Change in the Location of Amylomaltase Produced by Mutation in *Escherichia coli*

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(Received 29 April 1964)

The utilization of sugars, for example lactose or maltose, can take place in *Escherichia coli* only in the presence of two catalytic systems, namely permease and enzymes catalysing the breakdown of the sugars within the cell (Cohen & Monod, 1957).

The present paper reports mutants that are not capable of utilizing maltose, although amylo-maltase is present in the cell. The results, however, indicate that it is not crypticity to maltose that causes incapacity for utilization but a change in location that is connected with the binding of amylo-maltase in the cell structure.

α -Glucosidase in an inactive form bound to the cell structures of *Saccharomyces cerevisiae* was found by Robertson & Halvorsen (1957), and a similar observation was made by Axelrod (1962) in *Saccharomyces oviformis*. In the latter case it was shown that ribonuclease solubilizes bound α -glucosidase from the cell structures.

A preliminary account of this work was presented at the Third Congress of the Czechoslovak Biochemical Society, Olomouc, Czechoslovakia, 1–5 July 1963.

METHODS

Bacterial cultures. All experiments were carried out with *E. coli* strain K₁₂ from our collection numbered 3310 and with a mutant prepared from this strain denoted as 3310M. In one experiment (see Fig. 2) two further strains from our collection were used, Hb₁ and K₁₀–10.

If not stated otherwise, cultures of the above strains were used in the early exponential phase of growth, cultivated on broth [beef extract (Difco), 0.3%; peptone (Difco), 1% (w/v); NaCl, 1.5% (w/v); pH 7] with aeration. For the wild-type organism maltose (30 mM) was added to the medium. Growth was determined with a Pulfrich photometer at 550 m μ . After growth, the bacteria were centrifuged at 0° and washed three times in 0.1M-phosphate buffer. The 0.1M-phosphate buffer used in all experiments was prepared by adding 0.1M-Na₂HPO₄ to 0.1M-KH₂PO₄ to give a final pH of 7.

For the isolation of the mutant and some other experiments, minimal medium M 63 (Pardee, Jacob & Monod, 1959) and the minimal medium described by Davis (1950), were used. These media are referred to below as minimal media.

Preparation of the mutant. The mutants were obtained by essentially the same method as that used by Gorini & Kaufman (1960), with the differences that the concentration

of glucose during cultivation before and after irradiation with ultraviolet light was 1% (w/v) and that sucrose was not added to the medium before subjecting the bacteria to the action of penicillin.

Plating method. This was carried out to determine whether the examined strains could grow on a minimal medium, where the only carbon source was maltose. The bacteria were plated on the surface of a solid agar-medium gel, which contained minimal medium with maltose or glucose and 1.5% (w/v) of agar (Difco). In some cases there was a soft agar layer, containing minimal medium with sugar and 0.6% of agar (Difco) with the suspension of bacteria at 45° layered on solid agar-medium gel.

Amylomaltase activity. This was determined by a modification of the method of Keston (1956). The sample was incubated in phosphate buffer in the presence of 3 mM-maltose in a total volume of 2 ml. for 30 min. with shaking at 37°. After incubation the mixture was treated in a water bath at 100° for 5 min. and filtered, and 0.2 ml. of the sample was added to 2 ml. of glucose oxidase reagent and incubated for 60 min. at 37°. The colour produced was measured with a Pulfrich photometer at 425 m μ . The composition of the glucose oxidase reagent was: glucose oxidase, 6.5 mg.; peroxidase, 8 mg.; dianisidine, 2.5 mg. (dissolved in 1 ml. of ethanol); 0.4 M-phosphate buffer (pH 7), 50 ml.

When a suspension of bacteria or the sediment after the disintegration of cells was used for the determination of amylo-maltase, toluene was added before the determination. The sample was suspended in phosphate buffer and toluene (1 drop/ml.) added; it was then shaken and incubated for 10 min. at 37° in stoppered flasks.

The number of bacteria for determining amylo-maltase activity was chosen so that glucose formation took place at zero order of reaction. The requirement was fulfilled when the extinction of the colour that developed did not exceed 0.35. Under these experimental conditions this corresponded to about 5 mg. dry wt. of cells/ml. during incubation with maltose.

In control experiments the amount of glucose or other substances reacting similarly in the suspension of bacteria was determined on incubation with phosphate buffer from which maltose had been omitted. In a further control 3 mM-maltose solution was added directly to the glucose oxidase reagent. In all cases the results of these analyses were practically zero.

