tone) in the dog suggest that the butyl chain undergoes very little ω -oxidation, since 75.6% of the dose was excreted as the (ω -1)-oxidation product, 5-ethyl-5-(3-hydroxy-1-methylbutyl)barbituric acid, and only 4.5% as the ω -oxidation product, 5-ethyl-5-(3-carboxy-1-methylpropyl)barbituric acid.

Our present results, however, do not permit a decision as to whether ω - or (ω -1)-oxidation is the more important in the degradation of propyl- and butyl-benzenes. It is significant, however, that the degradation of the (ω -1)-carbinols is of the same order as that of the corresponding alkylbenzenes.

SUMMARY

1. A study has been made of the fate of ethylbenzene, *n*-propylbenzene and *n*-butylbenzene in the rabbit.

2. About 60–70% of ethylbenzene undergoes α -oxidation to yield methylphenylcarbinol glucuronide and hippuric acid, and 10–20% undergoes ω -oxidation to yield phenaceturic acid.

3. About 50% of propylbenzene is excreted as the glucuronides of ethylphenylcarbinol and benzylmethylcarbinol, and about 15% as hippuric acid. The main precursor of hippuric acid appears to be benzylmethylcarbinol.

4. About 50% of butylbenzene is excreted as the glucuronides of methyl-2-phenylethylcarbinol and phenylpropylcarbinol, and about 20% as phenaceturic acid. The precursor of phenaceturic acid appears to be methyl-2-phenylethylcarbinol; phenylpropylcarbinol is not a precursor. Derivatives of benzylethylcarbinol were not detected as metabolites of butylbenzene, and this carbinol is not a precursor of phenaceturic acid.

5. The three alkylbenzenes have been proved to undergo α -oxidation; propyl- and butyl-benzenes also undergo (ω -1)-oxidation of the alkyl side chain. No direct evidence for the ω -oxidation of propyl- and butyl-benzenes has been obtained, but its occurrence cannot be ruled out.

6. A method modified from Gaffney *et al.* (1954) has been described for the determination of hippuric acid in urine.

The expenses of this work were in part defrayed by a grant from the Medical Research Council.

REFERENCES

- Anon. (1955). *Chem. Engng News*, **33**, 3124.
 Beer, C. T., Dickens, F. & Pearson, J. (1951). *Biochem. J.* **48**, 222.
 Bray, H. G., Thorpe, W. V. & White, K. (1950). *Biochem. J.* **46**, 271.
 Browning, E. (1953). *Toxicity of Industrial Organic Solvents*, p. 63. London: H.M. Stationery Office.
 Burns, J. J., Rose, R. K., Chenkin, T., Goldman, A., Schultze, A. & Brodie, B. B. (1953). *J. Pharmacol.* **109**, 346.
 Burns, J. J., Rose, R. K., Goodwin, S., Reichenthal, J., Horning, E. & Brodie, B. B. (1955). *J. Pharmacol.* **113**, 481.
 Dakin, H. D. (1911). *J. biol. Chem.* **9**, 127.
 Gaffney, G. W., Schreier, K., DiFerrante, N. & Altman, K. I. (1954). *J. biol. Chem.* **206**, 695.
 Heilbron, I. & Bunbury, H. M. (1953). *Dictionary of Organic Compounds*. London: Eyre and Spottiswoode.
 Maynert, E. W. (1952). *Fed. Proc.* **11**, 625.
 Parke, D. V. (1956). *Biochem. J.* **62**, 339.
 Quick, A. J. (1928). *J. biol. Chem.* **77**, 581.
 Raper, H. S. & Wayne, E. J. (1928). *Biochem. J.* **22**, 190.
 Robinson, D., Smith, J. N. & Williams, R. T. (1953). *Biochem. J.* **53**, 125.
 Robinson, D., Smith, J. N. & Williams, R. T. (1955). *Biochem. J.* **59**, 153.
 Robinson, D. & Williams, R. T. (1955). *Biochem. J.* **59**, 159.
 Smith, J. N., Smithies, R. H. & Williams, R. T. (1954a). *Biochem. J.* **56**, 317.
 Smith, J. N., Smithies, R. H. & Williams, R. T. (1954b). *Biochem. J.* **56**, 320.
 Smith, J. N., Smithies, R. H. & Williams, R. T. (1954c). *Biochem. J.* **57**, 74.
 Thierfelder, H. & Klenk, E. (1924). *Hoppe-Seyl. Z.* **141**, 13.
 Titus, E. & Weiss, H. (1955). *J. biol. Chem.* **214**, 807.
 Weitzel, G. (1951). *Hoppe-Seyl. Z.* **237**, 254.

