XVI. GROWTH OF *ASPERGILLUS VERSICOLOR* ON HIGHER PARAFFINS.

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INTRODUCTION.

IN connection with some investigations which are being made on the metabolism of paraffins in the plant it became of interest to find out the chemical changes which these substances undergo when used as the sole source of carbon for the growth of moulds.

It was shown by Tausson [1925] that a mould resembling *Aspergillus flavus* could utilise paraffin wax for this purpose, and by Tausz and Peter [1919] that certain bacteria isolated from the soil could also utilise synthetic paraffins. In the present research we have been fortunate enough to isolate another mould which will grow on paraffin wax. It is a member of the *A. versicolor* group and we have investigated its action on certain synthetic long-chain paraffins, with the hope that some information would thereby be gained as to the manner in which the organism attacks a long carbon chain of this type, which has no polar group.

By analogy with the action of moulds on fatty acids we should expect the chain to be broken at some particular carbon atom by oxidation. Stokoe [1928] showed that *Penicillium palitans* growing on a gelatin medium containing deodorised coco-nut oil oxidised the fatty acids at the β -carbon atom. Normally the β -keto-acids thus formed broke down to give the fatty acid containing two carbon atoms less and acetic acid, but under certain conditions, as for instance when respiration of the mycelium was impeded, the keto-acid lost carbon dioxide with the formation of a methyl ketone. Similar evidence was obtained by Acklin [1929]. Walker *et al.* [1928], who grew *A. niger* on calcium butyrate, suggested that the initial product of the oxidation was the β -hydroxy-acid. Dakin [1908, 1, 2; 1909], by acting on the ammonium salts of

fatty acids *in vitro* with hydrogen peroxide, showed that oxidation took place at the β -carbon atom, and that small amounts of the corresponding methyl ketones were also formed. He did not consider, however, that a definite answer could be given to the question whether an unsaturated acid, β -hydroxy-acid or β -keto-acid, was the first product of oxidation, and suggested that in the animal body all three would be in equilibrium with each other and readily interconvertible. These experiments have been repeated more recently by Raper and Clutterbuck [1925], who showed that in addition oxidation also took place at the γ - and δ -carbon atoms, with the formation of the corresponding keto-acids.

A suggestion as to the possible course of biological oxidation of the long-chain paraffins can be obtained from the intensive study of Francis *et al.* [1922, 1, 2] on the air oxidation of paraffins. They examined the products obtained by treatment of paraffin wax with air and oxygen at 100° in the presence of small amounts of turpentine to hasten the onset of oxidation. Although the products obtained were very complex mixtures the presence of secondary alcohols and ketones was established; it was probable also that dihydric alcohols, hydroxyketones and diketones were present. Primary alcohols were not detected. The secondary alcohols and ketones obtained probably had the same number of carbon atoms in the molecule as the paraffins originally present in the wax from which they were derived. The ketones were considered to be derived from the secondary alcohols, which were the primary products of oxidation, and an X-ray examination of the ketones showed that the carbon chain was attacked by oxygen at the third or fourth atom from the end. In addition to these non-acidic substances, complex mixtures of acidic substances were obtained. These had in all cases shorter carbon chains than the paraffins present in the original paraffin wax. Later, Francis and Wood [1927] examined the products obtained by oxidation of synthetic *n*-triacontane, $C_{30}H_{62}$, under similar conditions, and were able to establish the presence of 4-triacontanone, $CH_3.(CH_2)_{25}.CO.(CH_2)_2.CH_3$, an acid containing 26 carbon atoms and succinic acid. Evidence was also obtained that the primary oxidation product was a secondary alcohol. Since the amounts of these substances obtained were very small it was thought probable that the monohydroxy-derivative first formed was largely oxidised further to polyhydroxy-derivatives, which on subsequent oxidation gave polyketones, and then acids and hydroxy-acids with shorter carbon chains.

Tausson [1925, 1928], as the result of his experiments on the growth of *A. flavus* on paraffin wax, concluded that one of the first steps in the oxidation of the paraffins by the mould was the production of the fatty acid esters of the higher aliphatic alcohols. He considered that this view was supported by the fact that the mould would grow on such esters under the same conditions as on paraffin wax. Since, however, it is hardly conceivable that the mould could produce an ester from paraffin wax direct, without the preliminary production of its constituent acid and alcohol, it does not seem possible for esters to be

directly concerned with the metabolism of paraffin hydrocarbons. Further criticism of Tausson's work is given later.