One unit of amylo-maltase activity was defined as the amount of enzyme liberating 1 μ g. of glucose/hr. under these standard conditions.

Determination of transport of maltose into the cell. A suspension of bacteria (320 μ g. dry wt./ml.) was shaken at 30° in the presence of chloramphenicol (40 μ g./ml.) in broth or

phosphate buffer containing [^{14}C]maltose (6 $\mu\text{C}/\text{ml.}$) and unlabelled maltose to give a final concentration of 1 mM. Samples (80 $\mu\text{g.}$ dry wt.) were taken at intervals, filtered through a membrane filter and washed three times in an excess of phosphate buffer at 0°. After drying with an infrared lamp radioactivity was measured on a Frieske-Hoepfner counter (Erlangen-Bruck G.m.b.H.).

Location of amylomaltase. This was determined after disintegration of the cells, effected in two ways:

(1) Broth cultures, in the exponential phase of growth, were centrifuged at 0°. The sediment was transferred to a mortar and ground with about twice its weight of carborundum powder. The product was resuspended in water.

(2) Broth cultures, in the exponential phase of growth, were mixed with sucrose solution [final concn. of sucrose, 20% (w/v)]. Penicillin (final concn. 2000 units/ml.) was added to this culture. It was incubated (stationary) at 37° for 90 min. The resulting spheroplasts were centrifuged off at 0° and subjected to osmotic shock by dilution with water.

In either case disintegrated cells, prepared from about 60 mg. dry wt. of cells, were suspended in 6 ml. of water and centrifuged at 7000–10000*g* for 15 min. at 0°. The sediment was made up with phosphate buffer to the original volume and, after the addition of toluene (1 drop/ml.), incubated for 10 min. at 37°. Amylomaltase activity was determined in the supernatant and in the sediment.

Solubilization of amylomaltase bound to insoluble structures. This was effected by incubating the sediment obtained from 13.6 mg. dry wt. of bacteria in phosphate buffer in the presence of various substances (see below), in a total volume of 5 ml., for 30 min. at 37° with shaking. The mixture was then centrifuged and amylomaltase activity was determined in the sediment and in the supernatant in the manner described above.

Utilization of maltose by intact cells. This was determined chromatographically (Burger, Hejmová & Kleinzeller, 1959) or by the conventional Warburg method measuring oxygen consumption at 30° (gas phase, air). In these experiments the bacteria were suspended in phosphate buffer and maltose was added to give a final concentration of 3 mM.

MATERIALS

Peroxidase was prepared by the method of Maehly (1955) to the second step of purification.

[^{14}C]Maltose was prepared by Dr J. Kopoldová in the Isotope Laboratory of the Biological Institute, Czechoslovak Academy of Sciences, Prague, and purified chromatographically on Whatman no. 4 paper.

Unless stated otherwise ribonuclease from bovine pancreas, once-crystallized (a commercial preparation of Reanal, Finomvegyszergyár, Budapest, Hungary), was used. A six-times-crystallized preparation of ribonuclease, also from bovine pancreas, a gift from Dr V. Liebl of our Institute, was also used. Glucose oxidase was a crude preparation (Sigma Chemical Co., St Louis, Mo., U.S.A.). Difco substances were a gift from Dr E. J. Ordal.

RESULTS

The mutant 3310M was obtained from the wild-type strain 3310 and differed from it in the following features. Unlike the wild-type strain it was

not capable of utilizing maltose either in phosphate buffer or during growth on minimal medium or broth. On minimal medium, where the only carbon source is maltose, the mutant did not grow either on submerged cultivation with aeration or on agar plates at 27° or 37°. It grew on minimal medium, however, with another source of carbon, e.g. glucose or glycerol. However, analysis of amylomaltase activity after treating intact cells with toluene (see the Methods section) showed that there was no essential difference between the activities of the enzyme in the two strains. There was, however, a difference in the regulation of amylomaltase formation in these two strains. In the wild-type strain amylomaltase was inducible, whereas in the mutant it was constitutive.

Fig. 1 gives the curve of amylomaltase formation in the wild-type strain and the mutant on growth in broth at 30°. Maltose (30 mM) was present in the culture medium of the wild-type strain. The increase in the amount of enzyme synthesized was approximately the same in both cases, but the activity of the enzyme was higher in the mutant than in the wild type throughout cultivation. Mutants with the same qualities were also obtained from other strains of *E. coli*, and the results given in the present paper were also qualitatively reproducible in these mutants.