Maleic Anhydride in the Study of Naturally Occurring Isomers of Vitamin A

By P. A. PLACK

National Institute for Research in Dairying, University of Reading

(Received 9 February 1956)

During the past ten years, several *cis* isomers of vitamin A have been described (Robeson & Baxter, 1945, 1947; Graham, van Dorp & Arens, 1949; Dieterle & Robeson, 1954; Wald, Brown, Hubbard & Oroshnik, 1955; Robeson *et al.* 1955b). Their formulae are shown in Fig. 1. It is now known that

some of these compounds occur naturally. Their presence can be detected by a comparison of chemical and biological assays of vitamin A extracts (Fisher, Kon & Thompson, 1952; Robeson *et al.* 1955b), this test being based on the finding that the *cis* isomers so far investigated have lower

biological activities than all-*trans* vitamin A (Harris, Ames & Brinkman, 1951; Ames, Swanson & Harris, 1955), whereas in the Carr-Price reaction their molar extinction coefficients are approximately equal (C. D. Robeson, personal communication to Dr S. K. Kon). Methods for the isolation, characterization and estimation of *cis* isomers are limited. Wald and co-workers (Hubbard & Wald, 1952-53; Wald & Burg, 1955) estimate certain of the isomers by conversion of the alcohol into the aldehyde form, and combination with opsin. With chromatography on calcium phosphate, Bro-Rasmussen, Hjarde & Porotnikoff (1955) have separated quantitatively 13-*cis* vitamin A (neo-

vitamin A) from all-*trans* vitamin A₁ and vitamin A₂. Robeson & Baxter (1947) obtained crystalline 13-*cis* vitamin A from fish-liver oil by molecular distillation, fractional chromatography and crystallization. The same isomer was prepared by Chatain & Debodard (1951) by the reaction with maleic anhydride, followed by chromatography and crystallization, and by Dalvi & Morton (1952), who used fractional chromatography only.

At present, the simplest quantitative method of measurement of a *cis* isomer is that of Robeson & Baxter (1947), which estimates 13-*cis* vitamin A in the presence of the all-*trans* isomer by a procedure involving reaction with maleic anhydride. This

(Wald, Brown, Hubbard & Orshnik, 1955)

(Graham, van Dorp & Arens, 1949; Robeson *et al.* 1955 b)

(Wald *et al.* 1955)

(Robeson & Baxter, 1947)

(Robeson *et al.* 1955 b)

(Robeson *et al.* 1955 a)

Fig. 1. *Cis-trans* isomers of vitamin A alcohol described in the literature, numbered according to the system of Karrer.

method has been applied by Meunier & Jouanneteau (1948), Hayes & Petitpierre (1952) and other workers. Dalvi & Morton (1952) used a similar method, but at a lower temperature. The method described here is an extension of this work.

The combination of maleic anhydride with dienes is well known in organic synthesis. The compound formed by this substance with vitamin A was first investigated by Kawakami (1935) and later by Robeson *et al.* (1955*b*). The latter authors have also given data on the rates of reaction of certain isomers, additional to those of Robeson & Baxter (1947).

In their work on the vitamin A of marine Crustacea, Fisher *et al.* (1952) found a discrepancy between chemical and biological assays on extracts from euphausiids, indicating that *cis* isomers were present. A comparison of the u.v.-absorption curves of an extract from *Meganyctiphanes norvegica* (M. Sars) and of all-*trans* vitamin A alcohol showed that 13-*cis* vitamin A was not one of these (Kon, 1954). A method of estimating other *cis* isomers was therefore required, and the reaction of crystalline all-*trans* vitamin A acetate, palmitate and alcohol, and of vitamin A extracts from natural sources, with maleic anhydride was studied. From it, a method for estimating certain *cis* isomers was evolved, and also a method for their isolation. Results obtained with vitamin A extracts of *M. norvegica*, *Thysanoessa raschii* (M. Sars) and *Pandalus bonnieri* (Caullery) have been used here to illustrate one of these methods, but an account of the isomers of vitamin A present in these and other marine Crustacea will be reserved for a future paper.

NOMENCLATURE

In this paper, vitamin A is used as a general term to cover all forms and isomers of vitamin A₁ when the particular form or configuration does not affect the issue. When reference to a particular isomer is made, the Karrer numbering given in Fig. 1 is used. Weights of vitamin A are given in terms of micrograms of the equivalent quantity of all-*trans* vitamin A alcohol, and were determined either by direct weighing, or by the Carr-Price reaction.