In the present work attempts were made to grow a number of moulds on pure synthetic paraffins. A strain of *A. versicolor* was eventually obtained which would grow on these substances as a sole source of carbon. The mould grew equally well on odd- or even-number paraffins, but there was an upper limit to the length of the chain which it would attack; for only with great difficulty could growth be obtained on *n*-tetratriacontane $C_{34}H_{70}$, and it was never found possible to obtain growth on *n*-pentatriacontane $C_{35}H_{72}$.

An investigation has been made into the products of metabolism when the carbon source has been *n*-heptacosane $C_{27}H_{56}$. Only extremely small amounts of metabolic products were obtained, and these were apparently for the most part complex mixtures. These results suggested that the carbon chain had been attacked at numerous points, with the ultimate production of carbon dioxide and, perhaps, water-soluble substances of quite low molecular weight. In this connection it is noteworthy that Raistrick and his co-workers [1931], in the course of an investigation of the metabolic products of a very large number of mould species grown on a glucose medium, found that by far the greater number produced practically nothing but carbon dioxide as a metabolic product.

It is possible that by restricting the oxygen supply the paraffin metabolism of the mould could be diverted from its normal course, so as to lead to the accumulation of either direct intermediary products or others derived from them; just as, for instance, Stokoe [1928] found that methyl ketones were produced from fatty acids when the respiration of the mould was hindered by the presence of excess of fat. Unfortunately the normal growth of *A. versicolor* on paraffins is very slow, and such an experiment might easily lead to so little growth that intermediary products would be produced in quantity insufficient for chemical identification. It seemed to us that information might be more easily obtained by growing the mould on products which might themselves be intermediaries in the metabolism of paraffins.

The following substances were chosen for this purpose:

2-pentadecanone $CH_3.(CH_2)_{12}.CO.CH_3$,
 12-tricosanol $CH_3.(CH_2)_{10}.CHOH.(CH_2)_{10}.CH_3$,
 14-heptacosanone $CH_3.(CH_2)_{12}.CO.(CH_2)_{12}.CH_3$,
 14-heptacosanol $CH_3.(CH_2)_{12}.CHOH.(CH_2)_{12}.CH_3$,
 15-nonacosanol $CH_3.(CH_2)_{13}.CHOH.(CH_2)_{13}.CH_3$,
d-10-nonacosanol $CH_3.(CH_2)_8.CHOH.(CH_2)_{18}.CH_3$,
 16-hentriacontanone $CH_3.(CH_2)_{14}.CO.(CH_2)_{14}.CH_3$,
 16-hentricontanol $CH_3.(CH_2)_{14}.CHOH.(CH_2)_{14}.CH_3$,
 and myricyl alcohol, m.p. 88° , from carnauba wax.

Normal growth occurred with the primary alcohol and the ketones, but no growth at all with the secondary alcohols. These results suggest that the biological oxidation of paraffins takes place in the first instance by the direct

introduction of a keto-group, perhaps at several points in the chain simultaneously. The oxidation then causes disruption of the paraffin chain with the formation of shorter fatty acids, which are then further metabolised through keto-acids, as discussed previously. It will be seen that the scheme of metabolism excludes the initial formation of secondary or polyhydric alcohols, and in this respect only does it differ from the air oxidation of paraffins observed by Francis. On general chemical grounds it is, of course, highly improbable that the paraffin chain could be oxidised at the terminal carbon atom with the formation of a primary alcohol or *n*-fatty acid containing the same number of carbon atoms.

We reject Tausson's [1925] conclusion—that one of the first steps in the oxidation is the production of fatty acid esters of the higher aliphatic alcohols—for the following reasons.

(1) In his experiments the amount of intermediary products formed was very small. From one culture after 12 weeks' growth on 3.65 g. of paraffin there were obtained 1.24 g. of mycelium and 1.29 g. of residual paraffin. The latter was extracted at 70° with 70 % alcohol, when an intense orange-yellow solution was obtained. This contained 0.234 g. of products which were stated to be non-acidic. Another sample of residual paraffin weighing 1.03 g. was saponified directly with 1 % aqueous potassium hydroxide, when 0.0178 g. of an acidic product was obtained. Without further characterisation this was assumed to be fatty acid derived from esters of higher alcohols. Such esters are not readily saponified by this mild hydrolysis and, as Tausson admits that much decomposition took place with evolution of gaseous products having the smell of terpineol, we suggest that this small amount of acid was perhaps derived from the pigments present, for in our own experiments these were acidic.