These properties could be explained most easily by crypticity to maltose (Wiesmeyer & Cohn, 1960), and therefore the uptake of [^{14}C]maltose by these strains was determined under different conditions.

Fig. 2 shows that there was a considerable transport of maltose into the mutant during incubation in broth. On incubation in phosphate buffer

Fig. 1. Formation of amylomaltase in *E. coli* 3310 (wild-type strain) and 3310M (mutant strain). Cells were cultivated aerobically at 30° in broth in the presence of maltose (30 mM) (strain 3310) or without maltose (strain 3310M). ○, Strain 3310; △, strain 3310M.

the speed of transport of maltose was the same as in broth but the extent of accumulation at equilibrium was lower. The maximum rate of maltose penetration into the mutant both in phosphate buffer and in broth was about 4.8 μ moles/mg. dry wt./hr. This would correspond to a $Q_{0.5}$ value of 1300. In fact, as stated above, the strain did not utilize maltose either in phosphate buffer or during incubation in broth, or even in the presence of a much higher concentration of maltose (30 mM) than was used in this experiment. Fig. 2 also shows the extent of accumulation in the two other mutants (Hb₁ and K₁₀-10) that do not possess amylo-maltase. The extent of accumulation in the mutant 3310M was between that of the other two mutant strains and the wild type, and the amount of accumulated maltose was about 0.197 μ mole/mg. dry wt. Although this concentration corresponded to about one-third of the accumulation in the wild-type strain, it represented accumulation against the concentration gradient. Approximate calculation shows that, on the assumption that maltose was evenly distributed in the cell, the average concentration within the cell was 50 times that in the medium.

To determine whether the inability to utilize maltose could be explained by a difference in the location of amylo-maltase in our mutant as against the wild-type strain, the following experiments were performed. Amylo-maltase activity was compared in two fractions of cell substance, i.e. in sediment and in the supernatant, obtained by centrifuging disintegrated cells. Table 1 shows that most and in some cases all of the amylo-maltase activity of the mutant was found in the sediment, whereas in the wild-type strain a considerable part of the amylo-maltase activity was present in the supernatant. Since the sediment was obtained in a relatively small gravitational field (see the Methods section) it would be assumed that, in the mutant, amylo-maltase was bound to some insoluble or easily sedimenting cell structures.

In a further experiment the effect of toluene on the activity of amylo-maltase of the sediment was studied. Table 2 shows that in the mutant strain amylo-maltase of the sediment is in inactive form and becomes active after the treatment with toluene. However, in the wild-type strain most of the amylo-maltase in the sediment is in an active form even without the treatment with toluene. Toluene had no effect on the activity of amylo-maltase in the supernatant of either strain.

Investigations were then made of the conditions under which amylo-maltase could be liberated from the sediment (solubilized) *in vitro* and how such treatments influenced the utilization of maltose in the mutant *in vivo*. Of the different substances studied, e.g. Mg²⁺, nitrilotriacetic acid plus EDTA,

sodium deoxycholate, papain plus cysteine, and ribonuclease, only the last liberated amylo-maltase into the medium under our experimental conditions (see the Methods section). In an experiment from a sediment containing 129 units/mg. of original cells, 160 units/mg. were liberated into the clear supernatant (obtained after centrifugation at

Fig. 2. Uptake of [¹⁴C]maltose in different strains of *E. coli*. The washed suspensions from the exponential phase of growth on broth (at 37° aerobically) were incubated in broth in the presence of chloramphenicol and [¹⁴C]maltose (see the Methods section). ○, Strain 3310; △, strain 3310M; □, strain Hb₁; ●, strain K₁₀-10.

Table 1. Amylo-maltase content in cell fractions of cells of *Escherichia coli* 3310 (wild-type strain) and 3310M (mutant strain)

Cells grown in broth in the presence of 30 mM-maltose (strain 3310) and without maltose (strain 3310M) were disintegrated with carborundum or by spheroplast shock (see the Methods section). Sediment and supernatant were obtained by centrifuging suspensions of disintegrated cells at 7000–10000*g* for 15 min. The sediment was treated with toluene. Total activity was measured after the addition of toluene to intact cells.