The terms 'fast' and 'slow'-reacting isomers of vitamin A alcohol refer to those isomers which react with maleic anhydride at a rate comparable respectively, to that of all-*trans* vitamin A alcohol, or of 13-*cis* vitamin A alcohol (Robeson *et al.* 1955*b*).

EXPERIMENTAL

Materials

Crystalline all-*trans* vitamin A alcohol, acetate and palmitate, and α -tocopherol, were obtained from Roche Products Ltd. Light petroleum, b.p. 40–60°, and benzene, pure, crystallizable, from May and Baker Ltd. were redistilled

before use. Maleic anhydride (May and Baker Ltd.) was recrystallized at least twice, from either benzene or chloroform, and dissolved in benzene to give a 10% (w/v) solution. Other chemicals were of A.R. quality, or the purest available. The commercial vitamin A concentrate from mixed fish-liver oils was obtained from Crookes Laboratories Ltd., and whale-liver oil was a gift from Hector Whaling Ltd.

Natural sources of vitamin A used were the euphausiid Crustacea *M. norvegica* and *T. raschii*, caught in Loch Fyne, and the decapod crustacean *P. bonnieri*, obtained from the river Clyde.

Methods

General. Previous work from this laboratory (Fisher *et al.* 1952) has shown that in *M. norvegica* and *T. raschii* the major part of the vitamin A is contained in the eyes. The eyes of *M. norvegica* and *P. bonnieri* were removed for use, but with *T. raschii*, owing to its small size, whole animals were extracted. The method of extraction was that given by Thompson, Ganguly & Kon (1949). Saponification was carried out by adding to each 2.5 g. of fat (or less) 5 ml. of ethanol and 1.1 ml. of 60% (w/v) aqueous KOH, and boiling gently for 3 min., adding 20 ml. of water and extracting with 20, 15 and 15 ml. of diethyl ether in a separating funnel. The combined extracts were washed with three lots of 20 ml. of water, dried over anhydrous Na₂SO₄, evaporated to dryness and taken up in a few millilitres of *n*-hexane. The vitamin A in the alcohol form thus obtained was purified by the chromatographic procedure of Thompson *et al.* (1949), except that the column was made up from a slurry of alumina in 8% (v/v) ethanol in *n*-hexane. The whale-liver oil and Crookes vitamin A concentrate were saponified directly and chromatographed as described above.

Ultraviolet-absorption curves were read on a Beckman model DU quartz spectrophotometer with 1 cm. cells, and *n*-hexane as solvent.

Carr-Price reaction. The spectrophotometer of Thompson (1949) was used to measure the intensity of the blue colour produced in this reaction at its absorption maximum, 618 m μ . Workers at Distillation Products Industries have found that the *p*-phenylazobenzoate esters of three *cis* isomers of vitamin A have the same absorption maxima (622 m μ .) and essentially the same $E_{1\text{ cm.}}^{1\%}$ values as this ester of all-*trans* vitamin A (C. D. Robeson, personal communication to Dr S. K. Kon). It is therefore assumed that the alcohol isomers give $E_{1\text{ cm.}}^{1\%}$ values similar to that of all-*trans* vitamin A alcohol. The different absorption maximum found by the American workers is probably instrumental in origin. The method used was as described by Thompson (1949), except that the sample was in either benzene or CHCl₃ solution, and 1 drop of acetic anhydride was always used. With vitamin A in benzene solution, the 'stable' reading was very transient, and occasionally it was necessary to use the maximum reading attained. The presence of maleic anhydride tended to stabilize the readings but had no effect on their value. Vitamin A that had combined with maleic anhydride did not give a blue colour in the Carr-Price test.

The calibration of the instrument has been described by Fisher, Kon & Thompson (1956). In the work which follows, instrument readings are given. These may be converted into $\mu\text{g.}$ of vitamin A/ml. of reaction mixture by dividing the readings by 50.

Reaction of vitamin A with maleic anhydride. A solution of the appropriate vitamin A in benzene was prepared at a

concentration of 10–20 $\mu\text{g./ml.}$ A 5 ml. sample was pipetted into an amber-coloured 125 ml. conical flask in a water bath at 25°. To it was added 5 ml. of the 10% solution of maleic anhydride in benzene, previously warmed to 25°. A 1 ml. sample of the mixture was taken immediately, diluted with an equal volume of benzene, and the vitamin A present determined by the Carr–Price test. Subsequent samples were taken at intervals of 0.5, 1, 2, 3, 4, 5 and 6 hr., and diluted as necessary before the Carr–Price test. The flasks were kept corked between the taking of samples. A control flask, containing equal volumes of vitamin A solution and benzene, was always set up, and samples and readings were taken at 0 and 6 hr. The control readings were constant within the experimental error of the Carr–Price test, which is of the order of $\pm 5\%$.