(2) No steps were taken to ensure that the paraffin wax used was free from traces of other substances, or to carry out blank estimations on the paraffin used. We have found that commercial paraffin wax blackens considerably when treated with concentrated H_2SO_4 at 120°, and therefore contains at least a small amount of material that is not true paraffin.

(3) As the dried mixture of mycelium and residual paraffin was extracted with ether, the extract possibly contained any ether-soluble material present in the mycelium, and hence any fats found may not have been intermediate products but essential constituents of the mycelium.

EXPERIMENTAL.

Preparation of paraffins, ketones and alcohols.

These were synthesised by the methods described in a previous paper [Piper *et al.*, 1931]. *n*-Heptacosane was the paraffin chosen for the chief experiments on the metabolic products because ample supplies of myristic acid, from which it is prepared, were available. The paraffins, ketones and alcohols were prepared for the cultures by slow crystallisation from hot benzene-alcohol

(1 : 9), the thin flat crystalline leaflets being filtered off by very gentle suction, so as to prevent them packing down, and air-dried. By this means material exposing a considerable surface to the action of the mould was obtained. The paraffin wax used in the later experiments was commercial wax melting at about 50° which had been treated with concentrated H_2SO_4 at 120° until no further blackening took place and then recrystallised from benzene-alcohol to obtain it in a finely divided state. Its melting-point after treatment was 53–55°.

Isolation of a mould which will grow on paraffin wax.

In Tausson's experiments cultures were prepared by adding sterile scrapings of paraffin wax (M.P. 78°) to a flask containing sterile mineral salt solution and then inoculating. The mould was grown at 23–25°. Development of mycelium was first noticeable after 10 days, and, as growth proceeded, yellow and finally red pigment was produced. Change of p_{H} of the medium was found to have little effect on the rate of growth, acid media tending to become more alkaline as growth proceeded. Nitrates and ammonium salts served equally well as sources of nitrogen.

The mould used by Tausson was stated by him to be similar to *Aspergillus flavus*, and as a preliminary experiment it was decided to attempt to grow this and a number of related moulds on paraffin wax. Through the kindness of Prof. Raistrick we were enabled to obtain cultures of the following moulds from Messrs Nobel's Ltd. of Ardeer: *A. flavus*, *A. parasiticus*, *A. oryzae*, *A. effusus*, *A. tamari* and an unnamed *Aspergillus*, numbered Ac 62.

Cultures of these were prepared by adding 2 g. of paraffin wax (M.P. 57°), cut into shavings by means of a microtome, to 50 cc. of mineral salt solution contained in each of a number of litre flasks. The mineral nutrient solution had the following composition: $\text{Ca}(\text{NO}_3)_2$ 0.1 g., KNO_3 0.025 g., KH_2PO_4 0.025 g., MgSO_4 , $7\text{H}_2\text{O}$ 0.025 g., $\text{Fe}_2(\text{SO}_4)_3$ 0.0025 g., H_2O 100 cc. Each flask was then heavily inoculated with the appropriate mould. The method of inoculation was to take a vigorously growing culture on a beerwort or glucose agar slope, add about 60 cc. of mineral nutrient solution, and scrape up as much of the mould as possible with a platinum wire without disturbing the agar. The suspension of mycelium and spores thus obtained was poured into the prepared flask, and distributed by gentle shaking. It was not thought necessary to carry out these experiments under strictly sterile conditions. The flasks were then incubated at about 28°.

Very slight growth appeared to take place in all cases, although *A. flavus*, *A. effusus* and *A. tamari* were the most promising. Further experiments were therefore made with these three moulds, using *n*-heptacosane as source of carbon instead of paraffin wax. Nine flasks were prepared, each containing 1 g. of *n*-heptacosane and 25 cc. of mineral nutrient solution; and 3 were inoculated with *A. effusus*, 3 with *A. flavus* and 3 with *A. tamari*. The paraffin was prepared in fine leaflets, as stated above, and was readily distributed over the whole surface of the liquid in the culture flask. After 3 weeks there was

little evidence of growth, except in the case of one flask inoculated with *A. tamari*, on the side of which had appeared a small green patch surrounded by some red pigment. The remaining flasks were therefore re-inoculated with the appropriate moulds, using for inoculation vigorously growing 6-day subcultures on beerwort agar. After 2 days the flasks re-inoculated with *A. tamari* showed signs of growth and continued to grow; none however of the other cultures showed any growth.