	Amylomaltase activity (units/mg.)		
	Total activity	Sediment	Super- natant
Wild-type strain 3310	129	35	48
	151	53	65
	—	92	77
	86	—	63*
Mutant strain 3310M	21	17	0
	93	112	0
	71	43	13
	77	—	8*

* Disintegration by spheroplast shock.

10000g in 15 min.) with ribonuclease, and 6 units/mg. without it. Only high concentrations of ribonuclease (3 mg./ml.) had this effect. Ribonuclease, however, did not liberate amylomaltase from living cells under the same experimental conditions. This indicates that, under our experimental conditions, there appears to be no lysis of intact cells under the influence of ribonuclease.

The effect of ribonuclease on living cells of the

Table 2. *Effect of toluene treatment on the activity of amylomaltase bound to the sedimenting fraction of Escherichia coli 3310 (wild-type strain) and 3310M (mutant strain)*

Penicillin spheroplasts from *E. coli* 3310 and 3310M were subjected to osmotic shock (see the Methods section). Sediment and supernatant were obtained by centrifuging suspensions of disintegrated cells at 10000g for 15 min. The sediment was made up with phosphate buffer to 10 ml., and 5 ml. was shaken with toluene (1 drop/ml.) at 37° and the remaining suspension (5 ml.) was shaken under the same conditions but without toluene.

	Amylomaltase activity (units/mg.)		
	Super- natant	Sediment treated with toluene	Sediment not treated with toluene
Wild-type strain 3310	194	136	104
Mutant strain 3310M	28	140	18

Fig. 3. Effect of ribonuclease on oxygen consumption during utilization of maltose in *E. coli* 3310M. The conventional Warburg method was used. Washed suspension of cells in phosphate buffer was present in all flasks. The gas phase was air. Additions: □, none; Δ, maltose (3 mM); ○, maltose (3 mM) + ribonuclease (3 mg./ml.).

mutant caused a change of the location of amylomaltase within the cells. This was proved in the following experiments. Washed mutant cells from the exponential growth phase were incubated in phosphate buffer at 37° for 15 min. with or without ribonuclease (3 mg./ml.). Then the cells were washed, centrifuged and disintegrated with carborundum (see the Methods section). Disintegrated cells were suspended in 10 ml. of water or phosphate buffer and centrifuged at 10000g for 15 min. at 0°. About 90–100 % of the total activity of amylomaltase was found in the supernatant of the ribonuclease-treated cells.

In a further experiment we examined the effect of toluene, and of toluene with ribonuclease, on bound enzyme in whole cells. Washed cells were shaken for 30 min. at 37° with toluene (1 drop/ml.) or with toluene and ribonuclease (3 mg./ml.). After centrifuging for 15 min. at 10000g we found that 71 % of the total cell activity had been liberated by toluene, and 72 % had been liberated by toluene and ribonuclease.

A study of the effect of ribonuclease on the utilization of maltose in intact mutant cells showed that, at a concentration at which ribonuclease liberated amylomaltase from the sediment *in vitro*, it strongly stimulated the utilization of maltose *in vivo* (see Fig. 3). In this experiment a Q_{O_2} value of 132 was obtained with maltose and ribonuclease. This corresponds to the maximum Q_{O_2} that was obtainable during incubation of this mutant with glucose.

In other experiments the effect of once-crystallized ribonuclease was compared with that of six-times-crystallized ribonuclease on the utilization of maltose in the mutant. No difference was found in the effect of these two substances.

The presence of ribonuclease in the medium enabled the mutant to grow on minimal medium with maltose as the only source of carbon. This was proved by plating the mutant in a soft agar layer (see the Methods section) containing ribonuclease (3 mg./ml.). The number of colonies corresponded to the number that, under the same conditions, grew on plates containing glucose as the only carbon source. In control plates without ribonuclease or with ribonuclease but without maltose no growth could be seen.

The effect of ribonuclease could be enzymic or it could act as a polycation. Therefore the effect of histone on maltose utilization by the mutant strain was studied. It significantly increased the utilization of maltose at the much lower concentration of 50 μg./ml. (Fig. 4).