By beginning with the control flasks, and staggering the initial readings at 5 min. intervals, it was possible to deal with six experimental flasks and six controls in one day.

Determination of 'fast' and 'slow'-reacting components in a mixture of vitamin A isomers. When the test just described was carried out on saponified vitamin A mixtures from natural sources, and the $\log_{10}$ reading plotted against time, curves such as those in Fig. 2 were obtained. During the first 2 hr., the fall in vitamin A concentration was due mainly to the removal of the 'fast'-reacting components by combination with maleic anhydride. At the end of this time, only 1% of the original 'fast' components should have remained (see results for all-*trans* vitamin A alcohol), and from 2 to 6 hr. the fall will have been due to the reaction of the 'slow' components. These values for the period 2–6 hr. lie on a straight line, and if this line is produced backwards to meet the axis, the intercept gives the $\log_{10}$ value of that part of the initial reading due to the 'slow' components.

Fig. 2. Reaction with maleic anhydride (5%, w/v) of 13-*cis* vitamin A alcohol (○), and of saponified extracts of vitamin A from whale-liver oil (□), *T. raschii* (△), *M. norvegica* (●) and *P. bonnieri* (■) at concentrations between 7 and 9 $\mu\text{g./ml.}$, in benzene at 25°. The reaction was followed by measurements of the absorption at 618 $\text{m}\mu$. in the Carr–Price test due to the unreacted vitamin A (see Methods section).

For whale-liver oil vitamin A, the intercept (see Fig. 2) is 2.170. The initial value was 2.606. Then

Percentage of 'slow' components

$$= \frac{\text{Initial reading due to slow components} \times 100}{\text{Initial reading}},$$

or,

$$\log_{10} (\text{percentage of 'slow' components}) = (2.170 + 2) - 2.606 = 1.564.$$

Hence, percentage of 'slow' components was 37, and percentage of 'fast' components was 63, by difference.

Isolation of the 'slow' components. A solution of the saponified vitamin A extract was prepared in benzene at a maximum concn. of 10 mg./ml. An equal volume of the 10% maleic anhydride solution was added, and the whole incubated for 3 hr. at 25° in a water bath. At the end of this time, some 99% of the 'fast' components would have combined with maleic anhydride. A volume of 2N-KOH in ethanol equal to that of the mixture was then added, to decompose the excess maleic anhydride and to render the maleic anhydride–vitamin A adducts water-soluble, followed 5 min. later by the same volume of water. The mixture was extracted in a separating funnel with three portions of light petroleum, each equal in volume to five times that of the mixture, and the combined extracts were washed three times with an equal volume of water and dried over anhydrous Na_2SO_4 . The light petroleum was removed and a small volume of *n*-hexane added. The solution was then usually chromatographed on weakened alumina by the method of Thompson *et al.* (1949).

Preparation of 13-cis vitamin A alcohol. This isomer was prepared from the saponified vitamin A extract from the Crookes vitamin A concentrate, which assayed at 36% of 'slow'-reacting isomers. The method described above was followed, with an initial vitamin A concentration of 3 mg./ml. in benzene. Chromatography was carried out on a column of weakened alumina (20 cm. $\times$ 1.8 cm.). The elution of vitamin A by 8% (v/v) ethanol in *n*-hexane was followed by observing its fluorescence with an ultraviolet lamp, and 2 ml. fractions were collected when the vitamin A band was 2 cm. above the base of the column. The first three fractions gave absorption curves in *n*-hexane with maxima at 328 $\text{m}\mu$.; they were combined and taken to consist of 13-*cis* vitamin A alcohol.

The biological assay of this preparation was carried out on rats by the vaginal-smear method of Pugsley, Wills & Crandall (1944). Solutions of the 13-*cis* vitamin A alcohol and of crystalline all-*trans* vitamin A acetate were made in arachis oil containing 0.1% α -tocopherol. The vitamin A concentration in each was determined chemically by the Carr–Price reaction, a suitable weight of the oil being dissolved directly in CHCl_3 for the test.

RESULTS

Reaction of pure vitamin A isomers with maleic anhydride

The method described for studying the reaction of vitamin A with maleic anhydride gives a series of readings corresponding to the amount of remaining vitamin A that had not reacted with maleic anhydride. For the pure isomers tested, a plot of these readings against time gave an exponential

curve, and a plot of $\log_{10}$ readings against time gave a straight line. Fig. 3 shows this plot for all-*trans* vitamin A acetate. All-*trans* vitamin A palmitate gave an identical straight-line relationship.

