tracted with ether, giving 0.10 g. of oil, which was dissolved in 4 ml. of ethanol and treated with 2:4-dinitrophenylhydrazine in HCl (Brady & Elmslie, 1926). A red precipitate, 0.13 g., m.p. 244–246°, was obtained. The m.p. was raised to 251–252° on recrystallization from ethyl acetate—brilliant red plates. A further amount of the dinitrophenylhydrazone was prepared directly from the oily portion of the distillate without hydrolysis. This was shown by analysis and comparison (mixed m.p.) with an authentic sample of m.p. 252° to be the 2:4-dinitrophenylhydrazone of anisaldehyde. (Found: C, 53.27; H, 3.86; N, 17.5; MeO, 9.9. Calc. for $C_{11}H_{12}N_4O_5$: C, 53.16; H, 3.82; N, 17.71; MeO, 9.8%.)

The ether-extracted neutral solution from the hydrolysis on acidification yielded crystals (0.31 g.) of m.p. 176–181°, consisting of impure anisic acid. Thus Fraction I of the distillate consisted mainly of methyl anisate together with a small amount of anisaldehyde.

(b) *Fraction II.* 0.64 g., b.p. 140–175°/c. 1 mm. This was a yellow oil containing a little crystalline material. As it was acidic it was dissolved in ether and washed with saturated aqueous $NaHCO_3$.

(c) *Acids from Fraction II.* On acidification of the $NaHCO_3$ solution, acids (0.05 g.) were precipitated. They were subjected to fractional sublimation in high vacuum, and gave two main fractions: (1) 12 mg., m.p. 102–104°; (2) 17 mg., m.p. 166–176°, after softening above 120°. Sublimates (1) was still a mixture which could not be satisfactorily separated into its components. Sublimates (2) on recrystallization from benzene gave 8 mg. of an acid of m.p. 180–181°, which was shown by comparison (mixed m.p.) with an authentic sample to consist of anisic acid.

(d) *Neutral portion of Fraction II.* The ether solution which had been washed with $NaHCO_3$ contained 0.47 g. of

oil which slowly developed crystals. A portion (0.19 g.) was subjected to alkaline hydrolysis by boiling with 5 ml. of $N-KOH$ in methanol for 1 hr. The crystalline K salt which formed on cooling was dissolved by addition of water (20 ml.), and the liquid after extraction with ether to remove unsaponifiable matter was acidified with HCl. The crystals (needles, 0.09 g.) which separated had m.p. 160–165°, raised to 180–182° on recrystallization from benzene. This was again anisic acid (mixed m.p., no depression).

Fraction II therefore contained an ester of anisic acid—doubtless methyl anisate—and a small amount of free anisic acid together with traces of other unidentified acids.

Neither Fraction I nor Fraction II showed any marked uptake of bromine when dissolved in CS_2 , which indicated the absence of appreciable amounts of cinnamic acid or derivatives.

SUMMARY

The volatile metabolic products of the fungus *Trametes suaveolens* (Linn.) Fr. grown on aqueous malt extract were examined. Methyl anisate was identified as the major product; anisaldehyde was also detected, but is present in much smaller amount. These two products are probably the chief contributors to the odour of the fungus in culture. A little free anisic acid was also detected; this may arise by atmospheric oxidation of the anisaldehyde.

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The Riboflavin Content of Food Served in Royal Air Force Messes

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As our knowledge of the role of riboflavin as an essential factor in human nutrition increases, the need for satisfactory estimation of the riboflavin content of food becomes more urgent. The microbiological method has been employed in many laboratories and has given reproducible results. However, if this method is to be accepted as reliable, it must give values in agreement with those obtained with other methods and in particular with those obtained with a reliable biological method. During the past two years food material prepared by a

method described by Macrae, Henry & Kon (1943) has become available. Since this consisted of a complex mixture of foodstuffs it appeared to be suitable for comparative tests from which the reliability of the microbiological method of estimation of riboflavin in foodstuffs might be ascertained. Any substances which might possibly interfere with such tests were likely to be present in a mixture of this kind. This comparative study allowed a determination of the daily riboflavin intake of R.A.F. personnel.