DISCUSSION

In micro-organisms cases quite often occur when an extracellular substrate is not utilized by the cell, although enzymes that catalyse the change in the

Fig. 4. Effect of histone on oxygen consumption during utilization of maltose in *E. coli* 3310M. Experimental conditions were as in Fig. 3. Additions: □, none; Δ, maltose (3 mM); ○, maltose (3 mM) + histone (50 μg./ml.) (the histone was added at 65 min.).

given substrate can be demonstrated in the cell. The discovery of permeases helped to explain this finding. However, cases are known where the substrate is the product of metabolism and is present in the cell, e.g. in the protein-protease system, and where this substrate is evidently 'protected' from enzyme action. This, of course, cannot be explained by crypticity (Mandelstam, 1963; Chaloupka & Liebster, 1959).

Attempts to localize amylo maltase show that in the mutant it had a different location from that in the wild-type strain. The different location of amylo maltase in the mutant was connected with the binding of the enzyme to insoluble structures. This fact gives a better explanation of the inability to utilize maltose in the mutant than crypticity alone, in spite of the fact that, as shown in Fig. 2, a significant fall in the accumulating power of maltose is produced by the mutation. The extent of accumulation of maltose in this mutant was about tenfold lower than in the experiments of Wiesmeyer & Cohn (1960). Despite this we are of the opinion that, on the basis of the facts given above (see the Results section), it is not possible to classify this mutant among typical strains cryptic to maltose. Unlike this mutant, the strains Hb₁ and K₁₀-10 have cryptic properties closer to those described for cryptic strains than the above mutant. For these reasons we do not consider it probable that insufficient uptake of maltose into the cell was the cause of the inability to utilize maltose.

However, the fact that, after disintegration of cells, different proportions of the enzyme were found in the different cell fractions is not necessarily a reflexion of actual differences in location in intact cells, because artifacts cannot be excluded. The

different proportions of amylo maltase found in the different cell fractions could mean either that the mutation caused a change in the structure of the enzyme or a change of insoluble structures of the cell. The fact that a substance that liberates the enzyme from insoluble structures *in vitro* and *in vivo* (ribonuclease) is also capable of removing the 'inactivity' of amylo maltase *in vivo* supports the concept that the inability of the mutant to utilize maltose is due to the mode of binding of amylo maltase to the structure of the mutant cell. On the basis of our experiments it is not possible to determine to what structure amylo maltase is bound. The ease of sedimentation suggests either the cell wall or the cytoplasmic membrane, but it cannot be excluded that the enzyme is bound in the mutants to ribosomes that are firmly connected with the cytoplasmic membrane (Cowie, Spiegelman, Roberts & Duerksen, 1961; Kihara, Hu & Halvorson, 1961; Proctor, 1961). Such an interpretation would fit with the report of Gardner (1963), who found that, during reactivation of irradiated *E. coli* cells with ribonuclease, disintegration of 23s and 16s ribosomes occurs to give 4s and 12s particles.

The mechanism of action of ribonuclease in our experiments is not clear. The fact that the six-times-crystallized preparation of ribonuclease had the same effect as the once-crystallized preparation is against the idea that the effect is produced by impurities contained in the preparation. The high concentration of enzyme, which is necessary both for solubilization and for stimulation *in vivo*, is evidence that ribonuclease does not have an enzymic function but rather that it acts as a polycation. In accordance with this are our results (Fig. 4) indicating that another basic protein, namely histone, caused the utilization of maltose *in vivo*.

Permeation of ribonuclease into the cell cannot be excluded. In fact, it was shown by Jerne & Maaløe (1957) that at a low ionic strength ribonuclease can cause the lysis of a bacterial culture. This is considered to be a result of an intracellular effect of the ribonuclease. Our results prove that during the treatment of intact cells with ribonuclease there was no liberation of amylo maltase. Moreover, the mutant grew on maltose as the only source of carbon only when ribonuclease was present. This serves as evidence against the assumption that ribonuclease caused lysis under our experimental conditions.

A series of papers has shown that the activity of enzymes in the cell is strongly influenced by conditions present at the site where the enzymes are localized (Kaplan, 1954; Mandelstam, 1960; Myrbäck & Willstaedt, 1955; McLaren, 1960; Tal & Elson, 1961; De Kloet, Van Wermeskerken & Koningsberger, 1961). It follows that the study of

the location of enzymes in the cell can contribute to the understanding of the control of their activity. Our results, however, indicate that it is impossible, without reservations, to generalize about the location of enzymes obtained in one organism even from results with strains of the same species, because the location of enzymes can be changed by mutation.

Davis & Leive (1963) have expressed the opinion that a diaminopimelic acid-incorporating enzyme of *E. coli* has specific sites concerned with location. If this is also valid for the amylomaltase system, then we consider that our system is particularly suitable for the genetic study of these possibilities.