All-*trans* vitamin A alcohol (Fig. 3) yielded a similar result but the rate of reaction was much greater. After 1 hr. only 11 % of the initial vitamin A was present as such, as against 75 % for the esters, and after 3 hr. about 1 % was found. From the curve it would be expected theoretically that only 0.13 % of the original vitamin A would be present at this time. This discrepancy was due partly to the presence of small quantities of 13-*cis* vitamin A alcohol, possibly produced by isomerization.

13-*cis* Vitamin A alcohol, prepared as described above, reacts very slowly with maleic anhydride, but again the values gave a straight line in a semi-logarithmic plot (Fig. 2).

The straight-line relationship for the pure isomers, between $\log_{10}$ readings and time, indicates that their reaction with maleic anhydride is pseudo-unimolecular, which is to be expected, since on a molar basis the initial concentration of maleic anhydride is some 14000 times that of vitamin A.

Fig. 3. Reaction of crystalline all-*trans* vitamin A acetate (○), and of crystalline all-*trans* vitamin A alcohol (●) at concentrations of about 10 $\mu\text{g./ml.}$ with maleic anhydride (5%, w/v) in benzene at 25°. The reaction was followed as in Fig. 2.

The velocity constant for the reaction may therefore be determined by the relationship $k = (1/t_1) \ln 2$, where k is the velocity constant of the reaction and t_1 is the time taken for the concentration of vitamin A to be reduced by one-half.

Under the standard conditions used [25° and 5% (w/v) of maleic anhydride], the following values were found: all-*trans* vitamin A acetate, k 0.280 hr.^{-1} ; all-*trans* vitamin A alcohol, k 2.24 hr.^{-1} ; 13-*cis* vitamin A alcohol, k 0.0081 hr.^{-1} .

These values are independent of the concentration of vitamin A, provided that 5% (w/v) maleic anhydride constitutes an excess, but dependent on the concentration of maleic anhydride. Fig. 4 shows this latter relationship, and indicates that the primary reaction determining the rate is bimolecular.

Determination of 'fast'- and 'slow'-reacting components

This method makes use of the difference in velocity constants of these two groups of isomers, illustrated by the figures for all-*trans* vitamin A alcohol and 13-*cis* vitamin A alcohol given above. As a test of the method, mixtures of saponified vitamin A extract from *M. norvegica* and all-*trans* vitamin A alcohol were made up as shown in Table 1. The curves obtained are shown in Fig. 5, and from these the percentages of 'fast' components were calculated (Table 1). The calculated values take into account the presence of 'fast' components in the vitamin A of *M. norvegica*.

Fig. 4. The reaction of crystalline all-*trans* vitamin A alcohol (9.2 $\mu\text{g./ml.}$) with various concentrations of maleic anhydride in benzene at 25°. Plot of velocity constants against the concentrations of maleic anhydride.

The reproducibility of the method was tested by repeated determinations of the percentage of 'fast'-reacting components in an *n*-hexane solution of vitamin A from whale-liver oil over a period of 1 month. This solution was stored in the dark at 0°. The values obtained were 62, 61, 64, 64, 64, 64, 62 and 63% of 'fast' components. The results for saponified vitamin A extracts from *M. norvegica*, *T. raschii*, *P. bonnieri* and whale-liver oil, derived from the curves in Fig. 2, are given in Table 2.

Isolation of the 'slow' components

As a test of this method, all-*trans* vitamin A alcohol was allowed to react with maleic anhydride for 30 min. and the remaining free vitamin A (about 32% of the initial) extracted. The u.v.-absorption curve of this recovered material was almost identical with that of the original all-*trans* vitamin A alcohol solution. It follows that all-*trans* vitamin A alcohol that has not reacted with maleic anhydride can be recovered substantially unchanged.

The preparation of 13-*cis* vitamin A alcohol described above gave an u.v.-absorption curve

Fig. 5. Reaction of mixtures of a saponified extract of vitamin A from *M. norvegica* and all-*trans* vitamin A alcohol with maleic anhydride in benzene at 25°. ○, 100% *M. norvegica*; □, 80% *M. norvegica* + 20% all-*trans*; △, 60% *M. norvegica* + 40% all-*trans*; ●, 40% *M. norvegica* + 60% all-*trans*; ■, 20% *M. norvegica* + 80% all-*trans*. The reaction was followed as in Fig. 2.

with a maximum at 328 mμ. By biological assay it had 0.68 times the activity of all-*trans* vitamin A alcohol, with true fiducial limits of 81–121% ($P=0.95$). Ames *et al.* (1955) give a mean value of 0.75 times. Application of the maleic anhydride reaction (Fig. 2) showed that the amount of 'fast' components present was too small to estimate.