EXPERIMENTAL

(a) *Materials*

The materials analyzed consisted of the total food available to five airmen or airwomen in their messes in this country at all meals during 7 days, or in the case of some of the airwomen's messes, the food available for breakfast, dinner and tea but not supper. The supper meal was collected separately because a large proportion of the personnel, mainly for social reasons, tended to take this meal away from their messes. The food was collected as served, stored at -20° and ground in the frozen state. The fine granules were then dried in a rapid current of air at 70° , and the dried material was again finely ground, well mixed and weighed before samples were taken for analysis. The final products contained approximately 5% of moisture. The average daily dry weight of the food served to one subject was calculated, and from this and the riboflavin content it was possible to determine the average daily intake of riboflavin by the personnel at the various R.A.F. Stations.

(b) *Methods*

Biological method. The biological technique used in estimating riboflavin was that originally devised by El Sadr, Macrae & Work (1940) and modified for use with cereal products by Copping (1943). The test animals were 21-day-old black and white rats receiving a basal synthetic diet of pure maize starch 60 parts, casein ('ashless, extracted, riboflavin-free, G.L.', supplied by Glaxo Laboratories Ltd.) 20 parts, hardened arachis oil 12 parts, pure lard 3 parts and McCollum's normal salt mixture 5 parts, mixed with 100 parts of water and steamed for 3-5 hr. in order to dextrinize the starch and prevent the possible development of refection (Fridericia, Freudenthal, Gudjonsson, Johansen & Schoubye, 1927). The R.A.F. diets were given as supplements to the basal diet; the daily dose was 1-3 g. of the dried material, and this amount was readily consumed by the rats. From some preliminary tests a rough estimate of the riboflavin content of the diets was obtained and a

suitable level selected for dosing which should yield satisfactory comparison with the standard daily doses of 6 and $12\mu\text{g.}$ of pure riboflavin, which have been found to give a suitable slope in the curve of growth response. The results were calculated according to the method of Irwin (1944) as set out in a report of results obtained by the Vitamin A Sub-Committee.

Microbiological method. The microbiological method of riboflavin estimation was that of Snell & Strong (1939) as modified by Barton-Wright & Booth (1943). An amount of 5 g. of the dried R.A.F. diet was used in each estimation.

Experience has led to the introduction of modifications of the method of extraction of the riboflavin when dealing with these mixed diets. Both the airmen's and airwomen's diets had a high fat content, and the dried food was therefore defatted with light petroleum. The samples were then autoclaved at a pressure of 15 lb./sq. in. for 30 min. with about 50 ml. 0.25 N-HCl. The mixtures were allowed to cool and adjusted to pH 4.6 with NaOH. They were then vigorously shaken with ether in a separatory funnel to remove all interfering lipids released by the HCl. The lower aqueous layer, which contained the riboflavin, was run off and the ether layer washed several times with water, the washings being added to the aqueous layer. Finally, the extracts were adjusted to pH 6.8 and made up to 500 ml. with water. Clear solutions were thus obtained and all centrifuging and filtering obviated. The remainder of the determination was carried out as previously described, except that the addition of asparagine to the medium has been abandoned.

RESULTS

The results given by the biological method were, with one or two exceptions, somewhat higher than those obtained by the microbiological method, but, on the whole, the agreement between the results with the two methods was good (Table 1). The microbiological method of determination would, therefore, appear to be reliable for the estimation

Table 1. *Riboflavin content of the diets served at various R.A.F. Stations, and the riboflavin intake of the personnel*