Three weeks after the second inoculation the amount of paraffin remaining in one of the *A. tamari* flasks was determined in the following way. The culture was washed on to a Büchner funnel, and the mixture of paraffin and mycelium then removed from the filter-paper and treated with successive portions of warm light petroleum (B.P. 40–60°) to remove all the paraffin. This solvent will not remove any fatty material from the fresh mycelium, whereas ether, the solvent employed by Tausson, might readily do so if employed in quantity sufficient to bring about partial dehydration of the mycelium. The weight of mycelium, dried at 100°, was 0.046 g. and of the recovered paraffin 0.92 g. This did not blacken when treated with concentrated H_2SO_4 at 120°, and was identical in all respects with the original paraffin.

This, and many similar results were so discouraging as to cause us to abandon the experiments with moulds of known history, and, as a last resort, to carry out experiments with the unknown green mould which had, as has been previously mentioned, appeared on the side of one of the culture flasks.

A subculture was made from this patch of mould direct into another flask containing 1 g. of *n*-heptacosane and mineral nutrient solution. After 6 days patches of red pigment appeared on the paraffin adhering to the walls of the culture flask, and later, green areas of mould. Growth also took place on the paraffin floating on the surface of the mineral nutrient solution, but was not noticeable so early as that on the walls, because red pigment was not at first produced. After some weeks, however, the mineral nutrient solution became pink in colour.

Glucose agar slopes were inoculated with material from this culture and incubated at 28°, and by this means subcultures were obtained which appeared to be fairly pure. From these Prof. Raistrick kindly prepared pure cultures for us by plating out on glucose agar. The course of development of the mould on this medium was as follows. After 2 days, white patches of mycelium appeared which turned yellow or orange as growth proceeded, the centres of the patches then turning green owing to production of spores. After about 2 weeks the agar, which had at first remained colourless, became dark red.

Through the kindness of Prof. Raistrick, a culture of the mould was sent to Prof. Thom, of Washington, D.C., U.S.A., who reported that it appeared to be a member of the *A. versicolor* group. This organism, which is best described at the present time as a strain of *A. versicolor* (Buillemin) Tiraboschi, appeared to be a suitable one for further experiments, and the products formed by its action on *n*-heptacosane were therefore examined.

Examination of products formed by the action of a strain of A. versicolor on n-heptacosane.

After 8 weeks, the first subculture of *A. versicolor* on *n*-heptacosane was examined for products of metabolism. The mineral nutrient solution was removed by filtration, and the residue was extracted several times with light petroleum (B.P. 40–60°). The extract was evaporated to dryness; weight 0.849 g. The mycelium was dried for some days *in vacuo* over CaCl_2 ; weight 0.068 g.

The product recovered was yellow in colour, and a sample blackened somewhat when heated with concentrated H_2SO_4 to 120°. It was redissolved in ether, and the solution extracted three times with a dilute solution of sodium hydroxide. The washings were bright pink. The ethereal solution was now much paler yellow than before, but further washing removed no more pigment. After removal of the ether the product melted at 58.8–59.1°, and obviously consisted largely of unchanged paraffin. It was therefore recrystallised from about 50 cc. of benzene-alcohol (1 : 2) yielding a colourless material similar in all respects to the original paraffin. The mother-liquor was evaporated to dryness and the residue, which weighed only 0.05 g., melted indistinctly and was completely molten at 58.5°, the melt being cloudy. It still probably contained much unchanged paraffin, and, as the total amount was small, it was not examined further until it had been combined with similar products from later cultures.

An experiment was carried out to determine whether change of p_{H} of the mineral nutrient solution towards greater alkalinity had any effect on the rate of decomposition of the paraffin by the mould. Two flasks, each containing 1 g. of *n*-heptacosane and 100 cc. of mineral nutrient solution, were inoculated with *A. versicolor* grown on glucose agar, and to one, excess of powdered CaCO_3 was added. The flasks were incubated at 28° for 5 weeks, and the amount of paraffin remaining was determined as previously described. With CaCO_3 , 0.64 g.; without CaCO_3 , 0.62 g.: the two products were similar.

Thus change of initial p_{H} from 5.8 to 7.9 had no effect on the rate of oxidation of the paraffin, and in all later experiments the original nutrient solution of p_{H} 5.8 was used. It was found that cultures on this nutrient solution slowly became more alkaline, the reaction at the end of the experiments being about p_{H} 8.