DISCUSSION

Kawakami (1935) suggested that the adduct of vitamin A acetate and maleic anhydride involved the combination of two molecules of the latter with the four double bonds of the vitamin A side chain. Robeson *et al.* (1955b), however, found that only the two terminal double bonds of the side chain reacted with one molecule of maleic anhydride, and the present work, which shows that the first and rate-determining reaction is bimolecular, supports their view.

According to Robeson *et al.* (1955b), an isomer with *trans* configurations at double bonds 11 and 13 reacts rapidly with maleic anhydride, whereas if one or both are *cis* the rate of reaction is slow. From Fig. 1 it will be seen that the all-*trans*, 7-*cis* (Wald *et al.* 1955) and 9-*cis* (Graham *et al.* 1949; Robeson *et al.* 1955b) isomers should comprise the 'fast'-reacting group, and the 11-*cis* (Wald *et al.* 1955),

Table 1. Percentage of 'fast' components in mixtures of a saponified vitamin A extract from *M. norvegica* and all-*trans* vitamin A alcohol

Solutions of saponified vitamin A extract from *M. norvegica* and of all-*trans* vitamin A alcohol in benzene, of the same strength by the Carr-Price reaction, were mixed in the proportions shown.

Percentage composition of mixture		Percentage of 'fast' components	
<i>M. norvegica</i> vitamin A	All- <i>trans</i> vitamin A	Calculated*	Found
100	0	—	19
80	20	35	35
60	40	51	51
40	60	68	64
20	80	84	80

* In the calculation of the percentage of 'fast' components, allowance has been made for the presence of these in the extract from *M. norvegica*.

Table 2. Percentage of 'fast' and 'slow' components in vitamin A from marine sources

Estimations were made as in the Methods section.

Source	Fast	Slow
<i>Thysanoessa raschii</i> , whole animals	17	83
<i>Meganyctiphanes norvegica</i> , eyes	19	81
<i>Pandalus bonnieri</i> , eyes	29	71
Whale-liver oil	63	37

13-*cis* (Robeson & Baxter, 1947), 9:13-di-*cis* (Robeson *et al.* 1955b) and 11:13-di-*cis* (Dieterle & Robeson, 1954; Robeson, Blum, Dieterle, Cawley & Baxter, 1955a) isomers the 'slow'-reacting group. There is one possible exception to this rule, namely 7-*cis* vitamin A. Wald *et al.* (1955) ascribe this formula to neovitamin Ab, which is 'slow'-reacting (Robeson *et al.* 1955a), but the latter authors suggest the 11:13-di-*cis* structure.* The relative amounts of these two groups may be determined by the method described.

The test of Robeson & Baxter (1947) does not allow for the possible presence of 'slow'-reacting isomers other than 13-*cis*, but these do occur naturally. Neovitamin Ab has been found in the eyes of lobsters (Wald & Burg, 1955); Fisher, Henry, Kon, Plack & Thompson (1955) found in *M. norvegica* small amounts of an isomer which resembles the 11-*cis* compound,* and Lambertsen & Braekkan (1955) have suggested that 9:13-di-*cis* vitamin A may be present in *Pandalus borealis*.

No account has been taken of the possible presence of vitamin A₂, which has been found, apart from fresh-water fish, in marine fish-liver oils (Edisbury, Morton, Simpkins & Lovern, 1938; Gillam, Heilbron, Jones & Lederer, 1938; Lovern, Morton & Ireland, 1939; Wald, 1938-39; Cama *et al.* 1952; Cama & Morton, 1953; Bro-Rasmussen *et al.* 1955). On theoretical grounds, it seems likely that the *cis-trans* isomers of this vitamin will react in the same way as those of vitamin A₁, but samples of definite configuration have not been available for testing.

The difference in the rates of reaction with maleic anhydride of all-*trans* vitamin A alcohol on the one hand and of all-*trans* vitamin A acetate and plamitate on the other is very marked. The behaviours of the alcohol and ester forms in adsorption chromatography are also markedly different, and the two effects are possibly due to dissimilar electron distributions of the alcohol and esters. The 'slow'-reacting isomers of vitamin A alcohol also have different rates of reaction. In Fig. 2 it will be seen that over the period 2-6 hr. the slopes of the curves for 13-*cis* vitamin A and saponified whale-liver oil (of which 13-*cis* is the main 'slow' component) correspond, whereas those for *T. raschii*, *M. norvegica* and *P. bonnieri* are greater, but roughly similar among themselves. These rates of reaction may possibly be due to different rates of isomerization to the 11:13-di-*trans* form, rather than to different rates of combination with maleic anhydride. The method is not sufficiently accurate at

the moment to enable individual components to be identified by this property.