R.A.F. Station	Riboflavin content of food (mg./100 g. dry matter)		Average daily intake of				Energy (e) (cal.)
	Method of assay		Food (g. of dry matter)	Riboflavin (mg.) Method of assay			
	Rat growth	Microbiological		Rat growth	Microbiological		
(a) Airmen's messes							
1 a	0.26	0.25	568	1.5	1.4	2675	
2 a	0.31	0.29	635	1.9	1.8	3045	
3 a	0.48	0.43	625	3.0	2.7	3060	
4 a	0.31	0.29	592	1.8	1.7	2740	
(b) Airwomen's messes							
5 a	0.45	0.37	532	2.4	2.0	2585	
6 e	0.23	0.27	459	1.1	1.2	2726	
6 s	—	0.38	106	—	0.4		
7 e	0.21	0.27	495	1.0	1.3	2622	
7 s	—	0.27	80	—	0.2		
8 e	0.38	0.37	510	1.9	1.9	2934	
8 s	—	0.38	89	—	0.3		
9 a	0.36	0.28	606	2.1	1.7	—	

a indicates all meals included; e indicates all meals except supper; s indicates supper only.

of riboflavin in foodstuffs, having been found to give values similar to those obtained by the more costly and time-consuming rat-growth method.

The average daily intake of riboflavin per person was calculated from the average daily intake of dry food (Table 1). Intakes of less than 2 mg. of riboflavin for all except three of the messes were found with the biological method, and for all except two with the microbiological technique. The average daily intake per head was about 2.0 mg. for the airmen and 1.8 mg. for the airwomen. At the stations at which the supper meals were collected and dealt with separately, a rather large proportion of the daily energy and riboflavin intake appeared to be derived from the suppers. It is of interest that the airwomen's mess with the lowest riboflavin intake was one serving very much greater numbers than the other three airwomen's messes.

DISCUSSION

If the standards for optimum requirement of vitamins, as set out by the Committee on Food and Nutrition of the National Research Council of the United States of America in 1941 (National Nutrition Conference), are taken as valid, the present values for riboflavin intake would appear to be suboptimal, since the daily requirement was there laid down as about 3 mg. for active men and 2.5 mg. for active women. The requirement for riboflavin has not, however, been finally established. Sebrell & Butler (1938), in a preliminary report of their findings on riboflavin deficiency in women, stated that cures were obtained with daily doses of 1-2 mg. in some cases, and with 0.025 mg./kg. body weight in others. These subjects, of course, received small amounts of riboflavin in their diets, but the total daily intake during the curing of the characteristic cheilosis could not have been more than 2 mg. in some instances. Since maintenance doses of any factor are usually considered to be less than curative ones, these experiments would indicate that the daily requirement of riboflavin is less than 2 mg. This is a lower estimate than the amount allowed in the National Research Council list, but it is confirmed by recent findings of Williams, Mason, Cusick & Wilder (1943), who attempted to induce riboflavin deficiency in human subjects by administration of a restricted diet, and then to restore the level of riboflavin in the tissues to normal again. From their studies they

concluded that 0.5 mg. riboflavin/1000 cal. was an adequate allowance. In view of these results it would seem that the diets examined from the R.A.F. messes did supply an adequate daily intake of riboflavin, since the figures given in Table 1 show that at least 0.5 mg./1000 cal. was present, and in most diets there was a safe margin over this amount. Since the estimations were made on the food as served, all losses due to cooking and keeping of the hot food before consumption were covered.

Lyle, Macrae & Gardiner (1944) have observed no sign of deficiency of riboflavin in R.A.F. personnel in this country, which further supports the view that the normal adult requirements of riboflavin must be less than 3 mg. daily. It is true that in addition to the average of 1.8-2.0 mg. received daily in their messes, the personnel must have received some additional riboflavin from food consumed outside, but this could not have amounted to more than a small fraction of a milligram in most instances. Since beer is a surprisingly good source of riboflavin, containing about 0.5-1.7 $\mu\text{g.}/\text{g.}$ (Barton-Wright, Moran & Sarson, 1943; Hopkins, 1943), beer drinkers would have a higher intake, but this would not affect the greater proportion of R.A.F. personnel as not more than 10% are regular beer drinkers.

SUMMARY

1. Biological and microbiological methods are described for estimation of the riboflavin content of the food served in R.A.F. messes.

2. The values obtained by the two methods were in good agreement, though the results obtained by the biological method tended to be somewhat higher than those obtained by the microbiological method.