SUMMARY

1. From certain strains of *Escherichia coli* were isolated mutants that contain amylomaltase, but despite this are not capable of utilizing maltose. This inability to utilize maltose is not due to crypticity of the mutants to maltose.

2. After disintegration of the cells the amylomaltase of the mutant is found to be associated with insoluble structures, whereas in the wild-type strain (capable of utilizing maltose) a considerable part of the enzyme is present in the supernatant.

3. Amylomaltase can be transferred from the insoluble structures to the supernatant by the action of ribonuclease or toluene.

4. The addition of ribonuclease or histone to intact cells of the mutant leads to the utilization of maltose.

The authors are indebted to E. Stejskalová (graduate chemist) for carrying out some of the analyses, and to Dr J. S. D. Bacon for valuable comments on some of the problems and for help in preparation of the manuscript.

REFERENCES

- Axelrod, B. (1962). *Biochim. biophys. Acta*, **55**, 269.
 Burger, M., Hejmová, L. & Kleinzeller, A. (1959). *Biochem. J.* **71**, 233.
 Chaloupka, J. & Liebster, J. (1959). *Folia microbiol., Prague*, **4**, 167.
 Cohen, J. N. & Monod, J. (1957). *Bact. Rev.* **21**, 169.
 Cowie, D. B., Spiegelman, S., Roberts, R. B. & Duerksen, J. D. (1961). *Proc. nat. Acad. Sci., Wash.*, **47**, 114.
 Davis, B. D. (1950). *J. Bact.* **60**, 17.
 Davis, B. D. & Leive, L. (1963). *Res. Commun. int. Symp. Mechanisms of Cellular Activities in Microorganisms, Marseille*, p. 9.
 De Kloet, S. R., Van Wermeskerken, R. K. A. & Koningsberger, V. V. (1961). *Biochim. biophys. Acta*, **47**, 144.
 Gardner, F. T. (1963). *Biochem. biophys. Res. Commun.* **10**, 454.
 Gorini, L. & Kaufman, H. (1960). *Science*, **131**, 604.
 Jerne, N. K. & Maaløe, O. (1957). *Acta path. microbiol. scand.* **40**, 362.
 Kaplan, J. G. (1954). *J. gen. Physiol.* **38**, 197.
 Keston, A. (1956). *Abstr. Pap. Amer. chem. Soc. 129th Meet., Dallas, Texas*, p. 31c; cited by McComb, R. B. & Yushok, W. D. (1958). *J. Franklin Inst.* **265**, 417.
 Kihara, H. K., Hu, A. S. L. & Halvorson, H. O. (1961). *Proc. nat. Acad. Sci., Wash.*, **47**, 489.
 McLaren, A. D. (1960). *Enzymologia*, **11**, 356.
 Maehly, A. C. (1955). In *Methods in Enzymology*, vol. 2, section 5, p. 801. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
 Mandelstam, J. (1960). *Bact. Rev.* **24**, 289.
 Mandelstam, J. (1963). *Ann. N.Y. Acad. Sci.* **102**, 621.
 Myrbäck, K. & Willstaedt, E. (1955). *Ark. Kemi*, **8**, 367.
 Pardee, A. B., Jacob, F. & Monod, J. (1959). *J. molec. Biol.* **1**, 165.
 Proctor, M. H. (1961). *Biochem. J.* **80**, 27r.
 Robertson, J. J. & Halvorson, H. O. (1957). *J. Bact.* **73**, 186.
 Tal, M. & Elson, D. (1961). *Biochim. biophys. Acta*, **53**, 227.
 Wiesmeyer, H. & Cohn, M. (1960). *Biochim. biophys. Acta*, **39**, 440.

Biochem. J. (1964), **93**, 606

Lipids of the Acetone-Insoluble Fraction from Red-Clover (*Trifolium pratense*) Leaves

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(Received 24 March 1964)

Recent investigations on plant lipids have revealed a number of new constituents, the most widespread being the galactolipids discovered in wheat flour by Carter, McLuer & Slifer (1956). Other workers (Wintermans, 1960; Kates, 1960) have shown that the galactolipids comprise the

major fraction of the total lipids in the leaves of a number of plant species.

Paper chromatography applied in conjunction with radioautography has provided the most effective method so far devised for the detection and estimation of the individual lipid components