The method of separating the 'slow'-reacting isomers from the 'fast'-reacting isomers is relatively simple compared with such processes as molecular distillation (cf. Robeson & Baxter, 1947), and is especially suitable when only small amounts of material are available. If only one 'slow'-reacting isomer is present, this method is capable of giving a high degree of purification, without resort to crystallization, which with small quantities of material is not possible. When more than one 'slow' reactor is present, application of this method provides a less complex mixture than the original for the application of fractional chromatography and other techniques.

SUMMARY

1. The reactions of crystalline vitamin A alcohol, acetate and palmitate with maleic anhydride in benzene solution have been studied. The alcohol form reacts much more rapidly than the esters.

2. A study of the reaction of maleic anhydride with saponified vitamin A extracts from various marine sources enables the percentages of 'fast'- and 'slow'-reacting isomers to be calculated. (The former comprise all-*trans*, 7-*cis* and 9-*cis* vitamin A, and the latter 11-*cis*, 13-*cis*, 9:13-di-*cis* and 11:13-di-*cis* vitamin A.)

3. The 'slow'-reacting isomers may be isolated from the reaction mixture simply by adding ethanolic potassium hydroxide, then water, and extracting with light petroleum. An example is given of the preparation of 13-*cis* vitamin A alcohol from a commercial vitamin A preparation.

This work was supported by a grant from the Development Commission. I am indebted to Dr W. Oroshnik, Dr J. W. G. Porter and Professor G. Wald for helpful discussions, to Dr C. D. Robeson for permission to make use of unpublished results and to Dr S. K. Kon for guidance in this work. Dr L. R. Fisher and Mr C. B. Cowey collected the specimens of *M. norvegica*, *T. raschii* and *P. bonnieri*, and I am grateful to them, to the Director and staff of the Marine Biological Station, Millport, for providing facilities, and to the captains and crews of M.V. *Calanus* and M.V. *Mizpah*. My thanks are due to Mr R. M. Brachi and Hector Whaling Ltd. for supplying samples of whale-liver oil. I would also like to thank Mrs P. Todd for the biological assay of neovitamin A.

REFERENCES

- Ames, S. R., Swanson, W. J. & Harris, P. L. (1955). *J. Amer. chem. Soc.* **77**, 4134.
Bro-Rasmussen, F., Hjarde, W. & Porotnikoff, O. (1955). *Analyst*, **80**, 418.
Cama, H. R., Dalvi, P. D., Morton, R. A., Salah, M. K., Steinberg, G. R. & Stubbs, A. L. (1952). *Biochem. J.* **52**, 535.
Cama, H. R. & Morton, R. A. (1953). *Analyst*, **78**, 74.

* Note added 29 May 1956. According to Oroshnik's latest work [Oroshnik, W. (1956) *J. Amer. chem. Soc.*, **78**, in the press], neovitamin Ab is 11-*cis* vitamin A and the isomer with $\lambda_{\max}$ 311 m μ . in *n*-hexane (11-*cis* vitamin A of Wald *et al.* 1955) is 11:13-di-*cis* vitamin A.