3. The daily intake of riboflavin by the personnel in their messes ranged from 1.5 to 2.6 mg. and averaged about 1.9 mg.

4. Since no sign of deficiency of riboflavin exists in the personnel receiving these diets, it is considered that the average riboflavin requirement of adults does not exceed 2 mg. daily.

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The Fluorimetric Estimation of 3:4-Benzpyrene in Whole Mice

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Previous methods for the quantitative estimation of carcinogenic hydrocarbons in animal tissues are based on spectrophotometric measurements of either the absorption spectrum (Chalmers, 1934; Jones, 1942; Lorenz & Shear, 1936; Lorenz & Shimkin, 1941; Lorenz & Stewart, 1940), or the fluorescence spectrum (Berenblum & Kendal, 1936; Berenblum & Schoental, 1942; Chalmers, 1938, 1940; Chalmers & Peacock, 1936; Hieger, 1936; Peacock, 1936). Although the greater simplicity of a fluorimetric estimation would result in a saving of time and money, its unspecificity, due to the presence of fluorescent impurities, was usually regarded as too great an obstacle. The present paper records methods of extraction and purification of 3:4-benzpyrene (henceforth called benzpyrene), as developed in this laboratory, and examines the possibilities and limitations of making allowance, in fluorimetric measurements, for the remaining impurities on a statistical basis.

In the course of investigations into the effect of lipid solvents on the carcinogenic activity of benzpyrene it was decided to measure the rate of elimination of the hydrocarbon from the animal body after subcutaneous injection. As previously emphasized (Dickens & Weil-Malherbe, 1942), small doses of carcinogen should be employed in experiments of this kind, since otherwise any cocarcinogenic or anticarcinogenic properties of the solvent are liable to be smothered. Benzpyrene is particularly favourable, as it combines, relative to 20-methylcholanthrene and 1:2:5:6-dibenzanthracene, the highest fluorescence intensity with the lowest carcinogenic potency after subcutaneous injection (Bryan & Shimkin, 1943). A single subcutaneous injection of 0.3 mg. benzpyrene was found satisfactory in our experiments. The method has therefore been devised for a maximum amount of 0.3 mg. benzpyrene; it is, however, easily adapted to the estimation of larger amounts by modifications of the procedure of chromatographic purification, which will be discussed.

SURVEY OF METHOD

The following three steps which are in principle, though not in detail, the essence of some previous methods, precede the actual estimation. (1) Lysis of the tissue in hot alcoholic NaOH. (2) Extraction of the hydrocarbon, together with non-saponifiable matter from the tissues, from alkaline 50 % ethanol by light petroleum. (3) Purification of the petroleum extract by concentration and cooling and then by chromatographic adsorption and fractional elution (so-called 'liquid chromatography'). Benzpyrene is first adsorbed on a column of silica gel from light petroleum and eluted by light petroleum-benzene mixture (4 : 1). It is then reabsorbed from this mixture on a column of alumina. After development by the same mixture it is eluted by benzene. (4) The concentration of benzpyrene is determined fluorimetrically.

DESCRIPTION OF METHOD

Reagents

Purification of solvents

Ethanol, 95% (v/v). Industrial methylated alcohol is mixed with 10N-H₂SO₄ (40 ml./l.) and rectified slowly through an efficient fractionating column (Clarke & Rahrs, 1923). This is followed by refluxing for 12 hr. over AgNO₃ (8 g./l.) + KOH (8 g./l.) and another rectification.

Light petroleum. The commercial product (b.p. 40–60°) is purified by the following successive extractions, each carried out with vigorous shaking for several minutes in a separator: nitrating mixture (125 ml. conc. H₂SO₄ + 100 ml. conc. HNO₃) (4 times), fuming H₂SO₄ (3 times), water and 25% NaOH. It is finally dried over CaCl₂ and rectified.

Benzene. Extraction of benzene (British Drug Houses Ltd., A.R.) with conc. H₂SO₄ (3–4 times), followed by washing with water and alkali, drying and rectification.

Ethanol may be recovered by fractional distillation, but before being used again it should be purified by the process described. The same applies to light petroleum recovered from light petroleum-benzene mixtures.