significant B12 and folate deficiency is a rare finding in patients with a normal MCV unless

cessing to xylol, and does not fade on storage after being mounted in DPX.

J. BURNS

Department of Pathology,
Radcliffe Infirmary
Oxford OX2 6HE
UK

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Early detection of folic acid deficiency in elderly patients

The recent paper by Raper and Choudhury (1978) is likely to lead to confusion and provoke unjustified requests for haematic assays. Their method of assigning a mean red cell volume (MCV) to a specimen of blood relies on an arbitrary calibration of their Coulter 'S' by adjusting the MCV potentiometer to give a mean value of 89 fl based on a study of 250 blood donors. No justification for this method of calibration is given and the comparability with MCVs measured in other laboratories is highly suspect.

The confusion is compounded by the patients being divided into groups with (a) red cell folate <170 ng/ml, (b) B12 <160 pg/ml and red cell folate >170 ng/ml, and (c) red cell folate >170 ng/ml and B12 >160 pg/ml. As the red cell folate