In another experiment, a culture on *n*-heptacosane which had begun to develop at 28° was removed from the incubator and allowed to develop at room temperature. Growth was visibly slower than in cultures kept at 28° and the latter temperature was therefore used in subsequent experiments.

Examination of a large culture. Eighteen cultures, each on 1 g. of *n*-heptacosane, were prepared in the same way as before and allowed to develop for 5–6 weeks. They were then filtered off and extracted with light petroleum as previously described. The weight of residual paraffin was 10.47 g. The mycelium was dried over CaCl_2 ; it weighed 3.416 g. Thus more than 4 g. of

n-heptacosane had been oxidised to CO₂ or water-soluble substances of low molecular weight.

The light petroleum extract was dark yellow. It was dissolved in ether and the ethereal solution washed with a 5 % solution of sodium hydroxide. The alkaline washings were deep purple. The washed ether solution, which was now pale yellow, was evaporated to dryness and the residue dissolved in 1300 cc. of a boiling mixture of benzene and alcohol (1 : 2), and the solution allowed to cool. The major part of the material crystallised out, and was shown by its melting-point and behaviour towards concentrated H₂SO₄ at 120° to be unchanged *n*-heptacosane. The mother-liquor, on evaporation to dryness, yielded a product weighing 1.7 g. which was not homogeneous, and contained brown gummy material. This was dissolved in 150 cc. of hot benzene-alcohol, and the solution allowed to cool. The product which crystallised out was again shown to be pure *n*-heptacosane. The mother-liquor on evaporation to dryness yielded 0.282 g. of material still containing much *n*-heptacosane. The fractionation was continued using first 30 cc. and finally 5 cc. of benzene-alcohol; 0.121 g. and 0.020 g. of practically pure *n*-heptacosane being obtained. The final mother-liquor yielded 0.14 g. of brown gummy material which could not be fractionated, and, owing to the small amount available, it was not examined further.

The purple alkaline washings from the above were acidified with HCl, when the solution turned orange. It was extracted with ether, and the ethereal solution evaporated to dryness. Rather less than 0.1 g. of red non-crystalline material was obtained.

The dried mycelium. This was powdered in a mortar and extracted with ether in a Soxhlet apparatus. The orange-coloured extract, after washing with a dilute solution of sodium hydroxide, yielded 0.318 g. of pale yellow material which was solid and largely crystalline. The washings were intense purple in colour, and, on acidification and extraction with ether, gave 0.16 g. of red acidic products, from which it was not found possible to obtain crystalline material.

From the yellow crystalline substance there was obtained, by recrystallisation from alcohol, 0.021 g. of material consisting of yellow needles, together with some unchanged *n*-heptacosane. This material was insoluble in ether and melted at 213.5–214.5°. It contained no nitrogen. (Found: C, 64.3; H, 4.14 %. Mol. wt. (depression of M.P. of camphor) 368. C₁₉H₁₄O₇ (Mol. wt. 354) requires C, 64.4; H, 3.95 %.) It is interesting to compare this rather unusual empirical formula with those of citromycetin (C₁₄H₁₀O₇; 2H₂O) and citrinin (C₁₃H₁₄O₅), two yellow crystalline compounds described by Raistrick *et al.* [1931] as having been formed by the fermentation of glucose by moulds.

A second batch of 16 cultures was examined after growing for 3–4 months and yielded similar results. The main paraffin fraction, however, was further divided into a number of fractions by crystallisation from a mixture of acetone and light petroleum (1 : 2) with a view to detecting any substance which had a greater or less solubility than *n*-heptacosane in this solvent. No such substance was found.

Experiments on the growth of A. versicolor on other paraffins.

Other paraffins available were *n*-tricosane, *n*-nonacosane, *n*-triacontane, *n*-tetratriacontane and *n*-pentatriacontane. Two cultures of each were made. In the case of *n*-nonacosane both were inoculated from glucose agar cultures, and in all other cases one was inoculated from a glucose agar culture, and one from a vigorously growing beerwort agar culture. The results of these experiments are given in Table I.