- Chatain, H. & Debodard, M. (1951). *C.R. Acad. Sci., Paris*, **232**, 355.
- Dalvi, P. D. & Morton, R. A. (1952). *Biochem. J.* **50**, 43.
- Dieterle, J. M. & Robeson, C. D. (1954). *Science*, **120**, 219.
- Edisbury, J. R., Morton, R. A., Simpkins, G. W. & Lovern, J. A. (1938). *Biochem. J.* **32**, 118.
- Fisher, L. R., Henry, K. M., Kon, S. K., Plack, P. A. & Thompson, S. Y. (1955). *Biochem. J.* **61**, vi.
- Fisher, L. R., Kon, S. K. & Thompson, S. Y. (1952). *J. Mar. biol. Ass. U.K.* **31**, 229.
- Fisher, L. R., Kon, S. K. & Thompson, S. Y. (1956). *J. Mar. biol. Ass. U.K.* **35**, 41.
- Gillam, A. E., Heilbron, I. M., Jones, W. E. & Lederer, E. (1938). *Biochem. J.* **32**, 405.
- Graham, W., van Dorp, D. A. & Arens, J. F. (1949). *Rec. Trav. chim. Pays-Bas*, **68**, 609.
- Harris, P. L., Ames, S. R. & Brinkman, J. H. (1951). *J. Amer. chem. Soc.* **73**, 1252.
- Hayes, E. & Petitpierre, M. A. (1952). *J. Pharm., Lond.*, **4**, 879.
- Hubbard, R. & Wald, G. (1952-53). *J. gen. Physiol.* **36**, 269.
- Kawakami, K. (1935). *Sci. Pap. Inst. phys. chem. Res. Tokyo*, **26**, 77.
- Kon, S. K. (1954). *Bull. Soc. Chim. biol., Paris*, **36**, 209.
- Lambertsen, G. & Braekkan, O. R. (1955). *Nature, Lond.*, **176**, 553.
- Lovern, J. A., Morton, R. A. & Ireland, J. (1939). *Biochem. J.* **33**, 325.
- Meunier, P. & Jouanneteau, J. (1948). *Bull. Soc. Chim. biol., Paris*, **30**, 260.
- Pugsley, L. J., Wills, G. & Crandall, W. A. (1944). *J. Nutr.* **28**, 365.
- Robeson, C. D. & Baxter, J. G. (1945). *Nature, Lond.*, **155**, 300.
- Robeson, C. D. & Baxter, J. G. (1947). *J. Amer. chem. Soc.* **69**, 136.
- Robeson, C. D., Blum, W. P., Dieterle, J. M., Cawley, J. D. & Baxter, J. G. (1955a). *J. Amer. chem. Soc.* **77**, 4120.
- Robeson, C. D., Cawley, J. D., Weisler, L., Stern, M. H., Eddinger, C. C. & Chechak, A. J. (1955b). *J. Amer. chem. Soc.* **77**, 4111.
- Thompson, S. Y. (1949). *Brit. J. Nutr.* **3**, 43.
- Thompson, S. Y., Ganguly, J. & Kon, S. K. (1949). *Brit. J. Nutr.* **3**, 50.
- Wald, G. (1938-39). *J. gen. Physiol.* **22**, 391.
- Wald, G., Brown, P. K., Hubbard, R. & Oroshnik, W. (1955). *Proc. nat. Acad. Sci., Wash.*, **41**, 438.
- Wald, G. & Burg, S. P. (1955). *Fed. Proc.* **14**, 300.

The Action of Culture Filtrates of the Fungus *Myrothecium verrucaria* on β -Glucosans

By R. A. AITKEN,* B. P. EDDY, M. INGRAM AND C. WEURMAN†
*Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge
 and Department of Scientific and Industrial Research*

(Received 28 September 1955)

The soil fungus *Myrothecium verrucaria* (*Metarrhizium glutinosum*) is well known for its ability to decompose cellulose, and it has been used in various investigations of that subject (cf. Siu, 1951). This paper describes some properties of a series of culture filtrates of the fungus and our observations seem interesting from two points of view.

(1) There is at present doubt about the nature of extracellular fungal cellulase. From comparisons of various fungi, including *M. verrucaria*, Reese, Siu & Levinson (1950) and Reese & Levinson (1952) concluded that an enzyme C_1 (cellulase; Siu, 1950) transforms insoluble complex cellulose into single molecules of water-soluble cellulose, and that a second enzyme C_2 , a β -1:4-glucosidase, breaks these glucose chains into fragments mainly of two units, i.e. cellobiose. They sometimes found glucose in

their reaction mixtures, but did not say clearly how they thought it might be formed. On the other hand Whitaker (1953), working with purified preparations from *M. verrucaria*, concluded that there is only a single enzyme, capable of degrading complex cellulose to glucose. Kooiman, Roelofsen & Sweeris (1953), also working with *M. verrucaria*, agreed with Whitaker save that they demonstrated the presence of a distinct cellobiase. Our work confirms that of Kooiman *et al.*

(2) We have found that *M. verrucaria* filtrates also attack β -1:3-glucosides such as laminarin and laminaribiose and, in addition, the β -glucosan isolated from barley by Preece & Mackenzie (1952). This has been analysed chemically by Aspinall & Telfer (1954), who concluded that it contains unbranched chains of β -D-glucopyranose units with approximately equal proportions of 1:3- and 1:4-linkages. The course of its breakdown by our filtrates confirms this view and leads to suggestions about the relative positions of these 1:3- and 1:4-linkages.

* Department of Brewing and Industrial Fermentation, Heriot-Watt College, Edinburgh.

† Central Institute of Nutrition Research, T.N.O., Utrecht, The Netherlands.