Table I. *Showing the growth of a strain of Aspergillus versicolor on a series of long-chain paraffins.*

Paraffin	Culture from which inoculated	Time in days before growth was observed	Description of growth
$C_{23}H_{48}$	Glucose	8	} Good growth with very little production of pigment
$C_{23}H_{48}$	Beerwort	6	
$C_{29}H_{60}$	Glucose	7	} Growth and pigment production similar to that using <i>n</i> -heptacosane
$C_{29}H_{60}$	Glucose	7	
$C_{30}H_{62}$	Glucose	8	} Growth and pigment production similar to that using <i>n</i> -heptacosane
$C_{30}H_{62}$	Beerwort	6	
$C_{34}H_{70}$	Glucose	—	No growth obtained
$C_{34}H_{70}$	Beerwort	12	Very slow growth
$C_{35}H_{72}$	Glucose	—	} No growth, even after re-inoculation
$C_{35}H_{72}$	Beerwort	—	
Paraffin wax	Glucose	8	} Good growth with very little production of pigment
Paraffin wax	Glucose	8	

It will be seen that mould growth has taken place on both odd- and even-number paraffins but that no growth has occurred on paraffins higher in the series than *n*-tetratriacontane.

Experiments on the growth of A. versicolor on ketones and alcohols.

These were carried out in the usual way with vigorously growing glucose agar cultures. A control experiment with *n*-heptacosane was made at the same time. Less pigment is produced in the case of the ketones, which makes the observation of initial growth more difficult; this is probably the reason for the longer latent period recorded for some of these substances in Table II. The results have been fully discussed in the Introduction.

Table II. *Showing the growth of a strain of Aspergillus versicolor on a series of ketones and alcohols.*

Substrate	Time in days before growth was observed	Description of growth
2-Pentadecanone	9	Normal growth, very little pigment observed
12-Tricosanol	—	No growth
<i>n</i> -Heptacosane (control)	14	Normal growth
14-Heptacosanone	21	Normal growth, very little red pigment formed
14-Heptacosanol	—	No growth
15-Nonacosanol	—	No growth
<i>d</i> -10-Nonacosanol	—	No growth
16-Hentriacontanone	21	Normal growth, very little red pigment formed
16-Hentriacontanol	—	No growth
Myricyl alcohol from carnauba wax, M.P. 88°	21	Growth not so good as on paraffins and ketones

SUMMARY.

A new mould has been found which will grow on synthetic higher paraffins as sole source of carbon. It is best described at the present time as a strain of *Aspergillus versicolor* (Buillemin) Tiraboschi.

It will grow on both odd- and even-number paraffins, but not on those with a longer chain than $C_{34}H_{70}$.

Experiments have been made to find out the way in which the paraffin chain is attacked by the mould, but a large scale culture on $C_{27}H_{54}$ did not yield any products of metabolism except carbon dioxide and mould mycelium. Indirect evidence has, however, been obtained from experiments with higher ketones on which the mould grew vigorously, and with secondary alcohols on which no growth was obtained at all. It appears that the primary product of oxidation of a paraffin is a ketone or polyketone, and that further oxidation results in the production of shorter fatty acids which are then metabolised in the usual way.

In conclusion we should like to express our cordial thanks to Prof. Raistrick for the gift of many species of *Aspergillus* and for advice and assistance in preparing the cultures.

We should also like to thank Prof. Garner for samples of *n*-triacontane and *n*-tetratriacontane, and Prof. Thom for the identification of the mould.

REFERENCES.

- Acklin (1929). *Biochem. Z.* **204**, 253.
Dakin (1908, 1). *J. Biol. Chem.* **4**, 77, 227, 419.
— (1908, 2). *J. Biol. Chem.* **5**, 173, 303.
— (1909). *J. Biol. Chem.* **6**, 203, 221.
Francis, Pope and Coysh (1922, 1). *J. Chem. Soc.* **121**, 496.
— Watkins and Wallington (1922, 2). *J. Chem. Soc.* **121**, 2804.
— and Wood (1927). *J. Chem. Soc.* 1897.
Piper, Chibnall, Hopkins, Pollard, Smith and Williams (1931).
Biochem. J. **25**, 2072.
Raistrick and others (1931). *Phil. Trans. Roy. Soc. Lond. B* **220**.
Raper and Clutterbuck (1925). *Biochem. J.* **19**, 385.
Stokoe (1928). *Biochem. J.* **22**, 80.
Tausson (1925). *Biochem. Z.* **155**, 356.
— (1928). *Biochem. Z.* **193**, 85.
Tausz and Peter (1919). *Centr. Bakt. Par.* **II**, **49**, 497.
Walker, Coppock and Subramaniam (1928). *J. Chem. Soc.* 1422.